SUPPLEMENTARY MATERIAL FOR THE MANUSCRIPT:

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

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The Supplementary material comprises:

Figure S1. Scanning electron micrograph of the ZnO nanopowder from the supply (Sigma Aldrich, product no. 544906) and the dried ZnO suspension after the application onto hazelnut leaves.

Figure S2. The distributions of the hydrodynamic radius of particles, R_h , in the supernatants from the dispersions of ZnO nanoparticles.

Figure S3. Cryosectioned digestive gland of *P. scaber*, treated with ZnO NPs in the concentration of 1500 $\mu\text{g Zn/g dry leaf}$, as seen under Hyperion 3000 Vis-IR microscope (SISSI Beamline, Elettra-Sincrotrone Trieste).

Figure S4. Spectral contribution of the Jung tissue freezing medium to the spectra of the *P. scaber* digestive gland tissue.

Figure S5. Spectral heterogeneity dendrogram of the sampled points of control animals as obtained by HCA on vector-normalized absorbance spectra in the 4000–900 cm^{-1} region.

Table S1. Zn concentration on the Zn-spiked leaves used for feeding experiments, before and after the experiments.

Table S2. Spectral band ratios and their attributions, accordingly to the clusters identified by HCA on controls and treated samples.

Method description S1: The dynamic light scattering analysis of the nanoparticle suspensions.

Method description S2: Quality control of ZnO NPs and ZnCl_2 application onto leafs.

Method description S3: FTIRM data collection, preprocessing and analysis.

Figure S1

Figure S1. Scanning electron micrograph of the ZnO nanopowder from the supply (Sigma Aldrich, product no. 544906) at the (A) 150,000x magnification (the size bar represents 100 nm) and (B) a piece of hazelnut leaf onto which ZnO nanoparticles at the concentration 4000 $\mu\text{g Zn/g}$ dry mass were applied (5000x; the size bar represents 1 μm). Photo A was taken with a scanning electron microscope (JSM-7500F, JEOL, Japan) at the Department of Biology, Biotechnical Faculty, Ljubljana, under the accelerating voltage of 5 kV. Photo B was taken with a thermal field-emission scanning electron microscope (JSM-6500F JEOL, Japan) at the Institute of Metals and Technology, Ljubljana, under the accelerating voltage of 15 kV.

Method description S1: The dynamic light scattering analysis of the nanoparticle suspensions

The DLS spectrometer was operated in the 3D cross-correlation mode (Urban and Schutenberger, 1998). In the latter geometry, two parallel beams (one above another) of the coherent incident light are obtained using a beam splitter situated between the laser and the sample. The beams are focused in the centre of the studied sample and the scattered light is collected with two detectors (also one above another). 3D cross-correlation is especially developed for studying strongly scattering samples, ZnO NP dispersions in our case, in order to suppress multiple scattering (Urban and Schutenberger, 1998). In our experiment, two coherent incident light beams were generated with a 20 mV He-Ne laser (Uniphase JDL 1145P), operating at $\lambda_0 = 632.8$ nm. The instrument is equipped with a laser attenuation system combined with an online incident laser intensity measurement that allows for the normalization of the static LS data. Correlation functions of the scattered light intensity, $G_2(t)$, were recorded at an angle of 90° .

$G_2(t)$ was then converted into a correlation function of the scattered electric field, $g_1(t)$, using the Siegert's relationship (Brown, 1993; Schärfl, 2007):

$$|g_1(t)| = \beta^{\frac{1}{2}} \left\{ \left(\frac{G_2(t) - G_2(\infty)}{G_2(\infty)} \right)^{\frac{1}{2}} \right\} \quad (\text{S1})$$

where $G_2(\infty)$ is the experimentally determined baseline and β is the coherence factor determined by the geometry of the detection.

For monodisperse particles small in diameter compared to the wavelength of light as well as for hard spheres of any size, the relaxation (or decay) time of $g_1(t)$, τ , is related to the relaxation rate of $g_1(t)$, Γ , and the translation diffusion coefficient, D , by the relationships:

$$|g_1(t)| = e^{-\frac{t}{\tau}} = e^{-\Gamma t} = e^{-Dq^2 t} \quad (\text{S2})$$

and

$$\Gamma = \tau^{-1} = Dq^2 \quad (S3)$$

The hydrodynamic radii, R_h , of colloidal particles are then obtained from the diffusion coefficient D via the Stokes-Einstein equation:

$$R_h = \frac{kT}{6\pi\eta_0 D} \quad (S4)$$

where k is the Boltzmann constant, T is the absolute temperature, and η_0 is the solvent (water) viscosity.

Equation S4 is strictly valid for monodisperse hard spheres. For polydisperse samples, the correlation functions are analyzed with an inverse Laplace transform program CONTIN (Provencher, 2001) by fitting the correlation curves with a sum of a limited number of exponents (in our case, up to 50).

$$g_1(t) = \sum_i A(\tau_i) e^{-\frac{t}{\tau_i}} \quad (S5)$$

where $A(\tau_i)$ is the weight of the corresponding exponent with decay time τ_i . Weight $A(\tau_i)$ is proportional to the LS intensity scattered by the i^{th} -particle. Decay times τ_i are converted into R_h using Equations S3 and S4 and presented as distributions over the hydrodynamic radii. Each scattering species of radius $R_{h,i}$ is represented in this distribution by its relative contribution, i.e. by the amplitude $A(R_i)$, to the total intensity of scattered light.

We collected 5 correlation functions with 60 s scan length. Each correlation curve was analyzed independently and compared with the averaged curve to ensure that the mathematical solution provided by CONTIN was correct. The results are presented in the **Figure S2**.

Figure S2. Unweighted (black line), mass-weighted (magenta line) and number-weighted (green line) distribution of hydrodynamic particle radius, R_h , in the supernatant from the dispersions of **(A)** ZnO nanoparticles with the concentration 1500 $\mu\text{g Zn/mL}$, and **(B)** ZnO nanoparticles with the concentration 4000 $\mu\text{g Zn/mL}$, measured using dynamic light scattering and analyzed by the CONTIN software.

Method description S2: Quality control of ZnO NPs and ZnCl₂ application onto leafs

Accuracy and precision of the AAS measurements

The certified concentration of Zn in the reference material was 180±6 mg/kg; our measurement was 180±4 mg/kg (mean±SD, n = 60), $Z' = -0.08$. Precision was always within 3% (relative standard deviation).

Metal spiking recovery

The measured Zn concentrations in the separately spiked leaves corresponded to the nominal values within 15 % for all test chemicals and concentrations. Recovery for ZnO NPs was ≥ 75 %, which we consider good, given the fact that a small share of NPs tends to fall off the leaves due to animal movement inside the Petri dishes and the manipulation of the leaves during the experiment (especially during the collection of fecal pellets). For the ZnCl₂-spiked leaves, the recovery appears to be >100 %; however, this occurrence is not a consequence of the methodological error. It is difficult to ensure an even application of test chemicals onto dry leaves as their surface is not completely flat. The liquid gathers somewhat more in the dents and around the leaf veins than on the protruding parts of the surface. Since *P. scaber* are able to sense metals in the food (Zidar et al., 2003, 2009), they avoid the parts with the highest metal concentration. The leaf remnants after the experiment therefore contain slightly higher metal concentration than the uneaten pieces of leaves before the experiment.

Table S1. Zn concentration on the Zn-spiked leaves used for feeding experiment, before and after the experiment.

Chemical	Nominal Zn concentration on leaves ($\mu\text{g/g}$ dry mass)	Measured Zn concentration in the separately spiked leaves^a ($\mu\text{g/g}$ dry mass, mean $\pm$ SD, n = 10)	Measured Zn concentration in the leaves after the experiment ($\mu\text{g/g}$ dry mass, mean $\pm$ SD, n = 10)	Recovery^b (%)
Control (deionized H ₂ O)	/	60 $\pm$ 5	56 $\pm$ 26	/
Nano ZnO	1500	1593 $\pm$ 340	1249 $\pm$ 366	78
	4000	4501 $\pm$ 840	3360 $\pm$ 1102	75
ZnCl ₂	1500	1734 $\pm$ 224	1845 $\pm$ 410	106
	4000	4121 $\pm$ 340	4767 $\pm$ 609	116

^a These leaves were prepared in the same way as for the feeding experiments (see the Experimental section of the manuscript), but they were not subjected to the experimental conditions. Instead, they were directly acid digested and measured by flame AAS.

^b The recovery was calculated as the fraction between the mean concentration of Zn in the leaves after the experiment and the mean concentration of Zn in the separately spiked leaves, and expressed in percentages.

Method description S3: FTIRM data collection, preprocessing and analysis

Figure S3. Cryosectioned digestive gland of *P. scaber*, treated with ZnO NPs in the concentration of 1500 μg Zn/g dry leaf, as seen under Hyperion 3000 Vis-IR microscope (SISSI Beamline, Elettra-Sincrotrone Trieste). (A) Section marked with 15x15 μm measurement areas (red squares with a plus sign). (B) The same section as in (A), where measurement areas have been categorized into the “peripheral” (green labels), “central” (blue labels) and “apical” (violet labels) regions. Numbers on the labels denote the sequence of marking. Dark blue line denotes the tissue border.

Quality criteria for subsetting the acquired FTIRM spectra for further analysis. Each collected spectrum was preprocessed using the atmospheric compensation routine of OPUS 7.0 (Bruker Optics GmbH, Ettlingen, Germany) to minimize the spectral contributions from water vapor and CO_2 . All of the cell spectra were separately subjected to quality tests described as follows.

The first quality criterion: signal intensity. Atmospheric corrected cellular spectra were separately subjected to a quality test based on an intensity criterion. To implement the

criterion, we chose the wavelength range 1707–1480 cm^{-1} . Amide I and Amide II bands dominate this spectral range, and they are related to the vibrational modes of amide linkage of cellular proteins. Only cells having an integral area intensity of those bands higher than the arbitrary chosen value of 8.0 arbitrary units (a.u.) were further post-processed. By applying this intensity criterion, cells showing a biomolecular content too low, possibly as a consequence of fracturing phenomena due to sample preparation (freezing and cutting) were excluded from the following data processing steps.

The second quality criterion: spectral contribution of the tissue freezing medium.

Another quality criterion was then applied in order to take into account the penetration of the tissue-freezing medium (TFM) within the sample. Penetration of the TFM is generally minimal; it depends on several parameters such as tissue type, dragging effects during cutting and tissue freezing conditions. However, this phenomenon cannot be ignored, as already demonstrated by the authors (Novak et al., 2013): Jung tissue freezing medium has several vibrational bands that overlap with sample diagnostic bands, and the extent of the penetration can bias the experiment results (**Figure S4A**). Among the TFM bands, the one centred at 2945 cm^{-1} , related to the stretching of CH moieties, can be easily discerned from the characteristic specimen bands, especially looking to the second derivative of the spectra (**Figure S4B, S4C**): a negative peak characterizes the pure freezing medium, while a positive one the pure sample. The height of the peak in the 2956–2932 cm^{-1} spectral region of the second derivative of the spectra (Savitzky-Golay algorithm, 13 smoothing point) was therefore evaluated for all the acquired spectra, and those spectra exhibiting a negative height value were excluded for the data analysis, since undoubtedly affected by TFM contamination. External regions were the ones more severely affected by the penetration and almost 40% of the collected spectra were excluded upon application of the two criteria, while more than 80% of the acquired spectra in the interior regions were taken into account for multivariate data analysis.

Figure S4. (A) Absorbance spectra of the pure Jung tissue freezing medium (TFM, black continuous line) of a sample region showing a clear contamination form TFM (black dotted line, Sample 2), and of a sample region virtually free from TFM contamination (gray continuous line, Sample 1). (B) Second derivative spectra (Savitzky-Golay algorithm, 13 smoothing points) of Sample1, Sample 2 and pure TFM. (C) Larger view of the

panel **(B)** in the spectral region 3050–2800 cm^{-1} . The spectral contribution arising from the TFM centred at 2945 cm^{-1} is highlighted.

Homogeneity of molecular profile among individuals of the same exposure regimen.

The selected sampled-areas of control animals were first analyzed in order to verify the influence of the inherent animal diversities on the variability of the spectroscopic data. HCA on vector-normalized absorbance spectra in the 4000–900 cm^{-1} region (Euclidean distances and Ward’s classification algorithm) gave a clear separation between peripheral, central and apical regions, independent on the animal (**Figure S5**). This result highlights that the spectroscopic signatures of peripheral, central and apical regions of hepatopancreatic cells are comparable among different animals. Therefore, the effects of both NPs and ions were analyzed independently for the three regions of the specimens.

Figure S5. Spectral heterogeneity dendrogram of the sampled points of control animals as obtained by HCA on vector-normalized absorbance spectra in the 4000–900 cm^{-1} region. Spectra belonging to the same sample areas classify in clearly separated clusters: black- peripheral region, light grey - central region, dashed pattern - apical region.

Tissue compositional analysis according to the relative intensities of vibrational bands. Data processing has been implemented by comparing the relative intensities of several diagnostic bands, in order to highlight the compositional modification at the level of apical region of hepatopancreatic cells induced by ingestion of Zn compounds. The intensity of a spectral band is related to the concentration of the vibrating group through the Beer-Lambert law. Once vector-normalized, the dependence of the spectral band intensity from the local sample thickness is minimized, and almost pure information on the abundance of biomolecules can be revealed. However, relative abundance of biomolecules is usually considered to further improve the reliability of data, obtained by rationing the intensity of the spectral bands of interest (**Table S2**).

Table S2. Spectral band ratios and their attributions, accordingly to the clusters identified by HCA on controls and treated samples. Mean value $\pm$ standard deviations are reported. The significance level, p , has been determined by Unpaired Student's t-test [*** $p < 0.001$]

Ratio of the area integral (A/A) or peak height ($H_{\max}/H_{\max}$) of FTIRM diagnostic bands	Attribution	Control (mean $\pm$ SD, n = 64)	ZnCl ₂ fed animals 4000 μ g Zn/g (mean $\pm$ SD, n = 203)	p	ZnO fed animals 4000 & 1500 μ g Zn/g (mean $\pm$ SD, n = 356)	p
A(1720-1485cm ⁻¹)/ A(3000-2830cm ⁻¹)	Protein to lipid relative content	2.0 $\pm$ 0.1	2.4 $\pm$ 0.3	***	2.343 $\pm$ 0.268	***
$H_{\max}$ (2945-2894cm ⁻¹)/ $H_{\max}$ (2971-2950cm ⁻¹)	Branching level of the aliphatic chains of lipids	1.73 $\pm$ 0.18	1.8 $\pm$ 0.3	Not sign.	1.8 $\pm$ 0.2	Not sign.
A(1770-1712cm ⁻¹)/ A(1580-1485cm ⁻¹)	Amide I to Amide II ratio (changes among structural and new synthesis proteins)	2.85 $\pm$ 0.07	2.9 $\pm$ 0.3	Not sign.	2.9 $\pm$ 0.4	Not sign.

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