FTIR microscopy reveals distinct biomolecular profile of crustacean digestive glands upon subtoxic exposure to ZnO nanoparticles

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Abstract

Biomolecular profiling with Fourier-Transform InfraRed Microscopy was performed to distinguish the Zn²⁺-mediated effects on the crustacean (Porcellio scaber) digestive glands from the ones elicited by the ZnO nanoparticles (NPs). The exposure to ZnO NPs or ZnCl₂ (1500 and 4000 µg Zn/g of dry food) activated different types of metabolic pathways: some were found in the case of both substances, some only in the case of ZnCl₂ and some only upon exposure to ZnO NPs. Both the ZnO NPs and the ZnCl₂ increased the protein (≈1312 cm⁻¹; 1720–1485 cm⁻¹/3000–2830 cm⁻¹) and RNA concentration (≈1115 cm⁻¹). At the highest exposure concentration of ZnCl₂, where the effects occurred also at the organismal level, some additional changes were found that were not detected upon the ZnO NP exposure. These included changed carbohydrate (most likely glycogen) concentrations (≈1043 cm⁻¹) and the desaturation of cell membrane lipids (≈3014 cm⁻¹). The activation of novel metabolic pathways, as evidenced by changed proteins’ structure (at 1274 cm⁻¹), was found only in the case of ZnO NPs. This proves that Zn²⁺ are not the only inducers of the response to ZnO NPs. Low bioavailable fraction of Zn²⁺ in the digestive glands exposed to ZnO NPs further supports the role of particles in the ZnO NP-generated effects. This study provides the evidence that ZnO NPs induce their own metabolic responses in the subtoxic range.

Introduction

There is a general consensus in the current nanotoxicological literature that the dissolved Zn²⁺ are the main factor in the toxicity of ZnO nanoparticles (NPs) for different organisms. On the other hand, several studies could not explain all the observed effects by the dissolution of ZnO NPs, which indicates that the particles themselves may play a role (Ivask et al., 2014; Ma et al., 2013). The clear differentiation between the roles of the dissolved ions and particles in the biological effects of NPs is expected to be detected at the molecular level. At this level the interaction mode between the ions and particles is expected to be different, while the responses at the higher levels of biological complexity are common for both particles and ions. For this type of information, the collective characterization and quantification of pools of biological molecules is needed.

One such method is the Fourier-Transform Infrared Microscopy (FTIRM), which provides spatially resolved information about the biochemical composition of a sample, allowing for the investigation of the functional groups that characterize a specimen. The infrared spectra of cells reflect molecular-level details regarding the concentrations, organizations, structures and (bio)chemical environments of the cellular constituents (Movasaghi et al., 2008). Analytical tools based on FTIR have been extensively employed for probing the molecular changes associated with abnormal tissues (Movasaghi et al., 2008); however, they have not been commonly implemented in the ecotoxicological studies until recently (Aja et al., 2014; Novak et al., 2013; Palaniappan & Pramod, 2010).

This study employs an experimental model, namely, the digestive glands of the terrestrial isopods Porcellio scaber, which have previously been used to investigate the effects of nanomaterials (WOx nanofibres) on the biomolecular profile using FTIR imaging (Novak et al., 2013). These organisms have also been used successfully to investigate the bioavailability of metals dissolved from NPs (Golobič et al., 2012; Romih et al., 2015).

The aim of this study was to use the FTIRM on the crustacean digestive glands to investigate whether subtoxic concentrations of ZnO NPs and ZnCl₂ (the source of Zn²⁺)
induce distinct biomolecular profiles. We hypothesize that Zn$^{2+}$ are not the only reason for the effects of ZnO NPs, but the effects are also governed by the ZnO particulate matter. Biomolecular profile data are accompanied by the information on the Zn bioavailability as measured by the Zn assimilation into the digestive glands.

**Methods**

**Preparation and characterization of the test chemicals**

We used the commercially available ZnO NPs, purchased from Sigma Aldrich (St. Louis, MO); the particles were <100 nm in diameter with the specific surface area of 15–25 m$^2$/g (product no. T9375, CAS 1314-13-2). ZnCl$_2$ as a source of free Zn$^{2+}$ was purchased from Merck (Darmstadt, Germany; product no. 1.08816.0250, CAS 7646-85-7, pro analysis grade). Stock suspensions of ZnO NPs and ZnCl$_2$ solutions at the concentrations of 1500 and 4000 $\mu$g Zn/mL were prepared in deionized water (Millipore, Billerica, MA). The concentrations were chosen on the basis of our previous study (Pipan-Tkalec et al., 2010). The ZnO NPs were dispersed by a magnetic stirrer at ambient temperature for 1 h (400 rpm) and further sonicated in the ultrasonic bath (Sonics 2 GT ultrasound cleaner, Iskra PIO, Šentjernej na Dolenjskem, Slovenia) for 1 h.

The characterization of the NPs was performed in ZnO nanopowder directly from the supply. Prior to the analyses, the ZnO nanopowder was attached to a holder with a double-sided adhesive carbon tape. The specimen was inspected by the field-emission scanning electron microscope (FE-SEM JSM-7500F, JEOL, Tokyo, Japan) under the accelerating voltage of 5 kV. The dispersions of ZnO NPs in deionized water (1500 and 4000 $\mu$g Zn/mL) were also inspected by the dynamic light scattering (DLS) using 3D-DLS-SLS spectrometer (LS Instruments GmbH, Fribourg, Switzerland). As the freshly prepared dispersions were unstable and turbid, they were allowed to settle for 1 h prior to the measurements. The measurements were performed using 1 mL of supernatants. Details on the instrument operating parameters and data analysis are presented in the Supplementary material (Method description S1).

**ZnO nanoparticle dissolution measurements**

Currently, the most widely used approach for estimating the extent of dissolution of metal oxide NPs to metal ion species in aqueous suspensions comprises ultracentrifugation or filtration followed by spectroscopic determination, such as atomic absorption spectroscopy (AAS) or inductively coupled plasma mass spectroscopy (ICP-MS) (Misra et al., 2012). The adequacy of all these methods is limited by imperfect separation by either filtering or centrifuge-assisted sedimentation, which leads to a positive error, and, on the other hand, to a loss of ions adsorbed on the filters (Xu et al., 2013). Other techniques, such as diffusion gradients in thin films and dialysis, can also give inconsistent results (Odjak et al., 2014). The free-metal-ion activity is most conveniently probed by ion-selective electrodes, but for Zn no such electrodes have been commercially available to date (Pesavento et al., 2009). In this study, we used an electrochemical method to determine the best possible approximation to free ion concentration (Jiang & Hsu-Kim, 2014). In addition to being sensitive exclusively to ion species, electrochemical methods facilitate direct determinations with minimal perturbation of the sample (Jiang & Hsu-Kim, 2014). Square-wave voltammetry (SWV) (Mirčeski et al., 2007) was used instead of the more common anodic stripping voltammetry (ASV), since the preliminary measurements featured concentrations which were well above trace level, where ASV is the method of choice.

Eight milliliters of ZnO nanopowder dispersions (1500 and 4000 $\mu$g Zn/mL) were ultracentrifuged at 100 000 × g for 30 min at 20°C (Beckman Coulter L8-70 M class H preparative ultracentrifuge with the Type 70.1 Ti rotor and 10 mL thickwall polycyliner tubes). The supernatants were then divided into two aliquots for measuring the Zn concentrations. The first aliquot was determined by flame AAS (Perkin Elmer AAnalyser 100, Waltham, MA) and the second by SWV. The Zn concentrations in the original ZnO NP dispersions were also determined by flame AAS after an overnight digestion in 1 M HNO$_3$ (sulfuric acid ratio 1:1 v/v). All of the values were compared and the percentages of dissolved Zn$^{2+}$ were calculated.

Square-wave voltammetry was applied using EG&G Princeton Applied Research Model 303A stationary mercury drop electrode assembly coupled with AUTOLAB PGSTAT 101 potentiostat via IME 303 interface. The working electrode was the hanging mercury drop electrode, the auxiliary electrode was a platinum wire, and the reference electrode was Ag/AgCl/3 mol/L KCl electrode (SSCE). In the electrolytic cell, 1 mL of 1 M, pH 7 acetate buffer was added to 4 mL of supernatant, yielding a 5 mL total volume. Before measurements, the solution was purged with N$_2$ for 4 min, and the headspace of voltammetric cell was continuously flushed with N$_2$ at all times to avoid O$_2$ interferences. The background Zn$^{2+}$ concentration was measured before each sample by substituting supernatant with deionized water. SWV was performed without any deposition step by scanning from −0.8 V to −1.3 V versus the reference electrode with 20 mV amplitude at 50 Hz and the scan rate of 100 mV/s. The scan gave rise to a peak at a median of −1.048 V versus SSCE and a median full width half maximum (FWHM) of 74 mV. The method of successive standard additions was then employed, adding 100 $\mu$L of 20 $\mu$g/mL Zn$^{2+}$ standard in each step. The Zn concentrations were finally calculated using peak heights by linear regression using the chemCal package (Ranke, 2013) for the R statistical software (R Core Team, 2013).

**Test organisms**

Terrestrial isopods Porcellio scaber Latreille, 1804 were chosen as model organisms. Being invertebrates, they are subject to no legal restrictions. The isopods were collected in September, 2011 from a compost heap in a non-polluted garden in Podutik, Ljubljana, Slovenia. The animals were kept in a controlled chamber at a constant temperature (20 ± 2°C) and a light regime (16 h of light and 8 h of darkness) and fed dry common hazel leaves (Corylus avellana) for 3 weeks before the Zn exposure. P. scaber adults of both sexes, including those at the intermoult and early premoult stages, were chosen for the experiments. The average fresh body weight of the animals was 44 ± 14 mg (mean ± SD; n = 100).

**Experimental design**

The experimental animals were divided into the following two groups: Group 1 (n = 50) was used for the analyses of Zn concentrations in digestive glands (flame AAS analyses), and Group 2 (n = 50) was processed for the FTIRM. The animals in both groups were fed Zn-spiked food for 14 days under the same experimental conditions; each group contained 10 individuals per each concentration of the tested chemical. At the end of the exposure period, Group 1 was switched to uncontaminated food for 1 day to deparute the Zn-spiked food from their digestive systems, while the animals in Group 2 were immediately dissected and processed for FTIRM.

The experimental set-up was performed as previously described (Golobič et al., 2012). During the experiment, the animals were fed dry common hazel leaves that were spiked with
ZnO NPs and ZnCl₂. All test chemicals were freshly prepared prior to the experiment at concentrations of 1500 and 4000 μg Zn/mL. 100 μL of the test chemical per 100 mg of leaf were applied onto the abaxial leaf surfaces, which resulted in the following two final concentrations of Zn: 1500 and 4000 μg/g of dry leaf. These concentrations were chosen based on our previous study, where they were found to be non-toxic (Pipan-Tkalec et al., 2010). The leaves were left to dry at room temperature.

We inspected the ZnO NP-spiked leaves to confirm that the NPs remained on the leaves prior to the experiment. Small pieces of the ZnO NP-spiked leaves were attached to a holder with a double-sided adhesive carbon tape. The specimens were sputtered with gold and inspected by the thermal field-emission scanning electron microscope JSM-6500F (JEOL, Japan) under the accelerating voltage of 15 kV (Supplemental Figure S1).

Each animal was placed individually in a plastic Petri dish with 9 cm in diameter, to which individual pieces of Zn-spiked leaves were added. No substrate was used. The Petri dishes were put in a covered glass container and moisture was maintained by sprinkling the walls of the container with deionized water. The container was kept under the same controlled conditions as during the animal cultivation stage. The food was not replaced during the exposure period and fecal pellets were collected weekly. At the end of the experiments, the remnants of leaves were collected, air-dried and weighed. Fecal pellets were also weighed after drying in a desiccator for 24 h.

After the experiment, the animals from Group 1 were dissected and the Zn concentrations in digestive glands (hepatopancreas) were measured. Each digestive gland was placed on a separate small piece of filter paper (approximately 4 mm × 7 mm in size) and was stored in a plastic tube until the analysis by flame AAS (Perkin Elmer AAnalyst 100, Waltham, MA). For FTIRM, only the hepatopancreas was isolated and processed for analysis.

Measurements of metal content in the food and in the animal tissues

The total Zn concentration in digestive glands of isopods from Group 1, in the uneaten leaf remnants after the experiments, and in the leaves spiked separately to serve as the accuracy check, was determined by flame AAS. Prior to the analysis, samples were digested by a “hot plate” method in a mixture of concentrated nitric (65% HNO₃, pro analysi, Merck, Darmstadt, Germany), and perchloric acid (70% HClO₄, pro analysi, Merck, Darmstadt, Germany) (HNO₃:HClO₄ = 7:1, vol/vol). After the evaporation of the acid, the residue was dissolved in 0.2% HNO₃. Within each measurement a certified reference material (TORT-2, National Research Council of Canada) was used to check the accuracy and precision of the analytical procedure. Along with the samples, also 20 replicates of a known amount of a certified reference material were acid digested and each sample was measured in triplicate. The calculations followed the approach described elsewhere (Phillips et al., 2007). The Zn spiking recovery was calculated on the basis of the Zn concentration in the separately spiked leaves and in the remnants of leaves after the experiment. All the data regarding the quality control of the experimental procedures are described and discussed in the Supplementary material (Method description S2).

Data analyses of feeding parameters and metal concentrations

Animals that died during molting and ovigerous females were excluded from further data processing (n = 7 in total). The numbers of analyzed animals are presented in the figures as part of the x-axis labels. The data are presented as the mean values, and uncertainties are expressed as standard deviations (SD). All of the data shown in the figures describe nominal concentrations of Zn (1500 and 4000 μg/g of dry leaf). The feeding rate of the isopods was calculated as the mass of consumed leaves in the 14 days of exposure divided by the mean mass of the animals used in the experiment. The statistical significances of the differences between the control and the exposed groups of animals were assessed by the Mann–Whitney U-test (*p < 0.05, **p < 0.01, ***p < 0.001), using the OriginPro 8.0 software (OriginLab Corp., Northampton, MA).

FTIRM: sample preparation, data collection, pre-processing and analysis

For the FTIRM, samples were prepared in the same way as in our previous study (Novak et al., 2013). The digestive glands of animals from Group 2 were shock-frozen in liquid nitrogen, using the Jung tissue-freezing medium (Leica Microsystems, Nussloch GmbH, Nussloch, Germany). Samples were sectioned transversally into 14 μm-thick sections using a Leica CM3050 cryotome (Leica Biosystems GmbH, Wetzlar, Germany), with the temperature of the microtome head and chamber maintained between −25 and −20 °C. Sections were placed onto 2 mm thick, IR-transparent, BaF₂ windows and transferred to the Christ Alpha 2-4 LSC Freeze Dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) using a cryo-transfer assembly cooled with liquid N₂, and freeze-dried at −30 °C and 0.4 mbar for 24 h. The samples were stored in a desiccator until the FTIRM analysis was performed.

The FTIRM measurements were carried out at the infrared beamline Synchrotron Infrared Source for Spectroscopy and Imaging (SISSI) of the Elettra-Sincrotrone Trieste (Lupi et al., 2007), using the Vertex 70 interferometer coupled with the Hyperion 3000 Vis-IR microscope (Bruker Optics GmbH, Ettlingen, Germany). Spectra were collected from cryosectioned digestive glands of animals from Group 2. Out of 10 exposed animals, up to 8 of them were selected for the FTIRM analyses. The following animals were selected: 5 control animals, 8 and 6 animals that were fed ZnO NPs containing 1500 and 4000 μg Zn/g of dry leaf respectively, and 1 and 5 animals that were fed ZnCl₂ containing 1500 and 4000 μg Zn/g of dry leaf, respectively. One to five lyophilized cryosections per animal were measured. For every cryosection, several measurement areas were chosen and divided into three regions: (1) the “peripheral” region, which contained the basal membranes of the hepatopancreatic cells and cytoplasmic organelles, (2) the “central” region, which included mostly the nuclear and perinuclear cellular regions and (3) the “apical” region, which contained the apical membranes of the hepatopancreatic cells, cytoplasm and cellular cytoplasmic organelles, such as the mitochondria and ribosomes (Supplemental Figure S3). A total of 512 scans acquired in transmission mode using the single point MCT-A detector were averaged for each measurement at 15 × 15 μm lateral resolution in the wavenumber region of 4000–900 cm⁻¹ to obtain one spectrum per measurement area.

Each collected spectrum was subjected to several pre-processing steps, described in detail in the Supplementary material (Method description S3), and only the spectra meeting the quality criteria described therein were subjected to further analyses. The selected spectra were then analyzed using a multivariate approach with hyperSpec (Beletes, 2012), the R (R Core Team, 2013) package for handling hyperspectral data. The hierarchical cluster analysis (HCA), based on the Euclidean distances and Ward’s classification algorithm, was performed on the vector-normalized absorbance spectra in the 3950–950 cm⁻¹ spectral region to highlight spectral similarities and dissimilarities among the different regions of hepatopancreatic cells that...
were induced by the ingestion of the different Zn compounds. Cluster centroids (the average spectrum of each identified spectral family) were then compared to reveal the biochemical differences that were responsible for the classifications in addition to their second derivatives (Savitzky–Golay algorithm, 17 smoothing points), which allowed for more precise extractions of peak frequencies. The relative intensities of diagnostic spectral bands were compared (Supplemental Table S2), and band assignment was carried out according to the literature (Movasaghi et al., 2008).

Results
Nanoparticle characteristics
Scanning electron microscopy revealed that the ZnO nanopowder was composed of particles of different sizes, ranging from tens of nanometers to several hundred nanometers (Supplemental Figure S1). The characterization of NPs in the aqueous suspension was done as suggested by the current nanoecotoxicological practice, although it is not relevant for our experimental set-up since the animals were exposed to dried NPs applied onto the leaf surface. ZnO NP size distributions were bi- or multi-modal for both concentrations after 1 h of settlement (Supplemental Figure S2). The average hydrodynamic diameters of the ZnO NPs in deionized water, 2 × Rs, were separated into 60–80 and 400–600 nm classes. The measurements also detected objects that were larger than 1000 nm for Rs, but any size estimation in this range is unreliable; besides, the number of such agglomerates was very small. The smaller sized classes were predominant in terms of mass and number (Supplemental Figure S2).

ZnO nanoparticle dissolution
ZnO NPs are among the most soluble NPs, which may significantly influence their biological effects. In this study, the measured values of dissolved Zn in aqueous ZnO NP suspensions (pH = 6.51, T = 20 °C) were lower in the case of SWV compared to the AAS method (Table 1). The ultracentrifugation under the chosen parameters was therefore not sufficient to sediment all the NPs, similarly as previously reported (Romih et al., 2015; Xu et al., 2013). The Zn concentration range that we obtained by voltammetry was similar to that reported by other researchers, namely 3.57 mg/L Zn²⁺ in 0.1 M KCl medium at pH 8 and 25 °C for uncoated ZnO NPs (David et al., 2012) or 0.3–1.3 mg/L Zn²⁺ in 81–82 mM KCl medium buffered with 20 mM 3-(N-morpholino)-propanesulfonic acid or piperazine-N,N’-bis(2-hydroxypropanesulfonic acid) at pH 8.6 and 25 °C for ZnO NPs coated with acetate, polyvinylpyrrolidone or 3-aminopropyl triethoxysilane (Jiang & Hsu-Kim, 2014). Therefore, we presume that the estimation of free Zn²⁺ shares by SWV was accurate. Based on the SWV, the free Zn²⁺ concentrations in the ZnO NP suspensions that were used for spiking the leaves for the feeding experiments represented less than 1% of the whole Zn content (Table 1).

Differences among the FTIRM spectra of the digestive glands of isopods from different exposure groups (control, ZnO NPs or ZnCl₂)
The HCA analysis revealed clear differences among the spectra from the controls and the Zn-fed animals only at the apical regions of the cells but not in the central or peripheral parts (Supplemental Figure S5), therefore only the apical regions of cells were subjected to further analyses. The HCA discriminated between the controls (Cluster 1 in Figure 1A) and the higher ZnCl₂ exposure concentration (Cluster 2 in Figure 1A), but no significant differences in comparison to 1500 μg Zn/g of dry food exposure were found. In the case of ZnO NP-fed animals, both concentrations induced differences in FTIRM spectra compared with the controls (Cluster 1 in Figure 1B), and result in a unique but heterogeneous cluster (Cluster 2 in Figure 1B).

In the case of ZnO NP exposure, FTIRM revealed differential spectroscopic patterns mainly in the 1320–1000 cm⁻¹ region, which is diagnostic for carbohydrates, nucleic acids and (when the Amide III region is discernible) proteins. In the case of ZnCl₂ exposure, the region around 3000 cm⁻¹ was changed as well. The identified discriminating spectral features and their biological significances are presented in Table 2.

Proteins
At the higher Zn exposure (4000 μg Zn/g of dry food), both ZnO NPs and ZnCl₂ elevated the relative concentration of proteins, while protein conformational changes compared to controls were found only in the case of ZnO NP exposure (Table 2). Proteins preserved the original folding pattern, dominated by the α-helix motive, upon exposure of animals to ZnCl₂ 4000 μg Zn/g of dry food, while ZnO NP exposure led to the formation of more extended random protein domains (Table 2).

Several vibrational modes are diagnostic for cellular proteins (Barth, 2007). Among them, Amide I and Amide II regions are considered especially relevant for describing protein content and structure in complex biological systems. The Amide I (~1700 to 1600 cm⁻¹) region represents mainly the C=O stretching of the peptide bond, with contributions from C–N stretching (~10%) and N–H bending (~10%), and its position/shape is sensitive to the network of hydrogen bonds involving the carbonyl group and consequently to protein secondary structure. The Amide II region (~1580 to 1480 cm⁻¹) represents mainly the N–H bending (~60%), with contributions from C–N stretching (~40%). Even if less diagnostic than the Amide I region, the Amide II region can also be deconvolved in contributions coming from helices, sheets, turns and random domains that are the folding motives of cellular proteins.

| Test chemical | Nominal Zn concentration (mg/L) | Total Zn concentration, measured by AAS (mg/L), average ± SD, n = 4 | Zn²⁺ concentration in the supernatant, measured by AAS (mg/L), average ± SD, n = 5 | Zn²⁺ concentration in the supernatant, measured by SWV (mg/L), average ± SD, n = 5 | Dissolved Zn fraction according to SWV (%) |
|---------------|-------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|-------------------------------------------|
| nano ZnO      | 1500                          | 1630 ± 200                                                   | 5.7 ± 0.4                                                    | 3.43 ± 0.06                                                  | 0.21                                      |
|               | 4000                          | 3080 ± 210                                                   | 8 ± 1                                                        | 3.9 ± 0.3                                                    | 0.13                                      |
| ZnCl₂         | 1500                          | 1430 ± 50                                                    | 1350 ± 80                                                   | Not measured                                                  | NA                                        |
|               | 4000                          | 3750 ± 90                                                    | 3820 ± 50                                                   | Not measured                                                  | NA                                        |

NA – not available.
Protein concentration

The area integral of the Amide I and II regions, $A (1720–1485 \text{ cm}^{-1})$, is conventionally considered diagnostic for the overall protein concentration. In the case of lipids, the total lipid concentration is estimated by the area integral of the stretching modes of aliphatic chains, $A (3000–2830 \text{ cm}^{-1})$. The relative variations of these two cellular constituents can be evaluated through the protein-to-lipid ratio $A (1720–1485 \text{ cm}^{-1}) / A (3000–2830 \text{ cm}^{-1})$, which increased upon the ingestion of both ZnO NPs and ZnCl$_2$ (Supplemental Table S2). This trend reveals either an increase of cellular proteins and/or a reduction of the digestive gland lipid content. In our case, both explanations are possible, but it appears more probable that proteins increased due to an intensity increase of the Amide III band (explained in greater detail in the following paragraph).

Protein structure

With respect to the proteome profile of the apical region of the hepatopancreatic cells, no significant structural differences could be detected comparing the spectral profiles of the Amide I and II spectral bands, which peaked at the same positions for both the control animals and those that were exposed to different Zn

Figure 1. Results of the FTIRM data analysis. (A) Dendrogram of the spectral heterogeneity of the apically sampled points of the control animals (cluster 1, black bar) and the animals that were fed ZnCl$_2$ in 4000 µg Zn/g of dry food (cluster 2, dashed bar) as revealed by the HCA using vector-normalized absorbance spectra in the 3950–950 cm$^{-1}$ region. The data for ZnCl$_2$ in 1500 µg Zn/g of dry food are not shown since no significant differences were found in comparison to the controls. (B) Dendrogram of the spectral heterogeneity of the apically sampled points of the control animals (cluster 1, black bar) and the animals that were fed ZnO NPs in 1500 and 4000 µg Zn/g of dry food (cluster 2, gray bar) as revealed by the HCA using vector-normalized absorbance spectra in the 3950–950 cm$^{-1}$ region. (C, D) Cluster centroids belonging to the apical regions of the controls (label: Control) and to the apical regions of the Zn-exposed samples, ZnCl$_2$ in 4000 µg Zn/g of dry food (label: ZnCl$_2$) and ZnO NPs in 1500 and 4000 µg Zn/g of dry food combined (label: ZnO NPs) for the spectral regions of 3050–2800 (C) and 1800–900 cm$^{-1}$ (D). (E, F) The second derivative of cluster centroids (17 smoothing points, Savitzky-Golay algorithm) for the spectral regions of 3050–2800 (E) and 1800–900 cm$^{-1}$ (F).
Changes in positions, shapes and/or relative intensity in comparison to the control

| FTIRM spectra | Spectroscopic annotation | Biological significance |
|---------------|--------------------------|-------------------------|
| ZnCl2 (4000 g Zn/g of dry food) | Amide III contribution | Conformation of proteins: mostly random domains a,b |
| ZnO NPs (4000 g Zn/g of dry food) | Protein-to-lipid ratio | Lipid content c |
| ν (C=O) of C–OH groups of ribose d | n(CH) | Desaturation of cell membrane lipid e, membrane fluidity f |
| ν (=CH) C–H stretching | C–O stretching of C–OH groups of glycogen, general g | Phospholipid relative concentration and conformational states |
| ν (C=O) | C–H stretching | Spectral invariance of the Amide III region |


d∗Huleihel et al., 2001.

Compounds (Figures 1D and F). The Amide I band was centered at 1637 cm−1, with two minor contributions at 1642 and 1688 cm−1, revealing that most of the proteins in this region were helical or randomly coiled. An analogous conclusion could be drawn from the spectral invariance of the Amide II band, where the most intense contributions centered at 1545 and 1516 cm−1 (Figure 1D and F). However, both treated groups showed the appearance of distinctive spectral features in the 1400–1200 cm−1 region, which could be ascribed to specific contributions of the Amide III bands. In the Amide III region, which originates from an N–H bending and C–N stretching of the peptide backbone, different secondary structures of proteins are more resolved than in the Amides I or II (Cai & Singh, 1999). Specifically, three secondary structure frequency windows can be identified for the Amide III region (Fu et al., 1994): α-helix, 1328–1289 cm−1; unordered, 1288–1256 cm−1; and β-sheets, 1255–1224 cm−1. However, the Amide III region is often neglected in data analysis, due to its much lower intensity when compared with Amides I and II, especially in studying complex biological systems, such as tissues or cells. Vibrational modes related to cellular carbohydrates and nucleic acids usually dominate the low wavenumber Mid IR spectral region, hiding the less intense Amide III region. However, in the investigated region of cells, the spectral contributions from nucleic acids is minor compared to other regions, which enables us to assign the contributions at 1312 cm−1 for all the spectral groups that underwent variation with respect to the controls upon ingestion of Zn and at 1274 cm−1 for ZnO NPs at both concentrations to Amide III, related to the α-helix and the random-coiled protein domains, respectively. It is to be noted that the possibility of discerning the Amide III contribution only in the treated animals supports the hypothesis of an increase in the protein concentration.

**Lipids**

Only in the experimental group fed ZnCl2 at the higher concentration, a relative and moderate increase of the spectral intensity of the band of vinyl stretching centered at 3014 cm−1 could be elicited (Table 2). The biological significance of this change is in the desaturation of membrane lipids (Table 2). However, no differential spectroscopic patterns in the lipid/phospholipid relative concentration and conformational states were revealed in the animals exposed to ZnO NPs or ZnCl2. Cellular lipids, and especially phospholipids, are characterized by the longest aliphatic chains and therefore their spectroscopic signatures are considered diagnostic of the cellular membranes' content, composition and order. Specifically, the shapes and positions of the methyl (–CH3), methylene (–CH2) and methylene (–CH) C–H asymmetric and symmetric stretching bands (spectral region 3000–2830 cm−1), in addition to the methyl and methylene bending modes (1480 and 1358 cm−1, respectively), were unchanged upon ingestion of both ZnO NPs and ZnCl2 in comparison to the control group (Figure 1C–F). Moreover, both the relative intensities and positions of the carbonyl ester band of the phospholipids, centered at 1740 cm−1, remained unchanged (Figures 1D and F), which is another confirmation that phospholipid concentrations and conformational states were unaffected. The ratio of the peak heights for the asymmetric stretching of methylene to that of methyl, $H_{\text{max}}(2945–2894\ \text{cm}^{-1})/H_{\text{max}}(2971–2950\ \text{cm}^{-1})$, which is diagnostic of the branching level of the aliphatic chains of lipids, did not vary significantly among the experimental groups (Supplemental Table S2).
The major prominence of the stretching band of the C–O group of ribose, discernible at $\sim 1115\,\text{cm}^{-1}$, which was common to both ZnCl$_2$ and ZnO NPs exposure, could reveal an increased RNA content upon exposure to both Zn sources.

Carbohydrates

While ZnO NPs did not induce detectable effects on the cellular carbohydrate pattern, ZnCl$_2$ induced an increase in the concentration of cellular sugars discernable at $1043\,\text{cm}^{-1}$ related to the C–O stretching of C–OH groups (Table 2). Changes in this peak are commonly related to glycogen alteration (Ozek et al., 2010).

Discussion

The FTIRM of the crustacean digestive glands after the exposure of animals to subtoxic concentrations of ZnO NPs and ZnCl$_2$ (the source of Zn$^{2+}$), revealed distinct biomolecular profiles. In the following paragraphs, we discuss the biological significance of observed changes in the biomolecular profile and conclude by linking them to the organism-level responses. Data on the biomolecular profile are compared to the assimilated amount of Zn in the digestive glands of $P$. scaber to elucidate the contribution of assimilated Zn$^{2+}$ to the observed changes.

Biological explanation of the differences in the spectra generated by the exposure to ZnO nanoparticles and/or ZnCl$_2$

The effects generated by both ZnO NPs exposures and the higher ZnCl$_2$ concentration

Altered protein-to-lipid ratios ($1720–1485\,\text{cm}^{-1}$) most probably due to the increase of protein content ($1312\,\text{cm}^{-1}$) and the increased RNA content ($\sim 1115\,\text{cm}^{-1}$), were present in both ZnO NPs exposures and the higher concentration of ZnCl$_2$. Increased protein content, accompanied by the elevation of RNA, indicates an intensification of the cellular metabolism.

Since there is no such effect present at the lower ZnCl$_2$ exposure and the concentration of the dissolved Zn$^{2+}$ in the case of ZnO was below that at the lower ZnCl$_2$ exposure, the consequences of ZnO NPs exposure must have been predominantly driven by the particulate matter (Table 2).

ZnO nanoparticle-generated effects

Some changes in the biomolecular profile of the digestive gland cells appeared only upon the ZnO NP exposure, but not in the ZnCl$_2$-fed animals. Among them, there are differences in the conformation of some proteins ($\sim 1274\,\text{cm}^{-1}$), which suggests that the activation of metabolic pathways is not activated either in the controls or in the ZnCl$_2$-exposed animals (Table 2).

ZnCl$_2$-generated effects

At the highest exposure concentration of ZnCl$_2$, additional distinctive features in the biomolecular profile were present, which were not detected in any of the ZnO NP exposures. An increased concentration of sugars ($\sim 1043\,\text{cm}^{-1}$), and a desaturation of cell membrane lipids (at $3014\,\text{cm}^{-1}$) were pointed out. An increased carbohydrate concentration implies alterations in carbohydrate metabolism, which is similar to some other studies on mice using the metabolomic approach (Lu et al., 2011). The desaturation of the cell membrane lipids indicates an altered membrane fluidity. Changes in membrane fluidity are common biological responses to temperature stress, salt stress, osmotic stress and/or desiccation (Los & Murata, 2004; Mahajan & Tuteja, 2005); therefore, it is likely that very high metal salt exposure concentrations caused a similar response (Table 2).

The link between biomolecular profile changes and organism-level responses

With the aim to link the biomolecular profile changes with the organism-level responses, we measured feeding parameters, body mass change and mortality. A reduced feeding rate was present only at the highest exposure concentration to ZnCl$_2$ (4000 µg Zn/g of dry food) (Figure 2), while in none of the exposure groups animal mass or mortality were affected. The detected changes in the biomolecular profile were not propagated along the levels of biological complexity, which has already been noted in the literature (Jemec et al., 2010).

A reduction of the feeding rate at 4000 µg Zn/g of dry food in comparison to the control is in line with our previous reports, where feeding behaviour (i.e. food assimilation efficiency) was also affected in a similar experimental set-up (Pipan-Tkalec et al., 2010). This exposure concentration is therefore regarded as an adverse-effect concentration. However, at this exposure, no evidence of lipid peroxidation could be discerned from the biomolecular profile, particularly from the ratios of carbonyl stretching to methyl and methylene deformations ($1740/1456\,\text{cm}^{-1}$) (LeVine & Wetzel, 1998; Vileno et al., 2010). Also, no occurrence of oxidative processes was revealed from the absence of downshifts of the carbonyl band at $\sim 1740\,\text{cm}^{-1}$ or the decreases in the methyl- and methylene-to-carbonyl ratios ($1456/1400\,\text{cm}^{-1}$) (Di Giambattista et al., 2011). Our results show that lipid peroxidation does not necessarily accompany adverse effects at the organism level, although lipid peroxidation is commonly interpreted as an indication of adverse effects at the bimolecular level (Lushchak, 2011).

Bioaccumulation studies were performed in order to assess the bioavailable fraction of Zn for isopods. In the animals that were fed ZnCl$_2$, the Zn concentration in hepatopancreas increased statistically significantly at both exposure concentrations. However, in animals fed ZnO NPs, no Zn assimilation was detected (Figure 3).
This is in accordance with the dissolution assessment, where the dissolved fraction of ZnO NPs in the aqueous suspension was less than 1% (at 1500 and 4000 ZnCl₂). The nominal exposure concentrations of Zn are provided on the x-axis. The symbols on the box plot represent maximum and minimum values (whiskers: ■), mean values ( ), outliers () and p < 0.01 (**); n = number of specimens in each test group.

Conclusions

Our results show that ZnO particulate matter induces biomolecular profile changes in the digestive glands of P. scaber and that Zn²⁺ is not the only reason for the effects of ZnO NPs. This was confirmed by the substantially altered biomolecular profile which was accompanied by a very low assimilated fraction of Zn in the animals exposed to ZnO NPs. The subtoxic ZnO NPs exposure induces digestive gland biomolecular profile changes that are in part particulate-matter specific (distinct protein conformation) and in part a non-specific response to the external stimulus, which is present in both ZnO NPs and ZnCl₂ exposures (increased protein and RNA content).

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Declaration of interest

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References

Aja M, Jaya M, Vijayakumaran Nair K, Joe IH. 2014. FT-IR spectroscopy as a sentinel technology in earthworm toxicology. Spectrochim Acta A 120:534–41.

Barth A. 2007. Infrared spectroscopy of proteins. Biochim Biophys Acta 1767:1073–101.

Beleites C. 2012. hyperSpec: a package to handle hyperspectral data sets in R. R package v. 0.98-20120725. Jena, Germany: Department of Spectroscopy and Imaging, Institute of Photonic Technologies. Available at: http://hyperspec.r-forge.r-project.org/index.html.

Cai S, Singh BR. 1999. Identification of b-turn and random coil amide III infrared bands for secondary structure estimation of proteins. Biophys Chem 80:7–20.

Cai S, Singh BR. 2004. A distinct utility of the amide III infrared band for secondary structure estimation of aqueous protein solutions using partial least squares methods. Biochemistry 43:2541–9.

David CA, Galceran J, Rey-Castro C, Puy J, Companies E, Salvador J, Monné J, Wallace R, Vakourov A. 2012. Dissolution kinetics and solubility of ZnO nanoparticles followed by AGNES. J Phys Chem C 116:11758–67.

Di Giambattista L, Pozzi D, Grimaldi P, Gaudenzi P, Morrone S, Castellano AC. 2011. New marker of tumor cell death revealed by ATR-FTIR spectroscopy. Anal Bioanal Chem 399:2771–8.

Fabian H, Jackson M, Murphy L, Watson PH, Fichtner I, Mantsch HH. 1995. A comparative infrared spectroscopic study of human breast tumors and breast tumor cell lines. Biospectroscopy 1:37–45.

Fu F-N, DeOliveira DB, Trumble WR, Sarkar HK, Singh BR. 1994. Region of the Fourier transform infrared spectroscopy: application to analyze calcium-binding-induced structural changes in calasequin. Appl Spectrosc 48:1432–41.
Movasaghi Z, Rehman S, ur Rehman I. 2008. Fourier transform infrared (FTIR) spectroscopy of biological tissues. Appl Spectrosc Rev 43: 134–79.

Novak S, Drobne D, Vaccari L, Kiskinova M, Ferraris P, Birarda G, et al. 2013. Effect of ingested tungsten oxide (WO₄) nanofibers on digestive gland tissue of Porcellio scaber (Isopoda, Crustacea): Fourier transform infrared (FTIR) imaging. Environ Sci Technol 47: 11284–92.

Odzak N, Kistler D, Behra R, Sigg L. 2014. Dissolution of metal and metal oxide nanoparticles in aqueous media. Environ Pollut 191: 132–8.

Ozek NS, Tuna S, Erson-Bensana AE, Severcan F. 2010. Characterization of microRNA-125b expression in MCF7 breast cancer cells by ATR-FTIR spectroscopy. Analyst 135:3094–102.

Palaniappan PR, Pramod KS. 2010. FTIR study of the effect of nTiO₂ on the biochemical constituents of gill tissues of zebrafish (Danio rerio). Food Chem Toxicol 48:2337–43.

Pesavento M, Alberti G, Biesuz R. 2009. Analytical methods for determination of free metal ion concentration, labile species fraction and metal complexation capacity of environmental waters: a review. Anal Chim Acta 631:129–41.

Phillips KM, Wolf WR, Patterson KY, Sharpless KE, Holden JM. 2007. Reference materials to evaluate measurement systems for the nutrient composition of foods: results from USDA’s National Food and Nutrient Analysis Program (NFNAP). Anal Bioanal Chem 389: 219–29.

Pipan-Tkalec Ž, Drobne D, Jemec A, Romih T, Zidar P, Bele M. 2010. Zinc bioaccumulation in a terrestrial invertebrate fed a diet treated with particulate ZnO or ZnCl₂ solution. Toxicol 269:198–203.

R Core Team. 2013. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing. Available at: http://www.r-project.org/.

Ranke J. 2013. chemCal: Calibration Functions for Analytical Chemistry, R Package Version 0.1-29. Bremen, Germany: University of Bremen. Available at: http://cran.r-project.org/web/packages/chemCal/index.html.

Romih T, Drašler B, Jemec A, Drobne D, Novak S, Golobič M, et al. 2015. Bioavailability of cobalt and iron from citric-acid-adsorbed CoFe₂O₄ nanoparticles in the terrestrial isopod Porcellio scaber. Sci Total Environ 508:76–84.

Vileno B, Jeney S, Sienkiewicz A, Marcoux PR, Miller LM, Forro L. 2010. Evidence of lipid peroxidation and protein phosphorylation in cells upon oxidative stress photo-generated by fullerols. Biophys Chem 152:164–9.

Whelan DR, Bambery KR, Heraud P, Tobin MJ, Diem M, McNaughton D, Wood BR. 2011. Monitoring the reversible B to A-like transition of DNA in eukaryotic cells using Fourier transform infrared spectroscopy. Nucleic Acids Res 39:5439–48.

Xu M, Li J, Hanagata N, Su H, Chen H, Fujita D. 2013. Challenge to assess the toxic contribution of metal cation released from nanomaterials for nanotoxicology – the case of ZnO nanoparticles. Nanoscale 5: 4763–9.

Supplementary material available online
Supplementary Figures S1 - S5, Method description S1 to S3 and Table S1 and S2