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**Proteomic analyses of early response of unicellular eukaryotic microorganism
Tetrahymena thermophila exposed to TiO₂ particles**

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List of abbreviations

2-DE: two-dimensional gel electrophoresis; CBB G-250: Coomassie Brilliant Blue G250;

DLS: dynamic light scattering; nLC-ESI-MS/MS: nano-scale liquid chromatography-electrospray ionization tandem mass spectrometry; R_h : hydrodynamic radius of colloidal particles; $g_1(t)$: correlation function of the scattered electric field; D : diffusion coefficient; q :

scattering vector; MAPK: Mitogen-Activated Protein Kinase; ERK: signal-regulated kinase;

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF:

phenylmethanesulfonyl fluoride or phenylmethylsulfonyl fluoride; TCA: trichloroacetic acid;

DTT: dithiothreitol; CHAPS: 3-[(3-Cholamidopropyl)dimethylammonio]-1-

propanesulfonate; IAA: Iodoacetamide; SDS: Sodium Dodecyl Sulfate.

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19 **Abstract**

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Key biological functions involved in cell survival have been studied to understand the difference between the impact of exposure to TiO₂ nanoparticles (TiO₂-NPs) and their bulk counterparts (bulk-TiO₂). By selecting a unicellular eukaryotic model organism and applying proteomic analysis an overview of the possible impact of exposure could be obtained. In this study we investigated the early response of unicellular eukaryotic protozoan *Tetrahymena thermophila* exposed to TiO₂-NPs or bulk-TiO₂ particles at subtoxic concentrations for this organism. The proteomic analysis based on 2DE + nLC-ESI-MS/MS revealed 930 distinct protein spots, among which 77 were differentially expressed and 18 were unambiguously identified. We identified alterations in metabolic pathways including lipid and fatty acid metabolism, purine metabolism, and energetic metabolism, as well as salt stress and protein degradation. This proteomic study is consistent with our previous findings (Rajapakse et al., 2012), where the early response of *T. thermophila* to subtoxic concentrations of TiO₂ particles included alterations in lipid and fatty acid metabolism and ion regulation. The response to the lowest TiO₂-NPs concentration differed significantly from the response to higher TiO₂-NPs concentration and both bulk-TiO₂ concentrations. Alterations on the physiological landscape were significant after exposure to both nano- and bulk-TiO₂; however no toxic effects were evidenced even at very high exposure concentrations. This study confirms the relevance of the alteration of the lipid profile and lipid metabolism in understanding the early impact of TiO₂-NPs in eukaryotic cells, e.g., phagocytosing cells like macrophages and ciliated cells in the respiratory epithelium.

44 Introduction

45 Holistic approaches, based on OMICs methodologies, can facilitate the study of molecular
46 mechanisms of interactions between different chemical, physical or biotic agents and cells
47 (Matranga, 2012). Nanoproteomics has easily found its way into the studies of nanotoxicity
48 and some new mechanisms of interaction are expected to be revealed (Ray, 2010; Tilton,
49 2014).

50 Indeed, in the case of exposure to TiO₂ nanoparticles (TiO₂-NPs), conventional *in vitro* and *in*
51 *vivo* studies provided evidence of oxidative stress, cytotoxicity, inflammation, and a series of
52 toxic responses (Ferin, 1992; Park, 2008; Monteiller, 2007; Johnston, 2009). Applying
53 proteomics to *in vitro* studies with BEAS-2B cell lines exposed to TiO₂-NPs has revealed a
54 novel antioxidant defense pathway or mechanism mediated by MAPK/ERK activated Nrf-2
55 signaling (Ge et al., 2011). In our previous study, we performed integrated OMICs approaches
56 including the proteomic and lipidomic analysis of *in vitro* primary mouse hepatocytes
57 exposed to TiO₂- ultra small NPs. Our data from hepatocytes also indicated an alteration in
58 lipids, such as a decrease of sphingomyelin as well as the up-regulation of ATP synthase and
59 electron transferring protein alfa (Tedesco, 2015). The proteomic analysis of BEAS-2B cell
60 lines exposed to TiO₂-NPs has shown that the proteomic analysis could reveal a novel
61 antioxidant defense pathway or mechanism mediated by MAPK/ERK activated Nrf-2
62 signaling (Ge, 2011). *In vivo* studies, analyzed by quantitative proteomics, have revealed the
63 early response in mice lymph nodes after TiO₂-NPs injection modulating the expression of
64 proteins involved in immune response and nucleosome assembly (Gao, 2011). More recently,
65 a quantitative proteomic analysis of the unicellular **eukaryotic** organism *Tetrahymena*
66 *thermophila* has revealed a deeper understanding of histone methyltransferases (Zhang, 2014)
67 or protein phosphorylation networks (Tian, 2013). Most of these effects could not be
68 elucidated using a conventional non-omic approach.

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69 In this paper, we present the complementary study to our previous work on the effects of the
70 unicellular eukaryotic organism *T. thermophila* on TiO₂-NPs (Rajapakse, 2012). The
71 aforementioned study provided evidence that neither TiO₂-NPs nor bulk TiO₂ particles at
72 concentrations up to 1000 µg/ml provoke adverse effects as assessed by the reactive oxygen
73 species ROS generation or lipid peroxidation. However, changes of the membrane fatty acid
74 profile were evident (Rajapakse, 2012).

75 The aim of the study presented here is to elucidate the early response of *T. thermophila* to
76 TiO₂-NPs by proteomic analysis. The genome of *T. thermophila* has been sequenced
77 (Tetrahymena Genome Database; www.ciliate.org), and its mitochondrial proteome (545
78 proteins) (Smith, 2007), phagosome proteome (73 proteins) (Jacobs, 2006), and ciliome
79 proteome (223 cilia proteins) (Smith, 2005) all well studied. In addition, *T. thermophila*
80 shares a higher degree of functional conservation with human genes than other microbial
81 eukaryotic microorganisms (Turkewitz, 2002). Therefore, it is an organism of choice for
82 studying the effects of particles at both the cellular level and the level of the organism.

83 Based on the results of our previous lipidomic research, where the membrane structure of *T.*
84 *thermophila* was altered but no toxic effects were evidenced (Rajapakse, 2012), we expect to
85 find alterations in fatty acid metabolisms also by proteomic tools. In this paper, we present the
86 quantitative analysis of the subproteom after exposure to TiO₂-NPs or bulk-TiO₂, where most
87 of the key proteins that regulate fatty acid synthesis as well as some responsible for energetic
88 metabolism are localized. We compare the data obtained with lipidomic and proteomic tools
89 and discuss the early response to TiO₂-NPs at subtoxic concentrations.

92 **Methods and materials**

93 **Chemicals**

Unless otherwise specified, reagents were purchased from Sigma Aldrich Co (St. Louis, MO, USA), Merck (Darmstadt, Germany) or Biolife (Milan, Italy). TiO₂-NPs with 99.7% purity were supplied in the form of a powder with an anatase crystalline structure, an average particle size of 15 nm and a surface area of 190 to 290 m²/g from Sigma Aldrich Co (St. Louis, MO, USA). Bulk-TiO₂ was purchased from Merck KGaA (Darmstadt, Germany).

Dynamic light scattering (DLS)

Dynamic light scattering (DLS) measurements were used to determine the hydrodynamic radius, R_h , of particles in suspensions. Methodological aspects of the DLS technique (Schärtl, 2007) are reported in Supporting Information. Measurements were conducted using a 3D cross-correlation spectrometer from LS Instruments GmbH (Fribourg, Switzerland) at 25 °C. The general idea behind the 3D cross-correlation mode is to suppress undesired contributions from multiple scatterings frequently encountered in practical systems (Urban, 1998). For this purpose, 2 coherent incident light beams are generated and the scattered light is collected by two detectors. The two scattered beams are then correlated with each other. DLS measurements were conducted at an angle of 90° using a He-Ne laser (Uniphase JDL 1145P, operating at a wavelength $\lambda_0 = 632.8$ nm) after equilibrating the samples at 25 °C for 15 minutes. 5 intensity correlation functions were collected at each angle and averaged. Each curve was analyzed independently and compared with the averaged curve to ensure accuracy of the mathematical solution. The powdered bulk-TiO₂ and TiO₂-NPs were dispersed in poor medium (PM) (Rajapakse, 2012), each at a concentration of 200 µg/ml, using an ultra-sound water bath for 30 minutes to disperse particle agglomerates. The stock suspensions were diluted in PM to 0.2 µg/ml and sonicated for 30 minutes. After sterilization at 121 °C and 1.1 bar for 20 minutes, 20 ml of the 200 µg/ml and 0.2 µg/ml bulk-TiO₂ and TiO₂-NPs stock suspensions were diluted in 20 ml sterile PM to make 40 ml of bulk or nanoparticle suspensions with final concentrations of 100 µg/ml and 0.1 µg/ml. The suspensions were sonicated for 30 minutes and shaken in the incubator at 32 °C for 24 hours at 90 rpm. The control suspensions were not incubated with TiO₂ particles. Because the

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suspensions were highly turbid, which prevented an accurate DLS analysis, they were diluted 1000-
times and analyzed without being filtered. Samples were analyzed before and after the exposure.

Zeta potential

Bulk-TiO₂ and 15 nm TiO₂-NPs were dispersed in a poor medium (PM) (Rajapakse, 2012),
which was adjusted with modifications (Schultz, 1997). A 30-minute bath sonication was used
to disperse particle agglomerates in stock solutions. The Zeta potentials of TiO₂-NP
suspensions (1000 µg/ml) were measured with ZetaPals (Brookhaven Instrument Corporation)
in the PM, which was used for the exposure to living cells.

ICP-MS analysis

Inductively coupled plasma-mass spectrometry (ICP-MS, Agilent 7500ce inductively coupled
plasma mass spectrometer for lower concentrations and ICP-OES, Varian 715-ES ICP
inductively coupled plasma optical emission spectrometry for higher concentrations) was used
to analyze the amount of Ti in the suspension of TiO₂-NPs particles.

The sample preparation for ICP-MS analysis is as follows: TiO₂ sample solutions were
dissolved in 10 mL of deionized Milli-Q water and 5 mL of concentrated H₂SO₄. After
dissolution the sample solutions were diluted to the mark with deionized Milli-Q water.
Because samples (TiO₂) were not completely dissolved in H₂SO₄, the samples were filtered
(paper filters, blue ribbon) and filters with precipitation transferred into the Pt crucibles. After
that the filters were dried and carefully ashed at 900 °C. After ashing, the amount of 2g of
KHSO₄ was added into the crucibles and fused (at about 900 °C). Fused samples were than
dissolved in 20 mL of diluted HCl (in a ratio 1:1 with deionized Milli-Q water) and gently
heated and mixed with magnetic stirrer during dissolution. When the fused samples were
dissolved, the solutions were transferred into to the 50 mL volumetric flasks and filled with
deionized Milli-Q water to the mark. Titanium was than measured in both solutions – in the

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3 146 solutions obtained in the first step of the procedure after dissolving in H₂SO₄ and in the
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5 147 second step in the solutions obtained by dissolving the fused samples. The final results
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7 148 represent the sum of both.
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11 150 TEM investigation

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14 151 For transmission electron microscopy (TEM) investigations, the materials were deposited by
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16 152 drying their water suspensions on a copper-grid-supported transparent carbon foil. The TEM
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18 153 analysis was performed with a JEOL 2100 microscope operated at 200 kV.
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21 155 *T. thermophila* growth and exposure conditions

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24 156 The axenic cultures of *T. thermophila* from the Protoxkit FTM (MicroBioTests Inc.) were
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26 157 grown for 24 hours in the dark at 32 °C in a semi-defined-proteose-peptone based rich
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28 158 medium (RM) (Schultz, 1997).
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31 159 The cells were harvested, washed, and resuspended in a poor medium (PM), which consisted
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33 160 of the semi-defined proteose-peptone based medium used by Schultz (Schultz, 1997), but
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35 161 lacking yeast extract and bacteriological peptone. The pH of the medium was adjusted to 7.4
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37 162 and the temperature to 32 °C for the entire experiment. All experiments were performed in
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39 163 100 ml batch cultures that were maintained in Erlenmeyer flasks and aerated by shaking at 90
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41 164 rpm in an incubator in the dark.
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45 165 After 1 hour in PM the cells were exposed to suspensions of bulk- or TiO₂-NPs. The final
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47 166 nominal concentration of particles in the medium, either bulk or nano-, was 0.1 and 100
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49 167 µg/ml. *T. thermophila* cultures were incubated at 32 °C for 24 hours. For each concentration
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51 168 of bulk- or TiO₂-NPs we used five independent replicates. A set of five replicates without
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53 169 TiO₂ was set up as a control for each assay.
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Two-dimensional gel electrophoresis and image analysis

After 24 hours, five replicate treatments with either i) no added TiO₂ particles, ii) bulk-TiO₂, or iii) TiO₂-NPs; 100 ml of cell suspension was harvested for the purpose of cell fractionation, which was slightly adapted by Guillemin and co-workers (Guillemin, 2005). Deeply frozen cells were homogenized in an ice-cold homogenization buffer containing 10 mM HEPES, 10 mM NaCl, 1 mM KH₂PO₄, 5 mM NaHCO₃, 5 mM EDTA, 1 mM CaCl₂, 0.5 mM MgCl₂, and the following protease inhibitors: 0.2 mM PMSF, 2 μM leupeptin, 2 μM pepstatin, and 1 mM ε-aminocaproic acid. Thereafter, 50 μl of 2.5 M sucrose was added to restore isotonic conditions. The homogenates were centrifuged at 6300 x g for 10 minutes to precipitate the pellet of the cell debris and nuclei. The supernatant was sedimented at 18000 x g for 200 minutes in a tabletop centrifuge. The Guillemin protocol was changed to increase cytosol purity by increasing the S1 centrifugation from 14000 g to 18000 g. Only the primary structured solubilized proteins remained in the sample due to the solubilization buffer step including a centrifugation at 1950 g pelleting down unsolubilized proteins. The subproteome is the same in all samples and consistent. The supernatant represented the cytosolic fraction, which was precipitated by 20% TCA and 100% cold acetone with 0.07% (v/v) β-mercaptoethanol. Proteins extracted by this method were solubilized in a solubilization buffer (7 M urea, 2 M thiourea, 4% CHAPS (w/v), 0.5% Triton X-100, 1% β-mercaptoethanol (v/v), 0.2% IPG buffer (3-10), 1% DTT (w/v), modified by Rabilloud (1998)). Afterwards, samples were alkylated with 30 mM IAA for 15 minutes in the dark and then mixed with the rehydration solution containing 8 M urea, 2% CHAPS (w/v), 15 mM DTT, 1% β-mercaptoethanol (v/v) and 1% IPG buffer (3-10), 1% DTT (w/v), modified from Rabilloud (1998). Afterwards, samples were alkylated with 30 mM IAA for 15 minutes in darkness and then mixed with rehydration solution containing 8 M urea, 2% CHAPS (w/v), 15 mM DTT, 1% β-mercaptoethanol (v/v) and 0.2 IPG buffer (v/v) (3-10). Solubilized

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3 196 samples were applied onto 11 cm IPG strips, pH 3-10NL (BioRad, Hercules, CA). Protein
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5 197 concentrations were measured according to Bradford (Bradford, 1976) using bovine serum
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7 198 albumin as a standard. The total protein applied per gel was 200 µg. Isoelectric focusing was
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9 199 performed on a Protean IEF Cell (BioRad) at 20 °C using the following program: passive
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11 200 rehydration for 12 hours, rapid voltage slope for all the steps, step 1: 250 V for 15 minutes,
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13 201 step 2: 8000V for 2.5 hours and step 3: at 8000 V until it reached 35000 Vh. After this first
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15 202 dimension protein separation the IPG strips were reduced (1% DTT (w/v)) and were then
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17 203 alkylated (4% IAA (w/v)) in an equilibration buffer containing 6 M urea, 50 mM Tris pH 8.8,
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19 204 30% glycerol (v/v), 2% SDS (w/v) and 0.002% CBB (w/v). The second dimension was
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21 205 carried out on homogeneous 12.5% T Criterion precast gels (Bio-Rad, Hercules, CA), at 120
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23 206 V for 2 hours using a Criterion Dodeca Cell (Bio-Rad). The second dimension was carried out
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25 207 on homogeneous 12.5% T Criterion precast gels (Bio-Rad, Hercules, CA), at 120 V for 2
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27 208 hours using a Criterion Dodeca Cell (Bio-Rad).
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29 209 The protein spots in the gels were visualized by staining with Coomassie Brilliant Blue G250
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31 210 (CBB G250), and gel images were obtained using the Image Scanner (GE Healthcare,
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33 211 Uppsala, Sweden). The Image Master 2D Platinum 6.0 software (GE Healthcare) was used for
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35 212 matching and analyzing visualized spots among differential gels to compare the level of
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37 213 protein expression between the cytoplasm of non-treated cultures of *T. thermophila* and
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39 214 nano- or bulk-TiO₂ treated cultures. The total protein amount was measured by loading
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41 215 alkylated and soluted proteins 200 µg/185 µL to a 11 cm IEF strip to normalize between
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43 216 samples.
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52 218 **Protein identification by nanoscale liquid chromatography-electrospray ionization**
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54 219 **tandem mass spectrometry (nLC-ESI-MS/MS) and database searching**
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The protein identification was performed by the nLC-ESI-MS/MS analysis and the *de novo* sequencing of the peptides produced by the proteolytic digestion of the excised spots from the 2-DE maps. The selection of spots is described in greater detail in the results section. The selected spots were excised from the gels and treated as described in the UCSF In-Gel Digestion Protocol (UCSF, 2009). Afterwards, the peptides were extracted, desalted and concentrated using the reversed-phase C18 solid phase extraction (TopTip, Glygen Corp., Columbia, USA), according to the manufacturer's protocol but with slight modifications. The peptides were loaded on the TopTip, pre-conditioned with a washing solution (50% of acetonitrile) and a binding solution (0.1% of formic acid). The peptides were then eluted with the washing solution (50% acetonitrile/0.1% formic acid), twice 35 μ L, dried and resuspended in 6 μ L of washing solution for the nLC-MS/MS analysis, which was performed with an on-line nano-flow HPLC system (EASY-nLC; Proxeon, Bruker Daltonics), coupled to a mass spectrometer HCTultra PTM Discovery System (Bruker Daltonics) and equipped with an electrospray ionization source. The peptides were eluted at a flow of 300 nL/min using a C18 reversed-phase pre-column (20 mm $\times$ 100 μ m, 5 μ m particle size, NanoSeparation, The Netherlands) and an analytical column (100 mm $\times$ 75 μ m, 5 μ m particle size, NanoSeparation, The Netherlands). Separation was achieved with a linear gradient of (A) water and formic acid (99.9%/0.1%, v/v) and (B) acetonitrile and formic acid (99.9%/0.1%, v/v) for 60 minutes as follows: first 0–100% B for 40 minutes, then 100% B for 20 minutes. A volume of 4 μ L was injected. The MS analysis was performed by the data dependent acquisition and exclusion of the MS² precursor for 1 minute (MS full scan within the range of 50–3000 m/z and MS² within the range 200–1500 m/z). The automated online tandem MS fragmentation of peptide ions was performed using alternating collision induced dissociation (CID) and electron transfer dissociation (ETD). The Data Analysis software (Bruker) was used to analyze the ESI-MS/MS spectra and perform a Mascot search, using the NCBI nr database for

protein identification. The Mascot search was done with the following settings: (i) taxonomy: other Alveolata; (ii) enzyme: trypsin allowing up to 2 missed cleavages; (iii) fixed modification: carbamidomethyl (c); (iv) variable modifications: acetyl (N-term) and oxidation (M); (v) peptides tolerance: 0.8 Da; (vi) MS/MS tolerance: 0.8 Da; (vii) peptide charges: 1+ 2+ and 3 + for CID and 2+ 3+ and 4+ for ETD); and (viii) instrument: ESI-trap. The identified peptides were then assembled to identify proteins, using a scoring scheme that measures their similarity – the one with the best score was designated as an identified protein. The peptides were considered reliable for identification if the MS/MS spectra had a Mascot score above 35 and an expected value below 0.01 (Ingelsson, 2012). In addition, we conducted the STRING network v10 (Szklarczyk et al. 2015) analysis of the 11 differentially expressed proteins with predicted network in evidence view, default settings and *T. thermophila* for species.

Statistical analysis

The experiment with five treatments (control; nano-group: 0.1 µg TiO₂-NPs/ml and 100 µg TiO₂-NPs/ml; bulk group: 0.1 µg bulk-TiO₂/ml and 100 µg bulk-TiO₂/ml) was conducted in five replications (five gels for each treatment). Hierarchical clustering, using Euclidean distance and Ward's method, was used as the main exploratory statistical analysis to detect groups of gels with similar protein expressions. A heatmap with dendrograms was used for the graphical presentation of the results, showing the relative expressions of spots for each gel. On the heatmap, the gels and the spots are reordered according to two dendrograms yielded by the chosen clustering method. A one-way ANOVA with Dunnett's multiple comparison tests with the probability value $p < 0.05$ considered significant was used to detect spots in which treatments with nano- and bulk-TiO₂ at two different concentrations statistically significantly differ in their expressions compared to the control group. The statistical analysis was done with the R program (R Core Team, 2013).

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Results

Characteristics of NPs in a suspension

Concentrations

The tested concentrations were selected on the basis of our previous studies and are considered as subtoxic concentrations for TiO₂-NPs for *T.termophila*. The nominal exposure concentrations were 0.1 µg/ml and 100 µg/ml of TiO₂-NPs. The ICP-MS analyses revealed that at a higher exposure concentration, the Ti concentration is in the expected range (63.1±32 Ti µg/ml). In the case of a lower exposure concentration, the analyses showed much lower concentrations of Ti than expected (0,013±0,008 µg/ml of Ti). The measured values in the case of a lower exposure concentration are within the expected environmental concentrations of TiO₂-NPs (Lou et al., 2014).

Primary particle size and shape

According to TEM, the TiO₂-bulk sample represents single-crystal particles with sizes ranging from approximately 50 to 300 nm (Fig. 1a). Electron diffraction revealed an anatase structure. HREM imaging confirmed good crystallinity of the particles, although a thin amorphous layer was frequently present at their surfaces (inset of Fig. 1b). The TiO₂-NPs sample deposited at the TEM-specimen support in the form of compact agglomerates of elongated nanoparticles, with sizes ranging between approximately 10 and 30 nm. In the same sample agglomerates of much smaller nanoparticles with sizes below 6 nm were also present (Fig. 1a). HREM (see inset of Fig. 1b) and electron diffraction showed that the nanoparticles were crystalline, with an anatase structure.

Size distribution

The measured $g_2(t)$ functions (for details on the correlation functions that are measured by DLS see SI) are shown in Figure 2 and the calculated size distributions are plotted in Figure 3. Clearly, two populations of particles are present in the suspensions, yielding two average R_h values, designated as $R_{h,1}$ and $R_{h,2}$, respectively. The average $R_{h,1}$ and $R_{h,2}$ values are reported in Table 1. The $R_{h,2}$ values for very large species (presumably aggregates) are only rough estimates, because their size is too large for a reliable determination with the laser light wave length used in this study (633 nm). The average value of the hydrodynamic radius of smaller particles, $R_{h,1}$, is 150 and 340 nm for 1N0 and 1B0 (1N0 = TiO₂-NPs concentration 0.1 µg/ml at 0h, 1B0 = bulk-TiO₂ concentration 0.1 µg/ml at 0 h), respectively, and decreases to 120 and 70 nm after 24 hours (see samples designated as 1N24 and 1B24 (1B24 = bulk-TiO₂ concentration 0.1 µg/ml at 24 hours, 1N24 = TiO₂-NPs concentration 0.1 µg/ml at 24 hours). Although average $R_{h,1}$ values in 1N suspensions seem to be somewhat lower after 24 hours, size distributions do not change considerably, only the number of aggregates decreases and the number of smaller species increases with time (Fig. 3). This is different with 1B suspensions, where $R_{h,1}$ is clearly lower after 24 hours, whereas $R_{h,2}$ is clearly higher. The aggregates in suspensions are very large ($R_{h,2}$ is considerably larger than 1000 nm) and therefore contribute significantly to the total scattering intensity. However, their number at the investigated concentrations is small (note that samples were diluted 1000-times prior to the DLS measurements). The predominant species in suspensions are particles with the size $R_{h,1}$. The electromobility measurements showed negative zeta potentials for all the suspended NMs (Table 2). Some difference in zeta potential could be seen only in the case of the lowest concentration of TiO₂-NPs.

Protein expression pattern of *T. thermophila* cytosolic proteins exposed to TiO₂ particles

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Applying a gel-based quantitative proteomics approach, we explored nano- and bulk-TiO₂ particle induced changes in the proteome of *T. Thermophila* after a 24-hour exposure to those particles at two different concentrations (0.1 µg/ml and 100 µg/ml). The cytoplasmic protein extracts were analyzed by 2D-DIGE and quantified by the imaging software and the selected differentially expressed proteins were identified by nLC-ESI-MS/MS. By using Coomassie brilliant blue staining, approximately 930 protein spots were detected on the 2-DE maps. Figure 4 shows representative 2-DE images of one (out of five replicates) of the *T.thermophila* in a control group and a group exposed to TiO₂ particles (0.1 µg TiO₂-NPs/ml, 100 µg TiO₂-NPs /ml, 0.1 µg bulk-TiO₂/ml, 100 µg bulk-TiO₂/ml).

Results of statistical analysis

On average, 600 spots were detected in at least four out of five replications (gels) per treatment. The cluster analysis of 25 gels, with the data for 600 spots (Fig. 5), showed two groups of gels: one group agglutinated the gels corresponding to cultures treated with 0.1 µg TiO₂-NPs/ml and in the second group gels from all the other treatments. Up to 266 spots were detected in five control gels as well as in at least one other treatment. The statistical analysis based on the one-way ANOVA revealed 94 spots that were differentially expressed at a significance level of 0.05, while further analysis with the Dunnett’s test showed 77 spots differentially expressed in comparison to the control gels. A heatmap analysis was performed on these spots (Fig. 6), which gave very similar results to the cluster analysis on the 600 spots: one group of gels from cultures treated with TiO₂-NPs at 0.1 µg/ml and a second group of gels from all the other treatments. Among the 77 spots selected as described above, we chose 18 for identification. Our selection of spots for identification was based on two characteristics of spots: a) their differential expression in comparison to the control in all four TiO₂ treatments (0.1 µg/ml and 100 µg/ml

344 nano- or bulk-TiO₂); b) a statistically significantly different expression in at least one of these
345 TiO₂ treatments when compared to the controls.

346 In this paper, we present also the STRING network analysis of 11 differentially expressed
347 proteins with predicted network in evidence view between triosephosphate isomerase
348 (ERA88549, up-regulated), enolase (EAR85197, down-regulated) and DNAk (EAR82836,
349 down-regulated) with 75% consistency for different NP exposures. Not the predicted
350 connections but consistent differentially expressed proteins over different sorts of NP
351 exposures were triosephosphate isomerase EAR88549, up-regulated, S-adenosylmethionine
352 synthetase family protein EAS03064, up-regulated, aconitate hydratase, EAR96511, down-
353 regulated, proteasome A-type and B-type family protein EAR85260, down-regulated, enolase
354 family protein EAR85197, down-regulated, peptidase family M20/M25/M40 containing
355 protein EAR97423, down-regulated, and phospholipase/carboxylesterase family protein
356 EAR82795, up-regulated. The inconsistency whether the differential expression was an up- or
357 down-regulation in the different NP exposures were purine nucleoside phosphorylase I
358 EAS00727, oxidoreductase EAR82825, aldo/keto reductase family protein EAR82825, Acyl-
359 CoA dehydrogenase EAR85826; the specific up- or down-regulation values are shown in
360 Supplementary material Figure S1 and Table 3.

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362 Identification of proteins

363 The differentially expressed spots selected for identification were: i) common to all four TiO₂
364 treatments (0.1 µg/ml and 100 µg/ml nano- or bulk-TiO₂) in comparison to the control; ii)
365 statistically significant in at least one of these TiO₂ treatments in comparison to the control.
366 We have identified two additional proteins that were differentially expressed only in TiO₂-
367 NPs treatments but not in bulk-TiO₂ treatments, in comparison to the control gels. The
368 expression levels of all identified proteins are shown in Figure 7.

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The identified proteins are related to lipid and fatty acid metabolism (Acyl-CoA dehydrogenase, triosephosphate isomerase), glucose metabolism (enolase family protein, aconitate hydratase, triosephosphate isomerase), Fe²⁺ homeostasis (aconitate hydratase), salt stress (S-adenosylmethionine synthetase family protein, peptidase family M20/M25/M40 protein, DnaK protein), protein degradation (Proteasome A-type and B-type family protein), purine metabolism (purine nucleoside phosphorylase I, inosine and guanosine-specific family protein), and metabolism *per se* (Phospholipase/Carboxylesterase family protein, oxidoreductase-aldo/keto reductase family protein) (Table 3).

Discussion

We examined the early response of eukaryotic protozoan *T. thermophila* exposed to TiO₂-NPs or bulk-TiO₂ at subtoxic concentrations by employing subcellular proteomics. At selected concentrations, no elevation of ROS content or lipid peroxidation occurred (Rajapakse, 2012). We used the cytosolic proteome and not the entire cell proteome. The cytosolic proteome research alone is something that had previously not been conducted in nanotoxicity studies. On average, we have detected 930 spots per gel (treatment), among which altogether 77 spots differed significantly in expression levels from those in the control samples. We selected 16 protein spots out of the 77, where the expression level was most significantly up- or down-regulated in comparison to control group (Fig. 7, Table 3). In addition, we identified two proteins out of these 77 that were differentially expressed only in TiO₂-NPs treatments, namely Acyl-CoA dehydrogenase and the DnaK protein (Table 3). The identified proteins are related to lipid and fatty acid metabolism (Acyl-CoA dehydrogenase, triosephosphate isomerase), glucose metabolism (enolase family protein, aconitate hydratase, triosephosphate isomerase), Fe²⁺ homeostasis (aconitate hydratase), salt stress (S-adenosylmethionine synthetase family protein, Peptidase family M20/M25/M40 protein, DnaK protein), protein

degradation (Proteasome A-type and B-type family protein), purine metabolism (purine nucleoside phosphorylase I, inosine and guanosine-specific family protein), and metabolism *per se* (Phospholipase/Carboxylesterase family protein, oxidoreductase - aldo/keto reductase family protein) (Table 3). Interestingly, the proteome of *T. thermophila* exposed to the low concentrations of TiO₂-NPs (0.1 µg nano-TiO₂/ml) differed from all other treatments (i. e. with a higher concentration of TiO₂-NPs and both concentrations of bulk-TiO₂) (Fig. 4, Fig. 5). The identified proteins were distributed into seven groups according to their function. The first group of proteins, the amount of which was significantly altered due to NP exposure, is involved in lipid and fatty acid metabolism. One of them, the mitochondrial flavoenzyme acyl-CoA dehydrogenase, catalyzes the initial step in fatty acid β-oxidation (GO:0016627). It was up-regulated in two different spots on the gels (Fig. 3, Fig. 6, Table 3). Although these two are mitochondrial proteins, their presence in the cytosolic fraction is expected because the synthesis of mitochondrial matrix enzymes takes place in cytosol (Hay, 1984). The cytosolic increase of acyl-CoA dehydrogenase could also be a consequence of an increase of peroxisomal activity where longer fatty acid chains, such as endocrine signaling prostaglandin and inflammatory signaling leukotriene, are metabolized. The changes in lipid and fatty acid metabolism were also described by Gao et al. (Gao, 2011) and Tilton et al. (Tilton, 2014) as an early response to TiO₂-NPs using the proteomic approach. Alterations in lipid and fatty acid metabolism are in line with our previous study, where a significant change in long chain fatty acid profiles was observed after the exposure of *T. thermophila* to TiO₂ particles (Rajapakse, 2012), and a study conducted by Mortimer et al. (Mortimer, 2011) testing Cu nanoparticles. These authors used lipidomic tools in testing Cu nanoparticles. In addition, the fatty acid β-oxidation pathway was also found significantly up-regulated in *E.coli* after having been exposed to TiO₂-NPs (Sohm et al. 2015). They explained the increased production of acetyl-CoA to be a protective mechanism against free fatty acids released from the altered

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membrane. In line with our previous lipidomic studies on *T. thermophila* and data on *E.coli* provided by Sohm et al. (2015), we also explain the alterations in fatty acid synthesis and degradation as being related to the membrane lipid profile. In addition, studying mouse liver, Jeon et al. (2011) discovered that TiO₂-NPs nanoparticles have the effect of up-regulating isovaleryl-CoA dehydrogenase, a member of the acyl-CoA dehydrogenase family.

Another protein involved in lipid and fatty acid metabolism, is triosephosphate isomerase. In addition to its role in glycolysis (discussed below), this enzyme is also involved in multiple metabolic processes, including gluconeogenesis, the pentose phosphate shunt, and fatty acid biosynthesis (Triosephosphate isomerase, 2014) (Menendez, 2007).

The second group of proteins, where the level of expression was significantly altered due to NP exposure, is composed of three glucose metabolism related proteins (the enolase family protein, aconitate hydratase, triosephosphate isomerase). Neither an increase nor a decrease of these enzymes led to an altered ATP content (Rajapakse, 2012). In our study, the enolase family protein was down-regulated in all treatments (Table 3), which is in agreement with a study conducted by Yang et al. (Yang, 2010). These authors also reported changes of proteins associated with energy metabolism, such as enolase 1 and phosphoglycerate mutase, due to SiO₂-NPs exposure. The other protein in the glucose metabolism protein group, aconitate hydratase, was identified in two different spots, where it was also down-regulated in all treatments (Fig. 3, Fig. 6, Table 3). Apart from its involvement in glucose metabolism, aconitate hydratase has a non-catalytic function in iron homeostasis (<http://www.ebi.ac.uk/interpro/entry/IPR006249>). The only identified protein related to energy production that was up-regulated was triosephosphate isomerase, a glycolytic enzyme. This enzyme plays an important role in several metabolic pathways and is essential for efficient energy production (Triosephosphate isomerase, 2014), as well as fatty acid metabolism, as discussed in the previous paragraph. Our results are partially in agreement

with those obtained for carbon-based NPs (CNP) (Blazer-Yost et al., 2011). These authors reported a general up-regulation of proteins involved in glycolysis/gluconeogenesis pathways. In contrast, our proteomic study only found triosephosphate isomerase to be up-regulated, while other proteins that belong to this pathway were down-regulated. This could be viewed as the natural consequence of an increase in cellular demand of triglyceride synthesis since triosephosphate isomerase equilibrates between the glycolysis output and the lipid synthesis from the early glycolysis steps. While enolase in this study is down-regulated and point to a decreasing in glycolysis output being an enzyme catalyzing a glycolytic step after the triosephosphatase isomeras activity. Triosephosphate isomeras is also an absolute enzyme maintaining instant equilibrium and therefore has a strong regulative impact between the glycolyse and the triclyceride synthesis. In line with this, we also observed the down-regulation of cytosolic aconitate hydratase that also metabolizes glycolyse products in citric acid cycle. By increasing flagellar synthesis, aconitate hydratas is also involved in unicellular organism motility (Tang et al 2004).

Another group of identified cytosolic proteins, the expression levels of which changed significantly after exposure to TiO_2 -NPs or bulk- TiO_2 , is a versatile group of proteins with diverse functions. However, they are all involved in ion-regulation, namely Fe^{2+} homeostasis, salt stress, and Ca^{2+} homeostasis (Table 3). These are: the S-adenosylmethionine synthetase family protein, the peptidase family M20/M25/M40 protein, and the DnaK protein (Fig. 3, Table 3). One of the physiological functions of the S-adenosylmethionine synthetase family protein in plants is the adaptation to salt stress (Espartero, 1994). This enzyme is also linked to polyamines, known to affect DNA bending and transition from B to Z DNA, causing frame-shifts and other infidelities at the RNA level, and modulating signal transduction (Wallace, 2003a; Wallace, 2003b; Thomas, 2003). The second protein in this group, the peptidase family M20/M25/M40 protein, includes a range of zinc metallopeptidases

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belonging to several families in the peptidase classification (Rawlings, 1995) (Table 3). These proteins are described as being associated with water deficit stress in *Arabidopsis thaliana*, Ca^{2+} ion homeostasis, and the inflammatory response (Dua, 2011). The DnaK protein, a member of the Heat shock protein 70 family (Hsp70), was found to be down-regulated in our study. This is in agreement with the previous report on salinity stress (Lockwood, 2011) and the uptake of metal oxide engineered NPs into the immune cells of the sea urchin *Paracentrotus lividus* (Falugi, 2012). A down-regulation of DNaK is also in line with the aconitate hydratase regulation of flagellas where several studies have shown how the impairment of aconitase hydratase results in less or no flagellas through the DNaK transcription regulative function (Shi et al. 1992). Tang et al. (2004) also show how a co-decrease in aconitate hydrates and DNaK is related in the flagella impairment of synthesis. Furthermore, Werner (Werner, 1999), concluded that both the down-regulation and regulation as well as the up-regulation of Hsp70 proteins are indicators of stress in *Potamocorbula amurensis*. In medical research, the down-regulation of mthsp70/GRP75 (mortalin, amitochondrial stress protein) was linked to Parkinson's Disease brains as well as to the cellular Parkinson's Disease model (Jin, 2006). In addition, some other authors reported the up-regulation of the Hsp70 family after the exposure of different organisms to different NPs which was accompanied by oxidative stress (Ahamed, 2010, Siddiqi, 2012, Jebali, 2014). The next group of differentially expressed proteins identified in our study is associated with protein degradation. The proteasome A-type and B-type family protein (Fig. 3, Table 3) was significantly down-regulated after TiO_2 -NPs exposure. Since in higher eukaryotic cells proteasomes are localized mainly in the nuclear envelope – the endoplasmic reticulum network compartment (Enenkel, 1998) – and since the amounts of nuclear proteasomes are increased in mitotic cells (Amsterdam et al., 1993); Palmer, 1996; Klein, 1990), the presence of proteasome proteins in cytosolic fractions is only possible during mitosis, when the nuclear

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3 494 envelope breaks down. Thus, we explain the down-regulation of proteasomal proteins in our
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5 495 study with the decreased mitosis in TiO₂ treated *T. thermophila* cultures. Our findings are in
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7 496 agreement with a previous study on TiO₂-NPs that reported an early cellular response effect
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9 497 on cell growth (Ge, 2011), although we did not resort to cell counting techniques in the
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11 498 present study. Another NP-related study reported a down-regulation of proteasome β 1
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16 500 The other two groups of differentially expressed proteins identified in our study are associated
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18 501 with Purine metabolism and Metabolism *per se* (Table 3). The last identified group of proteins
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20 502 with significantly altered levels of expression involved amino acid sequences belonging to
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22 503 hypothetical proteins (Table 3).

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25 504 To summarize, the proteomic study of cytosolic cell fraction has shown that the early
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27 505 response of *T. thermophila* to subtoxic concentrations of TiO₂ particles includes alterations
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29 506 mainly in (a) lipid and fatty acid metabolism, and (b) ion-regulation.

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32 507 (a) The proteomic tools confirmed the results obtained by conventional cytotoxicity
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34 508 biomarkers and lipid profiling reported by Rajapakse (Rajapakse, 2012), asserting that
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36 509 exposure to TiO₂-NPs affected lipid and fatty acid metabolisms. At the tested concentrations
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38 510 of TiO₂-NPs no oxidative stress occurred. Perturbations in lipid metabolism were also
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40 511 reported by Wang and Petersen (2013), who discovered a formation of lamellar bodies after
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42 512 exposing cells to gold-NPs. The concentric myelin-like structures, the so-called lamellar
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44 513 bodies, are the morphological hallmark of phospholipidosis (Anderson and Borlak, 2006).
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46 514 This issue requires further investigation. By conducting a proteomic study of the cytosolic cell
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48 515 fraction, we have also confirmed an alteration in the use of pyruvate, which was not used for
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50 516 energy metabolism but for the synthesis of fatty acids. This is suggested by the lack of
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52 517 increase in ATP under the same experimental set-up and particles tested (Rajapakse, 2012).
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55 518 The same, 6-ph, was observed by Sohm et al. (2015) in *E. coli* exposed to TiO₂-NPs.
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(b) In line with many other studies on NP effects, our proteomic study also shows ion-regulatory dysfunctions, including calcium homeostasis (Cao, 2013; Huang, 2010; Guo, 2013). For example, Simon et al. (Simon, 2011) clearly established the role of calcium homeostasis alteration in response to the presence of TiO₂-NPs. An alteration in the intracellular calcium ion concentration was observed as a primal signal response to a mechanical stimulus (Adachi, 2003; Verma, 2012). Furthermore, Ariano et al. (Ariano, 2011) provided evidence that silica nanoparticles change the intracellular calcium concentration and that the toxic effect is related to the degree of perturbed calcium homeostasis. Similarly, by using the transcriptomic and proteomic approach, Sohm et al. (2015) have shown that TiO₂-NPs caused a cellular response in *E. coli* indicating osmotic stress and explained that this is due to the adsorption of TiO₂-NPs onto the cell surface.

The novelty of our study is that we evidenced different proteomic responses to TiO₂-NPs as well as bulk-TiO₂ at both tested concentrations. At the tested concentrations, the particles did not provoke signs of toxic effects. The response to TiO₂-NPs at the lower concentration (0.1 µg TiO₂-NPs/ml) significantly differed from that at the higher concentration of both TiO₂-NPs (100 µg TiO₂-NPs/ml) and bulk-TiO₂. The response of *T. termophila* to a higher exposure concentration of TiO₂-NPs is similar to the effects of bulk-TiO₂ particles at both concentrations. From the results obtained in our study and the study published by Rajapakse et al. (Rajapakse, 2012), we suggest that nanoparticles either adsorb to the cell surface or interact with cell membranes and thus cause ion-regulatory dysfunctions, including calcium homeostasis, and alterations in the cell membrane composition. This leads to a variety of early, subtoxic cellular alterations. In *T. termophila*, ingestion of NPs is an important exposure route of NPs which is perhaps not the case in all other eukaryotic cells. In line with the data supplied in the literature (Sohm et al. 2015), our results strongly suggest that lipid

and fatty acid metabolisms are among the first biochemical signs of the cellular effects of nanoparticles, which most likely appear due to the adsorption of NPs on the cell surface and interactions with membranes of food vacuoles.

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Figure S1. STRING network analysis of the 11 differentially expressed proteins with predicted network in evidence view between Triosephosphate isomerase (ERA88549, upregulated), Enolase (EAR85197, downregulated) and DNak (EAR82836, downregulated). Default settings and T. thermophila. Not predicted connections but consisten differentially expressed proteins over the different sorts of NP exposures were triosephosphate isomerase EAR88549, upregulated, S-adenosylmethionine synthetase family protein EAS03064, upregulated, aconitate hydratase, EAR96511, downregulated, proteasome A-type and B-type family protein EAR85260, downregulated, enolase family protein EAR85197, downregulated, peptidase family M20/M25/M40 containing protein EAR97423, downregulated and phospholipase/Carboxylesterase family protein EAR82795, upregulated.

Supplementary Material

Dynamic light scattering, DLS, was used to determine the mean hydrodynamic radius, R_h , of particles in suspensions. DLS measurements were conducted using a 3D DLS Spectrometer from LS Instruments GmbH (Fribourg, Switzerland)¹ that operates at a wavelength of light $\lambda_0 = 632.8$ nm. Two coherent incident light beams were generated with a 20 mV He-Ne laser (Uniphase JDL 1145P) and the scattered light was collected by two detectors at a scattering angle, θ , equal to 90° .

Detailed methodological aspects of DLS can be found elsewhere^{2,3}. In DLS, fluctuations of the intensity of scattered light are monitored with time. Intensity fluctuations originate from the concentration fluctuations that arise from the Brownian motion of particles in solution. DLS measurements are presented as the correlation function of the scattered light intensity, $G_2(t)$, (t is the time on the relaxation time axis). The $G_2(t)$ function has the form:

$$G_2(t) = \langle I(0)I(t) \rangle \quad (1)$$

where $I(0)$ and $I(t)$ are the scattering intensities at time $t = 0$ and time t , respectively.

Similarly, the correlation function of the scattered electric field, $g_1(t)$, is:

$$g_1(t) = \langle E(0)E(t) \rangle \quad (2)$$

where $E(0)$ and $E(t)$ are the corresponding amplitudes. The $g_1(t)$ function is directly related to the diffusion coefficient, D , of a Brownian particle by:

$$g_1(t) = e^{-Dq^2t} \quad (3)$$

In Eq.3, $q (= (4\pi n_0/\lambda_0)\sin(\theta/2))$ is the scattering vector, depending on the intensity of incident light (λ_0), scattering angle (θ), and the refractive index of the medium, n_0 . Since the intensity and the amplitude of the electric field are related by $I = |E|^2$, the relation between the two correlation functions is usually written as:

$$G_2(t) = 1 + Bg_1^2(t) \quad (4)$$

where B is an experimental constant. Equation 4 enables transformation of $G_2(t)$ into the $g_1(t)$, from which D is calculated. Value of R_h is then obtained from D by applying the Stokes-Einstein equation:

$$R_h = \frac{kT}{6\pi\eta_0 D} \tag{5}$$

In Eq. 5, k is the Boltzmann constant, T the absolute temperature, and η_0 the solvent viscosity. Equation 3 is strictly valid for monodisperse spherical particles. For polydisperse particles, several exponents appear in the exponent of $g_1(t)$ and thus a multi exponential fit was used and values of D were determined by the inverse Laplace transform program CONTIN.⁴

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Figure 1: TEM micrograph of (a) nano-TiO₂/ml and (b) bulk-TiO₂/ml gel.

Figure 1. Normalized correlation functions, $G_2(t)$, for suspensions of nanoparticles at different times, t , after preparation: 1N0 = nano-TiO₂, concentration 0.1 µg/ml at $t = 0$ h; 1N24 = nano-TiO₂, concentration 0.1 µg/ml at $t = 24$ h; 1B0 = bulk-TiO₂, concentration 0.1 µg/ml $t = 0$ h; 1B24 = bulk-TiO₂, concentration 0.1 µg/ml at $t = 24$ h.

Figure 2. Normalized intensity weighted size distributions for suspensions of TiO_2 1N0 = nano- TiO_2 concentration $0.1 \mu\text{g/ml}$ at 0 h, 1N24 = nano- TiO_2 concentration $0.1 \mu\text{g/ml}$ at 24 h, 1B0 = bulk- TiO_2 concentration $0.1 \mu\text{g/ml}$ at 0 h, 1B24 = bulk- TiO_2 concentration $0.1 \mu\text{g/ml}$ at 24 h (designations of curves are the same as in Figure 1).

Figure 3: 2-DE reference gel images of control and TiO_2 treated cultures (**Control gel** = control group; **nano-group:** 0.1 $\mu\text{g nano-TiO}_2/\text{ml}$; 100 $\mu\text{g nano-TiO}_2/\text{ml}$; **bulk group:** 0.1 $\mu\text{g bulk-TiO}_2/\text{ml}$; 100 $\mu\text{g bulk-TiO}_2/\text{ml}$).

Figure 4: Cluster analysis on 606 spots present in at least four out of five replicates (gels) per treatment. (Control = 0.0 μg TiO_2 particles/ml gels; nano 0.1 = 0.1 μg nano- TiO_2 /ml gel; nano 100 = 100 μg nano- TiO_2 /ml gel; bulk 0.1 = 0.1 μg bulk- TiO_2 /ml gel; bulk 100 = 100 μg bulk- TiO_2 /ml gel).

Figure 5: Heatmap plot showing the relative percentage of 77 significantly expressed spots in different treatments. One square in a heatmap equals every spot's relative percentage volume in a single gel, with values expressed in heat colors (from white to yellow, orange and red).

Figure 6: Ratio between treatment and control protein expression in *T. Thermophila*, following exposure to TiO_2 particles (nano 0.1 = 0.1 μg nano- TiO_2 /ml gel; nano 100 = 100 μg nano- TiO_2 /ml gel; bulk 0.1 = 0.1 μg bulk- TiO_2 /ml gel; bulk 100 = 100 μg bulk- TiO_2 /ml gel). Protein expression levels were determined by relative volume, using image analysis. Mean spot intensities on at least 4 out of 5 gels per treatment are shown.

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Table 3: Identified differentially expressed proteins from *T. thermophila* cells treated with nano- or bulk-TiO₂ particles.

Spot nr	Protein	Putative identification, NCBI sequence identifier	Protein function	Biological process	0.1 µg nano TiO ₂ /ml	100 µg nano TiO ₂ /ml	0.1 µg bulk TiO ₂ /ml	100 µg bulk TiO ₂ /ml	MW (kDa)	Matched peptides	% coverage	Score	pI observed	Mr observed
Lipid and fatty acid metabolism														
355	Acyl-CoA dehydrogenase, C-terminal domain containing protein	gi 118377002	The Acyl-CoA dehydrogenases are mitochondrial flavoenzymes that catalyse the initial step in fatty acid β-oxidation (GO:0016627).	energy production, synthesis of new fatty acids, fatty acid oxidation	↑	↑	0	0	45506	9 (8)	30	278	6,5	55
356	Acyl-CoA dehydrogenase, C-terminal domain containing protein	gi 118377002	The Acyl-CoA dehydrogenases are mitochondrial flavoenzymes that catalyse the initial step in fatty acid β-oxidation (GO:0016627).	energy production, synthesis of new fatty acids, fatty acid oxidation	↑	↓	↓	↓	45506	7 (6)	24	211	7,2	60
597	triosephosphate isomerase	gi 146162132	Fatty acid biosynthesis, gluconeogenesis, glycolysis, lipid synthesis, cytoplasm. Triosephosphate isomerase (TIM) is a glycolytic enzyme that catalyzes the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate. The reaction is very efficient and requires neither cofactors nor metal ions.)	energy production	↑	↑	↑	↓	28210	13 (8)	38	294	7,6	32
Glucose metabolism														
14	aconitate hydratase	gi 118367081	iron regulatory protein, Krebs cycle, cytoplasm	energy production	↑	↓	↓	↓	99387	19 (16)	23	451	6,1	120
15	aconitate hydratase	gi 118367081	iron regulatory protein, Krebs cycle, cytoplasm	energy production	↑	↓	↓	↓	99387	30 (21)	31	607	6,2	120
220	enolase family protein	gi 118362946	9th step in glycolysis	energy production	↓	↓	↓	↓	50329	2 (1)	7	135	7,3	70
597	triosephosphate isomerase	gi 146162132	Fatty acid biosynthesis, gluconeogenesis, glycolysis, lipid synthesis, cytoplasm. Triosephosphate isomerase (TIM) is a glycolytic enzyme that catalyzes the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate. The reaction is very efficient and requires neither cofactors nor metal ions.)	energy production	↑	↑	↑	↓	28210	13 (8)	38	294	7,6	32

Ion regulation: Fe²⁺ homeostasis, Salt stress and Ca²⁺ homeostasis

14	aconitate hydratase	gi 118367081	iron regulatory protein, Krebs cycle, cytoplasm	energy production	↑	↓	↓	↓	99387	19 (16)	23	451	6,1	120
15	aconitate hydratase	gi 118367081	iron regulatory protein, Krebs cycle, cytoplasm	energy production	↑	↓	↓	↓	99387	30 (21)	31	607	6,2	120

Salt stress and Ca²⁺ homeostasis

30	dnaK protein	gi 118358030	Salt -stress indication. Downregulation observed in case of salinity stress. (Lockwood and Somero, 2011)	salt stress response	↓	↓	0	0	71682	4 (4)	7	260	6,1	100
445	S-adenosylmethionine synthetase family protein	gi 118380290	S-adenosylmethionine synthetase can be considered rate-limiting step of the methionine cycle.[2] S-adenosylmethionine is a methyl donor and allows DNA methylation.	cell growth, Ca+ homeostasis, SOS response	0	↑	↑	↑	44072	6 (5)	17	234	7	40
128	Peptidase family M20/M25/M40 containing protein	gi 118364457	Includes a range of zinc metallopeptidases belonging to several families in the peptidase classification (Rawlings and Barrett, 1995)	stress response	↑	↓	↓	↓	52932	14 (11)	36	516	6,2	80

Protein degradation

569	Proteasome A-type and B-type family protein	gi 118358232	Proteasome A-type and B-type family protein (The 20S proteasome multisubunit proteolytic complex, is the central enzyme of nonlysosomal protein degradation in both the cytosol and nucleus.)	protein degradation	↓	↓	↓	↑	27828	16 (12)	60	492	8,2	35
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Purine metabolism

561	purine nucleoside phosphorylase I, inosine and guanosine-specific family protein	gi 118375576	Family I consists of a group of plant stress-inducible proteins. The experimental results indicate PNP is an important route for the reduction of arsenate to arsenite in mammalian systems.	nucleoside metabolic process	↑	↓	↑	↓	30125	4 (3)	16	146	6,4	30
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Metabolism per se

578	Phospholipase/Carboxylesterase family protein	gi 118396232	In molecular biology, the alpha/beta hydrolase fold is common to a number of hydrolytic enzymes of widely differing phylogenetic origin and catalytic function. This family consists of both phospholipases and carboxylesterases with broad substrate specificity, and is structurally related to alpha/beta hydrolases PF00561 [2].	metabolism	↑	↑	↑	↑	33302	6 (5)	20	201	7,1	31
370	oxidoreductase, aldo/keto reductase family protein	gi 118397046	Aldo-keto reductases are a superfamily of soluble NAD(P)(H) oxidoreductases whose chief purpose is to reduce aldehydes and ketones to primary and secondary alcohols.	metabolism	↑	↓	0	0	39813	7 (6)	24	169	6,5	46
Hypothetical protein														
50	hypothetical protein THERM_00086780	gi 118358970	/	/	↑	↑	↑	↑	71762	2 (2)	4	130	6,8	90
96	hypothetical protein THERM_00864870	gi 146183651	/	/	↑	↑	↓	↓	60218	8 (6)	20	278	7,8	78
202	hypothetical protein THERM_00086780	gi 118358970	/	/	↑	↑	↑	↑	71762	3 (3)	6	146	6	72
223	hypothetical protein THERM_01002870	gi 229594034	/	/	↑	↓	↓	↓	48661	12 (9)	39	409	6,3	57
465	hypothetical protein THERM_01002870	gi 229594034	/	/	↓	↑	↑	↑	48661	2 (1)	7	97	7,1	38

Table 1. Average hydrodynamic radii for smaller particles, $R_{h,1}$, and for aggregates, $R_{h,2}$, in aqueous TiO_2 suspensions determined by DLS at 25 °C.

sample	$R_{h,1}$ / nm	$R_{h,2}$ / nm
1N0	150	>1000
1N24	120	>1600
1B0	340	>1000
1B24	70	>6000

Table 2. Zeta potential values of all tested particle suspensions before (0h) and after (24h) the exposure. Samples were dispersed in a test medium.

No of sample values	Sample	Zeta potential (mV)
1	nano-TiO ₂ : 0.1 µg/ml t = 0 h	-22.6 ± 0.4
2	nano-TiO ₂ : 0.1 µg/ml t = 24 h	-15.3 ± 2.0
3	nano-TiO ₂ : 100 µg/ml t = 0 h	-17.7 ± 1.2
4	nano-TiO ₂ : 100 µg/ml t = 24 h	-18.3 ± 0.9
5	bulk-TiO ₂ : 0.1 µg/ml t = 0 h	-16.7 ± 1.2
6	bulk-TiO ₂ : 0.1 µg/ml t = 24 h	-18.6 ± 0.8
7	bulk-TiO ₂ : 100 µg/ml t = 0 h	-20.8 ± 1.5
8	bulk-TiO ₂ : 100 µg/ml t = 24 h	-18.8 ± 0.9