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Transcriptomic responses to silver nanoparticles in the freshwater unicellular eukaryote *Tetrahymena thermophila*

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ABSTRACT

Currently, silver nanoparticles (AgNPs) are being increasingly used as biocides in various consumer products and if released in the environment they can affect non-target organisms. Therefore, understanding the toxicity mechanism is crucial for both the design of more efficient nano-antimicrobials and for the design of nanomaterials that are biologically and environmentally benign throughout their life-cycle. Here, the ciliate *Tetrahymena thermophila* was used to elucidate the mechanisms of action of AgNPs by analysing the gene expression profile by RNA-seq and the transcriptomic effects of AgNPs were compared to those induced by soluble silver salt, $AgNO_3$. Exposure to AgNPs at sublethal concentrations for 24 h induced phagocytosis, transport pathways, response to oxidative stress, glutathione peroxidase activity, response to stimulus, oxidation-reduction, proteolysis, and nitrogen metabolism process. Based on gene set enrichment analysis (GSEA), some biological processes appeared targets of both toxicants. In addition to many similarities in affected genes, some effects were triggered only by NPs, like phagocytosis, glutathione peroxidase activity, response to stimulus, protein phosphorylation and nitrogen metabolism process. This research provides evidence that AgNPs compared to $AgNO_3$ at the same concentration of dissolved silver ions dysregulate a higher number of cellular pathways. These findings confirm that AgNPs can induce toxicity not only due to soluble silver ions released from the particles but also to particle intrinsic features.

1. Introduction

Nanotechnology is a fast-developing field, and researchers are continuing to discover unique properties and applications of nanomaterials. There are different purposes for the use of nanoparticles (NPs) in improving human health, environmental quality, computer science technology and general devices. They have found their commercial applications in various products (Salata, 2004).

Among the metal NPs, silver NPs (AgNPs) have the highest degree of commercialization, which is due to their known antimicrobial properties (Gopinath et al., 2010; Sotiriou and Pratsinis, 2011; Youngs et al., 2012; Guo et al., 2014; Dubey et al., 2015). AgNPs are important in many industries, such as pharmaceuticals, cosmetics, textiles, surface coatings, electronic components and packaging for the food industry (Nadagouda et al., 2011; Benn et al., 2010). The use of silver as a bactericide and fungicide has been known for more than 20 centuries (Heiligtag and Niederberger, 2013). In the 19th century its use in medicine was widespread, but this declined with the advent of antibiotics (Hobman and Crossman, 2015). However, problems arising from the emergence of antibiotic resistant strains have led to renewed interest in silver as an antibiotic agent. Recent studies have confirmed that silver particles have effects against a wide spectrum of Gram-negative and Gram-positive bacteria and some also exhibit

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anti-fungal and antiviral activity (Rai et al., 2009). Currently, nanosilver is perhaps the most preferred anti-microbial nanomaterial and AgNPs coatings have been used to inhibit the unwanted growth of bacterial biofilms in medical catheters, prostheses, and heart valves (Ivask et al., 2014).

Even if AgNPs possess favourable properties for novel biomedical applications, their environmental toxicity is still a cause for concern (Burdușel et al., 2018). AgNPs may be released into the environment during the production, transport, erosion, washing and disposal of AgNPs products (Nowack and Mueller, 2008). There are two major classes of products which expedite the release of AgNPs into the environment: the cosmetics and healing lubricants, which via dermal exposure are eventually released into water bodies while bathing, and the nanomaterials incorporated into textile fabrics, which upon washing are released into the water stream (Wiesner et al., 2009). The broad industrial applications may potentially increase environmental exposure, and it may have negative impacts on the ecosystems (Fabrega et al., 2011; Anjum et al., 2013). AgNPs are of especially high concern, because some aquatic species are extremely sensitive to them (Kahru and Dubourguier, 2010; Bondarenko et al., 2013a).

Innovative omics approaches are increasingly used in environmental toxicology (Zhang et al., 2018) and have been recommended as one of the preferred tools in future nanotoxicology studies (Fadeel et al., 2018). Whole-genome profiling of gene or protein expression advances our understanding on mechanisms of action of NPs, which supports NPs risk assessment. Furthermore, omics techniques enable the development of biomarkers for NPs exposures and reveal NPs effects that are not apparent when assessed by traditional toxicity endpoints. For example, carbon and boron nitrite-based NPs induced significant transcriptomic responses in bacteria at nongrowth-inhibitory NPs concentrations, which helped to shed light on some subtle physiological effects of NPs (Mortimer et al., 2018). Transcriptomics has also been employed in studies evaluating AgNPs effects on eukaryotic unicellular organisms, such as freshwater green algae *Chlamydomonas reinhardtii* (Simon et al., 2013) and marine ciliate *Euplotes vannus* (Pan et al., 2018). Simon et al. (Anders et al., 2013) showed that AgNPs elevated the expression of genes encoding components of the cell wall and flagella in *C. reinhardtii*, indicating AgNPs damage to the outer surface of the cell, expected for a cell wall-encased organism without active uptake pathways for NPs. *E. vannus*, on the other hand, as an actively phagocytosing protozoan, was shown to differentially regulate transcripts involved in cell membrane trafficking – endo- and exocytosis upon exposure to AgNPs, suggesting AgNPs uptake and Ag efflux regulation (Pan et al., 2018). However, since neither of these two studies included a soluble Ag salt as a comparison, the effects of Ag ions to the transcriptome of protists and its role in AgNP-induced toxicity is still unclear. The important role of Ag ions in the toxicity of AgNPs to the freshwater ciliate *Tetrahymena thermophila* was demonstrated by Juganson et al. (2017), by using physiological assays and gene expression analysis of a selected set of genes (Juganson et al., 2017). The expression profiles of metal-binding, oxidative and general stress-related genes indicated that Ag ion-mediated toxicity mechanism prevailed over oxidative stress-related pathway in AgNPs toxicity to *T. thermophila* (Juganson et al., 2017).

Here, we used whole transcriptome sequencing (RNA-seq) to extend the understanding about AgNPs toxicity mechanisms in the ciliate *T. thermophila* beyond the regulation of metallothionines, oxidative and general stress-related genes. For that, we exposed *T. thermophila* to casein-stabilized AgNPs (Collargol) or $AgNO_3$ at two sublethal concentrations over 24 h. RNA-seq data was subjected to differential gene expression and gene set enrichment analysis to identify the pathways affected by AgNPs and $AgNO_3$ in the eukaryotic unicellular model at the transcription level. The findings contribute to the mechanistic understanding of AgNPs toxicity and support NPs hazard assessment.

2. Material and methods

2.1. Chemicals

Casein-coated colloidal silver NPs, called Collargol (batch N 297, from Laboratories Argenol S. L.) were dispersed in MilliQ water at 1 g/L and $AgNO_3$ was obtained as a 0.1 M solution (Sigma-Aldrich). Preparation of AgNPs stock dispersion and physicochemical characterization of AgNPs have been reported previously (Juganson et al., 2017; Blinova et al., 2013; Bondarenko et al., 2013b). AgNPs physicochemical characteristics are summarized in Table S1. Stock suspensions and solutions of silver compounds were stored at 4 °C in the dark.

2.2. T. thermophila strain CU428 culturing

T. thermophila strain CU428, whose macronuclear genome has been sequenced (Eisen et al., 2006; Coyne et al., 2008), was from IHB, CAS (Wuhan, China). 100 μl of cells were transferred to 5 mL of SPP medium (2% proteose peptone, 0,1% yeast extract, 0.2% glucose and 0.003% Fe-EDTA) supplemented with streptomycin sulphate and penicillin G, each at 250 μg/mL, and the fungicide amphotericin B at 1.25 μg/mL (all from CARLO ERBA Reagents). The cells were incubated at 30 °C under shaking (150 rpm) in an Erlenmeyer glass flask overnight. Cell concentration was determined by counting formaldehyde-fixed (5% v/v) cells in a haemocytometer (Neubauer) (Mortimer et al., 2010). When cells were in their logarithmic growth phase, with a doubling time of less than 3 h and at a concentration of ~3–4 × 10^5 cells/mL, they were harvested by centrifugation at 400×*g* for 5 min, at room temperature, and washed twice with MilliQ water. Final cell concentration was adjusted to 10^6 cells/mL in MilliQ water.

2.3. Toxicity testing

The dilutions of NPs and $AgNO_3$ were prepared in MilliQ water at the concentration ranges of 10–100 mg Ag/L for AgNPs and 0,5–20 mg Ag/L for $AgNO_3$. 100 μL of *T. thermophila* culture, at 10^6 cells/mL in MilliQ water, were added to 100 μL of test solutions (AgNPs and $AgNO_3$ at different concentrations in MilliQ water) in triplicate. After 24 h of exposure in 96-well plates at 25 °C in the dark (visible light was avoided since it promotes the biosynthesis of AgNPs from Ag ions by *T. thermophila* (Juganson et al., 2013)), the toxicity of AgNPs and $AgNO_3$ was evaluated by measuring the cellular ATP content using the luciferin-luciferase reaction based ATP assay kit (Sigma-Aldrich) as reported elsewhere (Juganson et al., 2017; Mortimer et al., 2010; Jemec et al., 2016).

For the toxicity test, the concentration-effect curves by the log-normal model were constructed and the concentration for 50% of maximal effect (EC_{50}) values (the effective concentration that induces a response in 50% of the population) with 95% confidence intervals were calculated based on nominal concentrations using REGTOX software for Microsoft Excel™ (Vindimian, 2001). All data were expressed as the average of three independent experiments ± standard deviation (SD).

2.4. Exposure of T. thermophila to silver compounds for RNA-seq

T. thermophila CU428 cells were exposed to two sub-lethal concentrations of AgNPs at 10 and 20 mg Ag/L. $AgNO_3$ was used as a control for dissolved Ag ions, expected to be present in AgNPs

dispersions, respectively at 0.76 mg Ag/L and 1.52 mg Ag/L of $AgNO_3$, as previously determined by Juganson et al. (2017).In this previous work two *T. thermophila* strains (CU427 and CU428) were tested. Among them, the strain CU428 appeared more sensitive to the AgNPs (the EC_{50} was lower in CU428 than in CU427). In addition, this strain has also been used to carry out the RNA Microarray and RNA-seq analyses of Tetrahymena Functional Genomics Database (TetraFGD) (Miao, 2017) and therefore it was considered appropriate for the transcriptomic analysis in the current study.

The exposures of *T. thermophila* to Ag compounds were conducted in MilliQ water to avoid interactions of media components with AgNPs and dissolved Ag ions. *T. thermophila*, is known to survive in water for at least a week (Koppelhus et al., 1994), and the starved cells undergo physiological, biochemical and molecular changes (Cassidy-Hanley, 2012). However, these were not crucial factors in interpreting the results because the exposures to AgNPs, $AgNO_3$ and unamended control culture occurred in the same conditions. Thus, any difference in gene expression or physiology of ciliates were assumed to be induced by the chemical exposures (Juganson et al., 2017).

Each exposure and unamended control were performed in triplicate, in non-tissue culture treated 6-well plates (Falcon), with each well containing 5 mL of *T. thermophila* culture, 5.5×10^5 cells/mL. 0.5 mL of Ag compounds or MilliQ water (controls) were added to the cells, which resulted in the final cell concentration of 5×10^5 cell/mL. The plates were incubated at 25 °C in the dark for 24 h. After this time, the ciliates were visualized using a light microscope (Olympus CX41equipped with DP71 camera) to monitor any changes in cell morphology and food vacuole filling with NPs in AgNP-exposed cultures.

2.5. RNA isolation

After 24 h exposure, *T. thermophila* exposed to AgNPs or $AgNO_3$, or incubated in MilliQ water (controls), were collected by centrifugation (5 min, 400×*g*). The cell pellet was dissolved in TRI Reagent® (Sigma-Aldrich) and RNA was isolated according to the manufacturer's protocol. Agarose gel electrophoresis (1%), Nanodrop™ 1000 UV–Vis Spectrophotometer (Thermo Fisher Scientific) and Agilent 2100 Bioanalyzer (Agilent Technologies) were used to check the integrity, concentration, and quality of RNA.

2.6. RNA sequencing and bioinformatic analysis

A total of 16 samples were subjected to RNA sequencing (RNA-seq): 4 unamended controls, 3 samples stressed with AgNPs at 10 mg Ag/L, 3 samples stressed with AgNPs at 20 mg Ag/L, 3 samples stressed with $AgNO_3$ at 0.76 mg Ag/L and 3 samples stressed with $AgNO_3$ at 1.52 mg Ag/L. Library preparation was conducted with the Illumina TruSeq Stranded mRNA Library Preparation Kit and RNA was sequenced using an Illumina NextSeq 500 deep sequencing system (producing paired-end reads with an Illumina 1.9 encoding and 75 bp of read length). The reads quality, obtained from the sequencing, was checked using the FastQC software (Andrews, 2010). Reads have been deposited to NCBI Sequence Read Archive database, SRA accession: BioProject: PRJNA641889, BioSample: SAMN15369556. Transcriptome assembly was performed using the STAR package included in the "RNA-seq Alligment" section of Blast2GO (Dobin et al., 2013), and paired-end reads were mapped to the *T. thermophila* SB210 coding sequences obtained from the *Tetrahymena* Genome Database (TGD) (Stover et al., 2006) with a yield per sample ranging from 84.7 to 85%. Then the experimental samples were compared with the control to evaluate quantitatively the inhibition or increase of gene expression due to NPs or silver ions. The Differential Expression Analysis (DEA) was carried out using the edgeR package (with Trimmed Mean of M-values (TMM) as normalization method and generalized linear model (GLM) likelihood ratio test as statistical test), included in the "Pairwise Differential Expression Analysis" section of Blast2GO (Robinson et al., 2009) to calculate normalized fold change and counts-per-million (CPM) ensuring that expression levels for different genes and transcripts can be compared across runs. Stringent default criteria were set: genes showing |log2 (fold change)| > 1 and false discovery rate (FDR) < 0.05 in all the 3 replications were defined as Differentially Expressed Genes (DEGs).

To assign gene function to contigs, the Gene Set Enrichment Analysis (GSEA) in Blast2GO (Götz et al., 2008) was performed.

2.7. Gene expression validation

Real-time quantitative polymerase chain reaction (RT-qPCR) was used to confirm the effects of AgNPs and $AgNO_3$ on the expression levels of selected genes listed in Table S2. Genes among the DEG list were selected not only by taking into consideration their biological role, but also, their physiological expression profile depicted in TetraFGD (Miao, 2017), giving priority to those genes that diverged from their expected profile. Primers (Thermo Fisher Scientific) were designed using the Primer-BLAST application included in the NCBI website. The specificity of each primer set and annealing temperature to optimize PCR conditions and the fluorescence signal specificity of PCR amplification were confirmed through assessment of the product melting curves and the efficiency was between 94.7% and 102.4% with $r^2 = 0.99$.

Each RNA sample (800 ng) was retrotranscribed to cDNA using an iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad) with iScript DNase to digest genomic DNA contamination. cDNA (0.3 μL or 12.5 ng) from each sample was used in RT-qPCR using SsoAdvanced Universal SYBR Green Supermix and the CFX Connect Real-Time PCR Detection System (both from Bio-Rad). All reactions were performed in triplicate in a final volume of 20 μL. Thermal cycling conditions were as follows: 3 min denaturation at 98 °C followed by 40 cycles for 15 s denaturation at 98 °C, 30 s annealing and elongation at 60 °C. 17S rRNA and HSP705 genes were used as references for normalization, and the relative amount of mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

For RT-qPCR experiments the statistical analyses were performed with CFX Maestro™ Software for Bio-Rad CFX Real-Time PCR Systems. One-way ANOVA analyses were used to assess significant differences ($p < 0.01$).

3. Results and discussion

3.1. AgNPs and $AgNO_3$ toxicity to T. thermophila and designing of the RNA-seq experiments

The viability measurements after exposing *T. thermophila* to AgNPs and $AgNO_3$ at a range of concentrations indicated that the minimal concentration of AgNPs lethal to *T. thermophila* was about an order of magnitude higher than the respective concentration of $AgNO_3$, based on Ag mass concentrations (Figure S1). This result was consistent with previously published data (Juganson et al., 2017). The EC_{50} values [mean (95% confidence intervals)] for AgNPs were 28 (25–32) mg Ag/L at 2 h and 38 (33–46) mg Ag/L at 24 h and for $AgNO_3$ 0.96 (0.87–0.98) mg Ag/L at 2 h and 1.1 (1–1.2) mg Ag/L at 24 h. Although the EC_{50} values, based on the total Ag concentration, differed for the two Ag compounds by an order of magnitude, the dissolved Ag concentrations at EC_{50} were almost equal in the two cases (Juganson et al., 2017), thus supporting the importance of released Ag ions in AgNPs toxicity. These toxicity

experiments also showed that for AgNPs, 2 h EC_{50} value did not differ significantly from the 24 h EC_{50} value ($p > 0.05$), meaning that exposure beyond 2 h did not increase AgNPs toxicity to *T. thermophila*. Furthermore, cells exposed to both Ag compounds at sublethal concentrations showed internalization of Ag in the vacuoles at optical microscope observation (Figure S2). This suggested that *Tetrahymena* cells were able to detoxify Ag in the exposure medium by internalizing Ag ions and AgNPs into food vacuoles and either sequestering toxic ions by biomolecules or agglomerating NPs into larger aggregates (Figure S2) thus probably expelling them from the cells as has been previously reported (Juganson et al., 2017; Juganson et al., 2013).

Detoxification may explain the higher tolerance towards Ag compounds in *T. thermophila* than other freshwater invertebrates (Juganson et al., 2017; Juganson et al., 2013). Such results are remarkable considering that the toxicity assays were performed in MilliQ water, where the effects of the medium components on the Ag ions complexation and speciation were eliminated. These features are presumably a result of the adaptation to high environmental concentrations of pollutants. Indeed, protozoa are also present in the wastewater purification process (Esteban et al., 1991).

Based on the results of the viability assay, two sub-lethal concentrations of AgNPs, 10 and 20 mg Ag/L which caused no reduction in the viability at 24 h exposure (Figure S1), were chosen for *T. thermophila* RNA-seq experiments. $AgNO_3$ concentrations of 0.76 and 1.52 mg Ag/L were selected to be used in parallel RNA-seq experiments, since, according to Juganson et al. (2017), these concentrations are equal to the levels of dissolved Ag in the suspensions of AgNPs at 10 and 20 mg Ag/L, respectively. Therefore, *T. thermophila* were separately exposed to the two Ag compounds at the above-mentioned concentrations for 24 h and RNA-seq experiments were performed to obtain gene expression profiles. The selection of 24 h as exposure time and not 2 h was supported by RT-qPCR experiments performed at both times of AgNPs exposure on a set of genes selected from the previous study (Juganson et al., 2017). Most changes in the gene expression at 2 h were similar to the changes at 24 h, but occurred at increased level after 24 h for most analysed genes (Figure S3). Therefore, 24-h exposure was selected for the transcriptomic investigation of the AgNPs stress in *T. thermophila*.

3.2. Differentially expressed genes (DEGs) analysis

To explore the overall differences between the *T. thermophila* expression profiles at different exposure conditions (AgNPs at 10 or 20 mg/L, $AgNO_3$ at 0.76 or 1.52 mg/L, or MilliQ water as Control), all samples were clustered in multi-dimensional scaling (MDS) plots (Fig. 1). The distance between each group of samples can be interpreted as the leading log-fold change between the samples for the genes that best distinguish that group of samples (Chen et al., 2016). The MDS plot showed that replicates of the same treatment clustered together, while samples from different treatments were well separated (Fig. 1). This indicates statistically significant differences between treatments. This result confirmed the reproducibility and reliability of the RNA-seq data.

The reads obtained were used in the downstream analyses to compare experimental samples with the controls to evaluate quantitatively the inhibition or increase of gene expression. The expression profiles of unamended *T. thermophila* (control samples) were in agreement with the data available on TetraFGD (Miao, 2017) after 24 h of starvation. The numbers of differentially

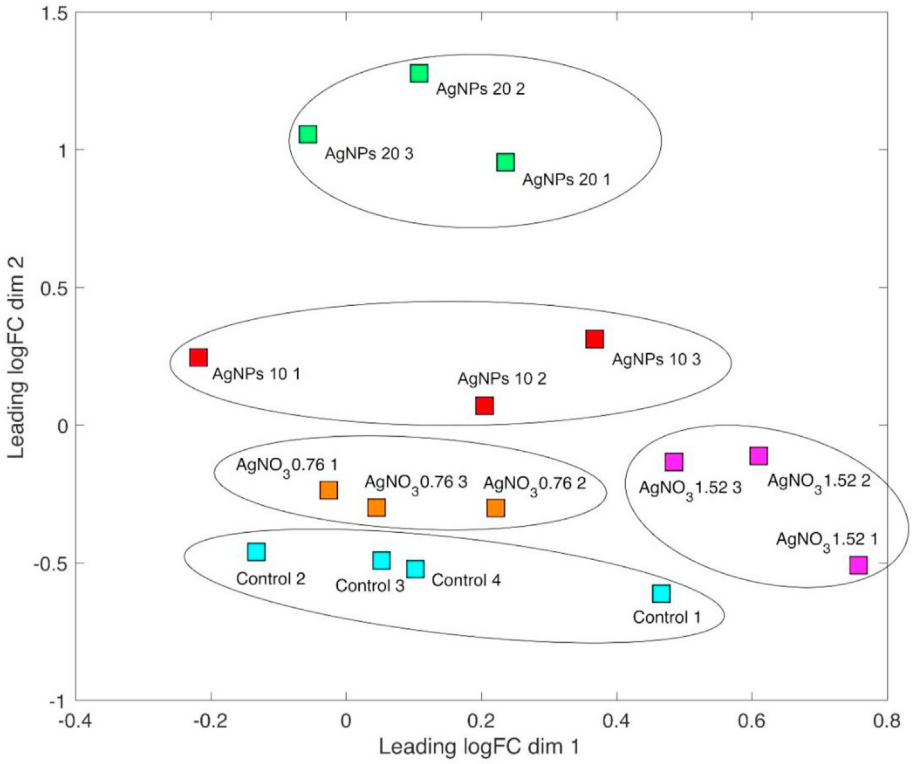

Fig. 1. Multi-dimensional scaling (MDS) plot showing the distances between the expression profiles of different samples: *T. thermophila* exposed to AgNPs at 10 or 20 mg/L (AgNPs 10 and AgNPs 20, respectively). $AgNO_3$ at 0.76 or 1.52 mg/L ($AgNO_3$ 0.76 and $AgNO_3$ 1.52, respectively) or incubated in MilliQ water (Control). The plot shows similarity between samples in which distances correspond to leading log-fold-changes (Leading logFC) between each pair of RNA samples. The leading logFC is the average (root-mean-square) of the largest absolute logFC between each pair of samples. Each colour indicates a different treatment and each square represents one treatment replica.

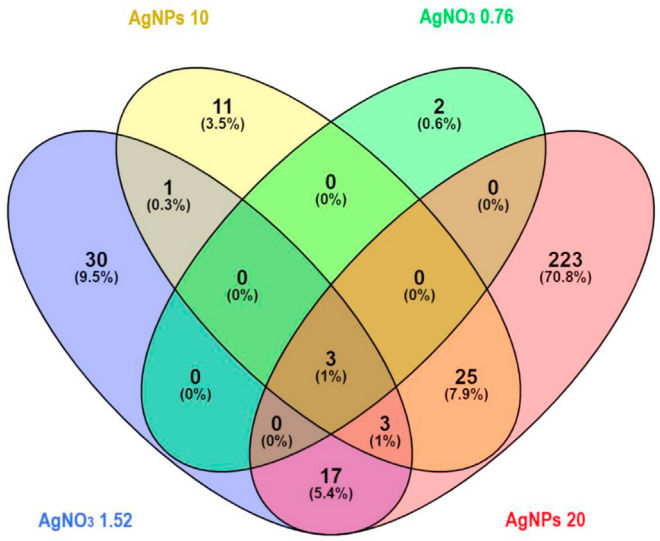

Fig. 2. Venn diagram showing numbers of differentially expressed genes (and their percentage over the total number of the DEGs) in *T. thermophila* exposed to AgNPs at 10 or 20 mg/L (AgNPs 10 and AgNPs 20, respectively) or $AgNO_3$ at 0.76 or 1.52 mg/L ($AgNO_3$ 0.76 and $AgNO_3$ 1.52, respectively). Venn diagram was drawn using a publicly available online tool (Oliveros, 2015).

expressed genes (DEGs) in *T. thermophila* exposed to AgNPs or $AgNO_3$ with respect to controls are shown in Fig. 2. AgNPs clearly affected a higher number of genes than $AgNO_3$. There are 43 DEGs (37 up- and 6 down-regulated) in the samples exposed to AgNPs at 10 mg Ag/L and 271 DEGs (201 up- and 70 down-regulated) in the samples exposed to AgNPs at 20 mg Ag/L. Only 5 genes were up-regulated in *T. thermophila* exposed to $AgNO_3$ at 0.76 mg Ag/L and 54 (52 up and 2 down-regulated) in the samples exposed to $AgNO_3$ at 1.52 mg Ag/L. While 32 DEGs were detected only in $AgNO_3$-exposed *T. thermophila* (30 DEGs by $AgNO_3$ at 1.52 mg Ag/L and 2 DEGs by $AgNO_3$ at 0.76 mg Ag/L), 24 genes were affected by both Ag compounds (17 DEGs only by the two highest concentration of both compounds, 3 DEGs by both AgNPs concentrations and $AgNO_3$ at 1.52 mg Ag/L, 3 DEGs by all the compounds regardless of their concentration, and 1 DEG by AgNPs at 10 mg Ag/L and $AgNO_3$ at 1.52 mg Ag/L) and 259 genes are differentially affected only by AgNPs. Most of the changes (223 genes) are triggered at the highest AgNPs concentration, 25 DEGs are affected by both AgNPs concentrations and 11 genes are dysregulated only by AgNPs at 10 mg Ag/L (Fig. 2, Figure S4 and Table S3). In many cases there is a linear tendency (for up or down regulation) that is concentration dependent, i.e. a gene which is dysregulated at a low compound concentration is dysregulated at a higher level at the corresponding higher compound concentration.

The absolute number of DEGs observed by Simon et al. (Anders et al., 2013) in a unicellular alga *C. reinhardtii,* after 2 h of exposure to AgNPs, is quite comparable to the data reported here after 24 h exposure to AgNPs at 20 mg Ag/L; 141 up-regulated and 86 down-regulated genes, with more up-regulated genes respect to the down-regulated, even though they represent a lower percentage over the total number of genes in the genome, 0.017% (in Simon et al. (Anders et al., 2013)) against 0.988% (in Table S3). Conversely, the number of DEGs observed by Pan et al. (2018) in a marine ciliated protozoan *E. vannus* after 1 and 12 h of exposure to AgNPs was much higher than detected in the current study and, differently from *T. thermophila*, the number of up-regulated genes was not higher than the number of down-regulated genes. Partially it can be due to the fact that the Pan et al. (2018) study did not include time-matched controls, RNA-seq samples exposed to AgNPs for 1 h and 12 h were compared to control samples processed at time 0, thus the differences observed might also be due to the natural changes of gene expression over time and not only to the AgNPs effects.

For global visualization of the results, for all DEGs under each stress condition, heatmaps, using CPM values, that allow to compare RNA-seq results among different runs, were generated (Fig. 3 and Table S4).

3.3. Gene set enrichment analysis (GSEA)

The GSEA of DEGs indicated their involvement in various pathways. Most of the DEGs reported here, are induced by exposure to AgNPs. These genes are involved in various biological processes (BP) and molecular functions (MF), *e.g.*: transport, ion transport, response to stress and to stimulus, protein phosphorylation, oxidation-reduction process, response to oxidative stress, glutathione peroxidase activity and cellular nitrogen compound metabolic process (Fig. 4 and Figure S5).

$AgNO_3$ treatment, at the higher concentration, was specifically associated with the up-regulation of genes involved in the vesicle-mediated transport biological process and associated to different cellular components (CC) like clathrin adaptor complex, clathrin coat, membrane coat, AP-type membrane coat adaptor complex, membrane protein complex and coated membrane (Fig. 4 and Figure S6). Therefore, the vacuoles which are visible in the *T. thermophila* treated with $AgNO_3$ (Figure S2) can be formed by the fusion of pinocytotic vesicles (Nilsson and van Deurs, 1983; Elde et al., 2005). This means that cells are exposed not only through their cell surface but also via internal membranes, as suggested previously (Juganson et al., 2017) in the case of phagosomes (Juganson et al., 2017; Mortimer et al., 2010; Kahru et al., 2008). NPs are internalized by *T. thermophila* mainly by phagocytosis (Mortimer et al., 2014a) as was the case also here with AgNPs accumulated in the food vacuoles (phagosomes) visible in the optical micrographs (Figure S2). Accumulation of NPs in the phagosomes increases the cellular membrane exposure to NPs and dissolved metal ions which can induce potentially harmful effects such as membrane composition changes (Mortimer et al., 2011). Here, some annotated genes related to phagocytosis in *Tetrahymena* genome database (TGD) (Stover et al., 2006; Jacobs et al., 2006) were found to be highly expressed in AgNP-exposed *T. thermophila*, but not dysregulated by NPs since these genes are probably constitutively expressed because phagocytosis is a common physiological process in *T. thermophila*. Possibly, other dysregulated genes are affected but they are not yet annotated, and so for this reason not identifiable. Nevertheless, a high number of genes involved in the processes called transport activity, ion transport via transporters or pores, and localization were up-regulated (Fig. 4 and Figure S5) and they can be related either to the AgNPs internalization, accumulation in the vacuoles and their movement through the cell.

Also, the toxic effect specifically produced by NPs, particularly at the higher AgNPs concentration, is evident by the down-regulation of several genes involved in vesicular movement along microfilaments and microtubules, such as those encoding kinesins. The active movement of kinesins supports several essential cellular functions including mitosis, meiosis, and transport of cellular cargo.

The relevant gene encoding ribosome biogenesis protein BOP1 (TTHERM_00532610), specifically associated with the rRNA processing pathway and responsible for the maturation of rRNA molecules, was down-regulated by AgNPs at the higher concentration (20 mg Ag/L), thus limiting the efficiency of the essential process of ribosome production. The Cyclin Dependent Kinase 6 (CDK6)

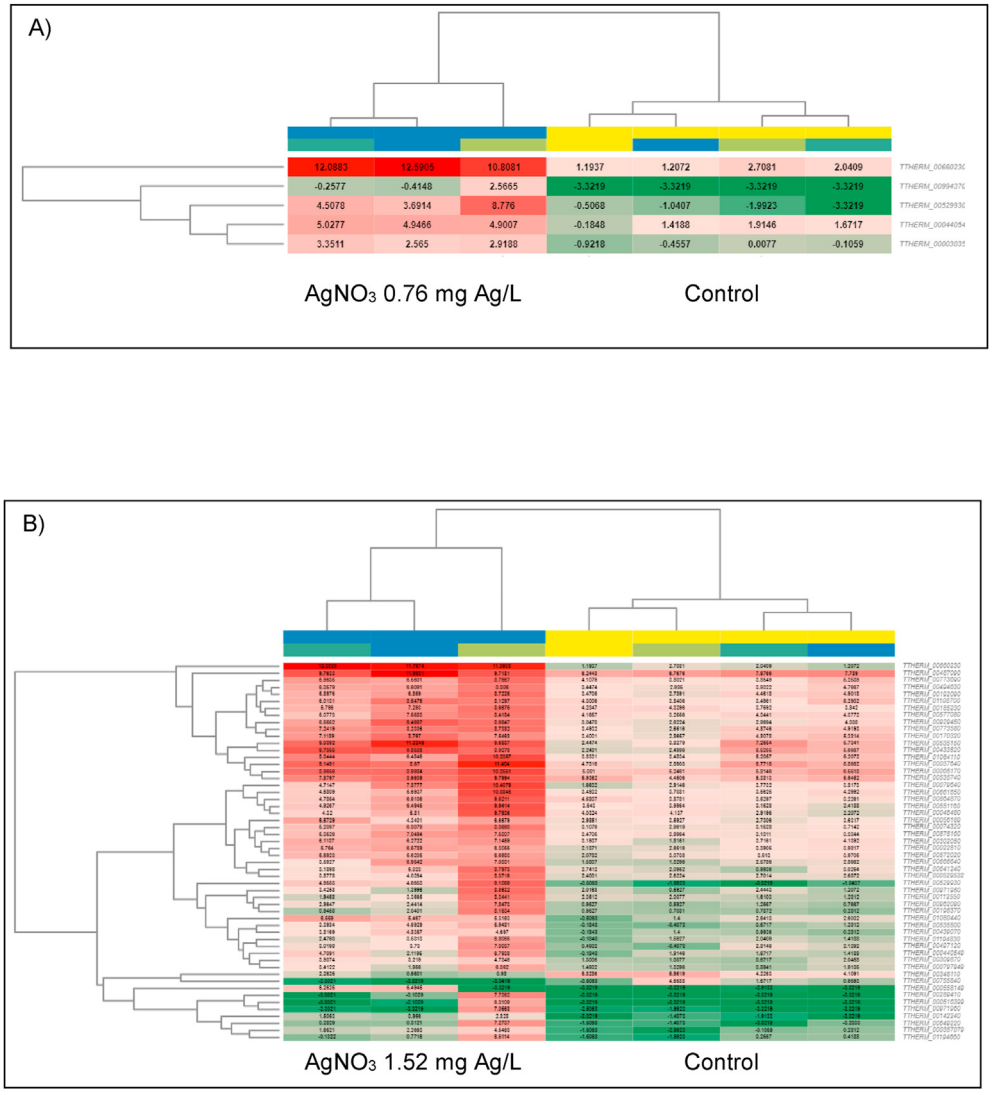

Fig. 3. Heatmaps of differentially expressed genes (DEGs) in different treatment groups (A, $AgNO_3$ 0.76 mg Ag/L; B, $AgNO_3$ 1.52 mg Ag/L; C, AgNPs 10 mg Ag/L; D, AgNPs 20 mg Ag/L) compared to unamended control. CPM values are used to generate the heatmaps. The numerical values are represented by a range of colours, from red (highly expressed) to green (lowly expressed). Each row corresponds to a gene. The three columns on the left represent the experimental replicates, the four columns on the right represent the control replicates. The replicates are quite homogeneous in the level of their expression as it is shown by the colours. The dendrograms on the left and top side are produced by a hierarchical clustering method of the Euclidean distance computed between genes (left) and samples (top) (Robinson et al., 2009; Glazko and Mushegian, 2010). Blue and yellow colours on the top indicate a graphical representation of the Euclidean distance between samples. In D, genes are divided in groups indicated by letters. The codes of the genes for each letter are listed in Table S4.

hypothetical protein, described as a protein kinase domain containing protein with sequence similarity to the CDKL protein kinase family which is involved in DNA replication (Stover et al., 2006; Yan et al., 2016), was up-regulated only upon exposure to AgNPs at the higher concentration (Fig. 4). The protein regulates the exit from the G1 phase of the cell cycle, but it is also a transcriptional regulator and is a moonlighting protein that is upregulated under stress conditions (Huberts and van der Klei, 2010; Tigan et al., 2016). AgNPs effect on the cell cycle was also described by Pan et al. (2018) in *E. vannus* stressed by AgNPs where cyclin E was up-regulated and other genes involved in the cell cycle progression were down-regulated. Moreover, the SRP54-type protein, that is the signal recognition particle (SRP) subunit (homolog of mammalian SRP54) (Stover et al., 2006), a key protein involved in the secretory pathway that both recognises and binds to the sequence of nascent proteins (Ramos et al., 1997), and also the YRS1 (tyrosyl-tRNA synthetase 1) predicted protein, a tRNA binding domain containing protein (Stover et al., 2006) were up-regulated by AgNPs at the higher concentration. These findings, together with the up-regulation of a considerable number of genes involved in kinase, phosphotransferase activities, and tRNA binding suggest an impairment in the normal cell cycle progression and transcriptional regulation (Fig. 4).

Both AgNPs and $AgNO_3$ treatments caused differential expression of genes involved in ion binding mechanisms and in the proteolysis pathway (Fig. 4 and Figure S7). The upregulation of the genes of the proteolysis pathway is likely indicative of protein damage caused by the oxidative stress generated by the Ag compounds (Pan et al., 2018).

3.4. Differential expression of oxidative stress-related genes upon exposure to AgNPs and $AgNO_3$

The expression of oxidative stress genes, like superoxide

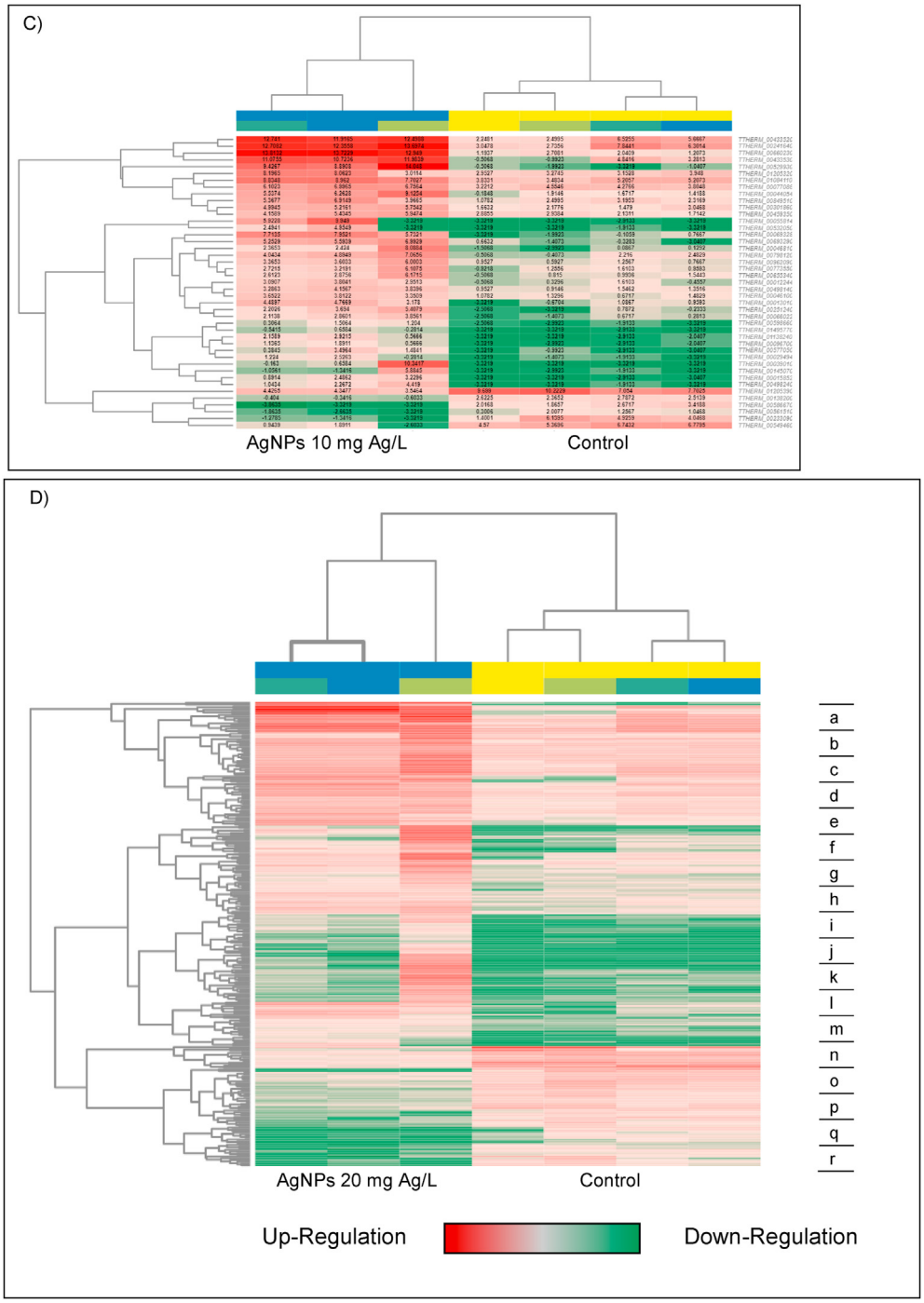

Fig. 3. (*continued*).

dismutases (SOD), catalases (CAT), peroxidases, glutathione, thioredoxin and heat shock proteins is induced by changes in the concentration of reactive oxygen species (ROS) (Koduru et al., 2018; Espinosa-Diez et al., 2015). According to Juganson et al. (2017), AgNPs induced ROS in MilliQ water time- and dose-dependently and AgNPs exposure caused dysregulation of the expression of oxidative stress genes in *T. thermophila* CU428. The DEGs analysis performed in our study confirms these results. The results of the current study suggest that AgNPs induce oxidative stress response in the cells in a concentration dependent way, since, thioredoxin and glutathione reductase family proteins and GPX were significantly up-regulated compared to untreated cells at the higher but not the lower AgNPs concentration (Fig. 5, Table S5). Glutathione S-transferase (GST) genes were up-regulated under the effect of AgNPs at the higher concentration (GST3,4,5,7,8,10,11,19,28, 29,55,57) and, in a few cases, also under the effect of $AgNO_3$ at the higher concentration (GST34 and GST58). These results are also in line with those obtained with *E. vannus* exposed to AgNPs (Pan et al., 2018). Furthermore, the citrate synthase I gene, a rate-limiting enzyme of the tricarboxylic acid (TCA) cycle, that catalyses the condensation of acetyl coenzyme A and oxaloacetate to form citrate (Numata et al., 1991; Kispal et al., 1988), was up-regulated by AgNPs only at the higher concentration (Fig. 4). This finding suggests that the induction of TCA cycle is included in the

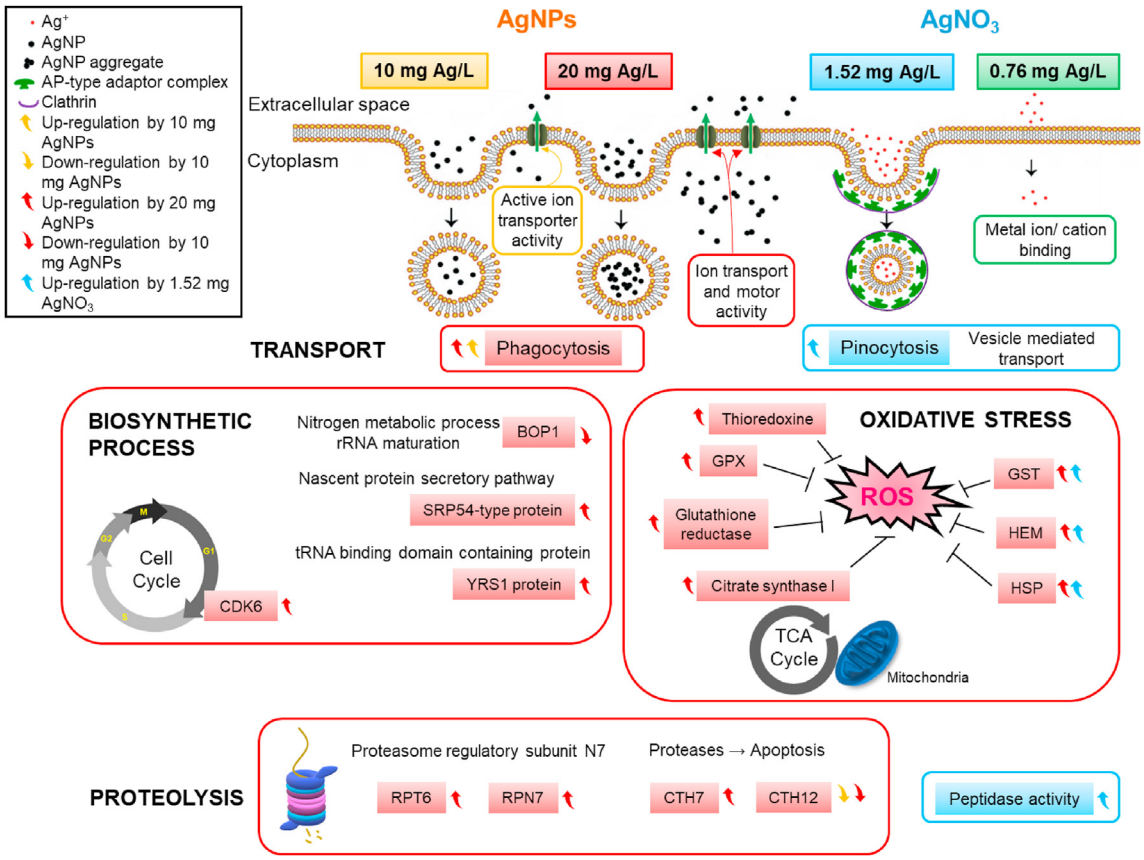

Fig. 4. Schematic representation of biological pathways affected by AgNPs and $AgNO_3$ in *T. thermophila*. Yellow, red, blue and green colours are associated to AgNPs 10 mg Ag/L, AgNPs 20 mg Ag/L, $AgNO_3$ 1.52 mg Ag/L, and to $AgNO_3$ 0.76 mg Ag/L, respectively. The small colored arrows that are oriented up or down indicate up-regulated or down-regulated processes. Their colours are associated to the affected treatments.

attempt to counteract the oxidative stress occurring in the *T. thermophila* cells (Mailloux et al., 2007; Smith et al., 2007).

Tetrahymena hemoglobin is a small oxygen-binding hemoprotein evolved with a truncated structure. Various biochemical functions, in addition to the conventional oxygen transport or storage, have been proposed for this primitive or ancient hemoglobin, but the exact in vivo activity is still unclear (Wittenberg et al., 2002). The HEM1 gene was not only up-regulated by AgNPs stress, but also by $AgNO_3$ at 1.52 mg Ag/L (Fig. 5, Table S5). In addition, also HEM12 gene, which is involved in heme biosynthetic pathway (Chelstowska et al., 2015), was up-regulated under AgNPs stress at the higher concentration (Fig. 4), thus indicating that these genes play an important role in the oxidative stress response.

AgNPs could also activate an inflammatory response, or a response to stimulus. Although the exact mechanism whereby NPs induce pro-inflammatory effects is not known, it has been suggested that phagocytized NPs can induce an inflammatory response, consequently leading to the generation of ROS and reactive nitrogen species. The oxidative stress results in the release of pro-inflammatory mediators or cytokines in higher organisms (Yang et al., 2012).

Interestingly, almost 30% of DEGs were not associated with any gene ontology (GO) term nor with an entry in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and are annotated in the TGD (Stover et al., 2006) as hypothetical or putative proteins, without identified related proteins in other organisms (Figure S8). This means that AgNPs do not only activate known processes such as response to stress, response to stimulus, oxidation-reduction and transport pathways but also regulate other proteins which are not characterized yet. These results open the way to the discovery of new mechanisms of stress response by metals. Considering that *T. thermophila* is a model organism easily manipulated by gene transfection and reverse genetics, it is well suited for the investigation of new gene functions.

3.5. Gene expression validation

To validate the expression data obtained from RNA-seq, 8 mRNAs that showed different expression patterns and are known to be involved in important biological functions were selected for further analysis using RT-qPCR (Figure S9). The RT-qPCR results showed a strong correlation with the RNA-seq analysis ($R^2 = 0.9506$, Fig. 6). For each gene, the expression count values obtained by RT-qPCR for all tested treatments (Figure S10) confirmed the data from RNA-seq.

17S rRNA and HSP705 were used as housekeeping genes. HSP705 was selected as a control housekeeping gene based on previously published data (Juganson et al., 2017), which stated that there was no change in HSP705 expression in *T. thermophila* strain CU428 after 24 h incubation with AgNPs or $AgNO_3$. Also the expression of HSP703, MTT5, and MTT1 was previously quantified by RT-qPCR upon exposure to AgNPs and $AgNO_3$ (Juganson et al., 2017) which allowed us to compare the RT-qPCR results of the previous and current studies with the RNA-seq data of the present study. Both Ag compounds induced significant up-regulation of MTT5. Moreover, MTT1 and MTT4 were up-regulated under these conditions, but with a higher fold change in the AgNPs stressed samples. The five *T. thermophila* metallothioneins (MTTs) are

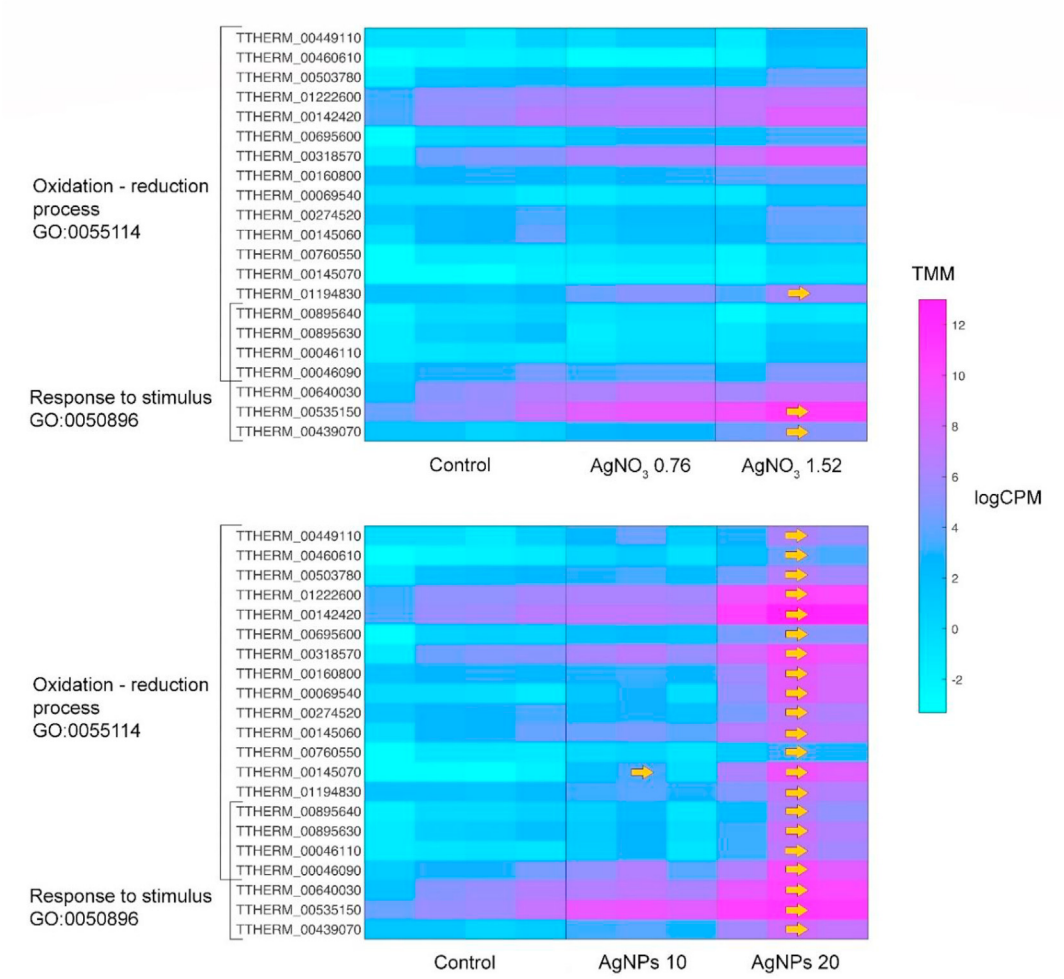

Fig. 5. Heatmaps of logCPM values of differentially expressed genes (DEGs) in two top scored biological processes (oxidation−reduction process and response to stimulus) in *T. thermophila* exposed to AgNPs or $AgNO_3$ or incubated in MilliQ water (control). Yellow arrows indicate genes that are significantly up-regulated with at least 4-fold change. The colour range indicates the gene expression levels from purple (high expression) to light blue (low expression) The gene common names are listed in Table S5.

usually divided in two groups according to the metal binding preferences that they show. MTT4 responds better to copper, while MTT1 and MTT5 (cadmium induced) respond to a more general stress (Diaz et al., 2007; Espart et al., 2015).

Cathepsin 12 (CTH12) DEGs validation confirms that its expression is down-regulated in cells exposed to AgNPs. *T. thermophila* has 125 CTH genes. Two of them, CTH 12 and CTH 7, were among the DEGs in *T. thermophila* exposed to AgNPs at 20 mg Ag/L: CTH12 was down-regulated and CTH7 was up-regulated (Fig. 4). Cathepsins are proteases that, in response to certain signals, are released from the lysosomes into the cytoplasm where they trigger apoptotic cell death via various pathways (Chwieralski et al., 2006). CTH12 is functionally associated to the proteolysis biological process together with other DEGs which were dysregulated upon exposure to AgNPs.

The RPT6 and RPN7 genes encode components of the proteasome regulatory subunit N7 as it is shown in the schematic representation resulting from the KEGG Mapper – Search Pathway tool (Kanehisa et al., 2016; Xiong et al., 2011) (Fig. 4 and Figure S11). These genes were up-regulated only in *T. thermophila* exposed to AgNPs at 20 mg Ag/L, suggesting that such an induction in proteasome regulatory subunit expression is justified by the oxidative damage caused by AgNPs that may produce protein fragments that need to be removed, as this has been hypothesized for *E. vannus* (Pan et al., 2018).

4. Conclusions

This study provides new evidence that AgNP-induced changes in the global gene expression of the protozoan *T. thermophila* are different from the changes induced by $AgNO_3$, suggesting distinct mechanisms of action of the two Ag compounds in the ciliate. This is different from the results of the previous study where a targeted approach was used to assess the expression of a small set of genes in *T. thermophila* in response to AgNPs and $AgNO_3$ exposure and no significant difference was detected between the effects of the two Ag compounds (Juganson et al., 2017). The latter study concluded that AgNPs exerted toxicity *via* Ag ions without causing significant oxidative stress. However, the current study employed a global gene expression analysis approach to provide a more comprehensive understanding on the impact of AgNPs exposure in the ciliate. It is not surprising that the number of DEGs was considerably higher in response to AgNPs than to $AgNO_3$ considering that the main feeding mode of *T. thermophila* is phagocytosis of suspended particulate matter (Mortimer et al., 2014b). The current study identified a wide range of dysregulated pathways in the AgNP-exposed ciliate. AgNP-specific changes in cellular processes included phagocytosis, glutathione peroxidase activity, response to

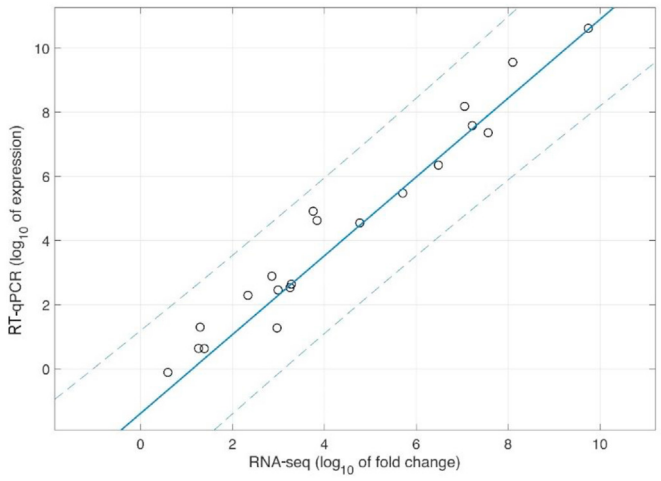

Fig. 6. Correlation between RT-qPCR and RNA-seq results for 7 up-regulated selected genes. Each gene is analysed in two or four treatments, each represented by one dot. Details of the treatments are in Figure S10. $R^2 = 0.9587$, with prediction bounds of 95%.

stimulus, protein phosphorylation and nitrogen metabolism process. Since AgNPs suspensions also contained dissolved Ag, there was expectedly some overlap in the induced gene expression changes with *T. thermophila* exposed to $AgNO_3$ at the concentration equal to dissolved Ag in AgNPs suspension. Specifically, both Ag compounds upregulated oxidative stress-related pathways. However, the overall transcriptional response to $AgNO_3$ was less pronounced than in the case of AgNPs. This may be explained by the ability of the ciliate to detoxify $AgNO_3$ by reducing Ag ions into metallic Ag which then agglomerate into nano- or microsized particles (Juganson et al., 2013). Since engineered AgNPs are commonly stabilized by a surface coating (casein in the current study) to avoid extensive agglomeration or fast dissolution, AgNPs may act as long-term sources of Ag ions, increasing Ag toxicity in freshwater environments. In addition, once taken up into the phagosomes, various physico-chemical transformations may affect the toxicity of AgNPs. Thus, based on the global gene expression analysis conducted in this study, NPs risk assessment requires special scrutiny and cannot be predicted based on the metal ion effects.

Further investigations of the genes affected at different times of exposure would be helpful to characterize time-dependent gene expression responses. Moreover, considering that previous analyses (Juganson et al., 2017) suggested differences in gene expression among *T. thermophila* strains, and variability likely occurs also at an individual cell level, further investigation applying new techniques of single cell transcriptomics would help to contribute to the understanding of the toxic effects of AgNPs.

The results reported here also validated the protocol of DEGs analysis as very suitable for detecting genes involved in stress response (both inhibited and induced). Genes specifically induced by AgNPs or $AgNO_3$ can be further studied to identify their transcriptional promoters, which would allow the development of biosensors by fusing the promoters to reporter genes.

Well characterized and standardized AgNPs were used to provide a good reference for future work. Recent studies highlight the capacity of many organisms, specifically that of bacteria to produce NPs when exposed to soluble silver ions (Koduru et al., 2018). In any future study it would be useful to compare the results of this work with those obtained by the exposure of the same model organism to biologically produced AgNPs.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.115965.

Credit author statement

Angela PIERSANTI: Conceptualization, Methodology, Data curation, Writing- Original draft preparation, Writing- Reviewing and Editing; **Katre JUGANSON :** Conceptualization, Methodology**; Matteo MOZZICAFREDDO :** Software, Validation, Formal analysis, Data curation, Writing- Reviewing and Editing**; Wei WEI, Jing ZHANG, Kangping ZHAO, Patrizia BALLARINI**: Visualization, Investigation. **Wei MIAO, Monika MORTIMER, Sandra PUCCIARELLI**: Supervision, Writing- Reviewing and Editing; **Cristina MICELI** Conceptualization, Methodology, Supervision, Writing-Reviewing and Editing

Statement

We identify for the first time the pathways affected by AgNPs in a freshwater eukaryotic unicellular model at transcription level and contribute to the understanding of AgNPs toxicity.

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