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Neurotoxic potential of ingested ZnO nanomaterials on bees

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HIGHLIGHTS

- ZnO NMs and $ZnCl_2$ increase mortality and also AChE and GST activity in bee brains.
- $ZnCl_2$ increases feeding rate in honey bees.
- AChE activity was the highest and survival the lowest after exposure to $ZnCl_2$.
- Elevated activity of AChE is a compensatory adaptation to ZnO NMs or Zn^{2+} exposure.
- We relate the observed effects primarily to Zn^{2+} ions.

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ABSTRACT

The honey bee is among most important pollinators threatened by environmental pollution, pest control and potentially, by products of nanotechnologies. The aim of the current study was an analysis of the neurotoxic potential of ingested zinc oxide nanomaterials (ZnO NMs) or zinc ions (Zn^{2+}) on honey bees. We analysed a variety of biomarkers, including metabolic impairment, feeding rate, and survival, as well as the activities of a stress-related enzyme glutathione *S*-transferase, and the neurotoxicity biomarker acetylcholinesterase. Acetylcholinesterase activity was found to be elevated in bees exposed to either of the tested substances. In addition, we observed increased feeding rate in the group treated with Zn^{2+} but not with ZnO NMs or control group. The observed effects we relate primarily to Zn^{2+} ions. Here we provide evidence that zinc ions either originating from Zn salt or Zn-based NPs have a neurotoxic potential and thus might contribute to colony survival.

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1. Introduction

Honey bees (*Apis mellifera*), are important pollinators and producers of honey and wax. Environmental pollution and pest control are known to enhance colony collapse disorder and cause massive death in honey bee colonies (van Engelsdorp et al., 2009), and this poses a threat to global agriculture (Grossman, 2013). For example, compounds with neurotoxic potential in bees are suspected of causing devastating consequences at low exposure concentrations. Farooqui (2013) hypothesised that chronic exposure of honeybees to low doses of certain pesticides disrupts neuronal cholinergic and octopaminergic signalling, affecting orientation and navigation abilities. He linked exposure to pesticides, olfactory learning and memory to colony collapse disorder. Similarly, Williamson et al. (2013) related altered activity of the cholinergic nervous system to physiological alterations and behavioural changes in honey bees.

The recent introduction of nanotechnology products and their inevitable release into the environment may also result in harmful effects on honey bees (Mueller and Nowack, 2008). Many of the currently available nanomaterial (NM)-containing consumer products contain ZnO or TiO_2 NMs (Nowack et al., 2012) and the likelihood that ZnO NMs are released into the environment is constantly increasing (Gottschalk et al., 2009; Miller et al., 2010; Ates et al., 2013). Zinc oxide NMs can be found in many different products

Abbreviations: AChE, acetylcholinesterase; NM, nanomaterial; GST, glutathione *S*-transferase; GSH, glutathione.

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such as optoelectronic devices (Zheng and Li, 2009), sunscreens, paint pigments, rubber components, cosmetics, food additives, and medicines (Zhu et al., 2013). Such abundance of products in global use which are containing ZnO NMs raises sensitivity concerning their toxic potential (Chang et al., 2012). A review of the literature concerning the toxicity of manufactured ZnO NMs to different taxa including bacteria, algae and plants, aquatic and terrestrial invertebrates and vertebrates (Ma et al., 2013) suggests a relatively high measured acute toxicity of ZnO NMs (in the low mg L^{-1} levels), dependent upon test species, physicochemical properties of the material and the test methods. It has been reported that generation of reactive oxygen species by dissolved ionic zinc from NMs, or by NMs themselves, is a common mode of the action of ZnO NMs in all species tested. We could not find reports on neurotoxic effects of ZnO or Zn ions on bees.

A key enzyme in the cholinergic nervous transmission is acetylcholinesterase (AChE; EC 3.1.1.7.). This is a serine protease that hydrolyses the neurotransmitter acetylcholine. Consequently, its activity is used as neurotoxicity biomarker for anticholinesterase chemicals such as organophosphorus and carbamate insecticides as well as other classes of environmental contaminants including complex mixtures of metals, detergents and other organic pollutants (Badiou et al., 2008; Badiou-Bénéteau et al., 2012; Abdel Rasoul et al., 2013; Carvalho et al., 2013; Boily et al., 2013). In insects, AChE is widely distributed in the brain, the thoracic and abdominal segments and the abdominal ganglia (Thany et al., 2010). Measurement of AChE activity is the most frequently used method for estimating the cholinergic function of an organism's nervous system. According to numerous published reports, alterations in AChE activity in invertebrates and vertebrates can be linked to oxidative stress, alterations in membrane biophysical characteristics, deregulation of cell signalling or impairment of neurotransmission (Badiou et al., 2008; Bainy et al., 2006; Carageorgiou et al., 2003; Gonçalves et al., 2012; Kaizer et al., 2005; Kumar 1998, 1999; Romani et al., 2005, Wilczek et al., 2003).

An intrinsic feature of all organisms exposed to pollutants is the ability to adapt to certain amounts of pollutants by turning on a variety of compensatory or detoxification mechanisms or enzymes, including glutathione-*S*-transferase (GST, EC: 2.5.1.18). Therefore, GST activity is a frequently measured marker of metabolic state of organism (Van der Oost et al., 2003; Diao et al., 2006). GST is a phase-II detoxifying enzyme that can be induced by numerous chemicals because of its active role in the detoxification of exogenous as well ad endogenous substances. It can metabolize insecticides by facilitating their reductive dehydrochlorination or by conjugation reactions with reduced glutathione, to produce water-soluble metabolites that are more easily excreted (Papadopoulos et al., 2004; Enayati et al., 2005). GST activity in bees was already used as an enzymatic biomarker to assess environmental quality (Carvalho et al., 2013; Badiou-Beneteau et al., 2013).

In the work presented here we examined the toxic potential of ZnO NMs and Zn^{2+} in honey bees after chronic dietary exposure by analysing survival and feeding rate as well as the activities of a stress-related detoxifying enzyme, glutathione *S*-transferase (GST), and the neurotoxicity biomarker acetylcholinesterase (AChE). The toxic potential of ZnO NMs and Zn^{2+} to bees is discussed along with actual threat of nanoparticles to bees.

2. Materials and methods

2.1. Test animals

Bee colonies (*Apis mellifera carnica*, Pollman 1879) were maintained in the field at the Biotechnical Faculty, University of Ljubljana, Slovenia according to standard commercial techniques. Adult bees were collected randomly during autumn of 2012 inside the hive using a mouth aspirator and then transferred to wooden cages. Before the treatments, the bees were subjected to a starvation period for 1 h. Groups of 36–44 adult bees were transferred to wooden cages and supplied *ad libitum* with water and 1.5 M sucrose solution in gravity feeders. The bees were maintained in cages for 1 h before treatment with ZnO NMs or Zn^{2+}. We used 244 bees for the chronic experiment.

2.2. Chemicals, solutions and suspensions

The following chemicals were purchased from Sigma Aldrich (Germany): ZnO nano powder (particle size < 100 nm), sucrose, dibasic and monobasic potassium phosphate, 1-chloro-2,4-dinitrobenzene, *L*-glutathione (reduced form), 5,5′-dithiobis-2-nitrobenzoic acid, sodium hydrogen carbonate, acetylthiocholine chloride, sodium sulphate and ethylenediamine tetraacetic acid. BCA Protein Assay Reagent A and Reagent B were purchased from Pierce (USA). All chemicals were of the highest commercially available grade, typically 99% or higher.

A suspension of the zinc oxide nanomaterials (ZnO NMs) was prepared by adding ZnO nano powder to milli-Q water with sonication (PIO Iskra, Sonnys 2GT; 40 kHz, 2 × 100 W) of the suspension for 24 h. Sucrose ZnO NMs suspension was prepared in 1.5 M sucrose in a final concentration of 1 mg ZnO mL^{-1}, corresponding to 0.8 mg Zn mL^{-1}. An aqueous solution of zinc ions (Zn^{2+}) with a known toxic potential for invertebrates (Muyssen et al., 2006) was used for comparison to the Zn NMs treatment. $ZnCl_2$ was dissolved in 1.5 M sucrose to obtain a final concentration of 1 mg $ZnCl_2$ mL^{-1}, corresponding to 0.48 mg Zn mL^{-1}. The Zeta-potential and conductance of a control sucrose solution, a Zn^{2+} solution and suspension of ZnO NMs were measured using Zeta-Pals–Brookhaven Instruments Corporation, US (Table 1). Zeta-potential is the electrokinetic potential in colloidal systems (McNaught and Wilkinson, 1997), and this parameter is used for the measurements of the stability of suspensions. These two parameters, zeta potential and conductivity were used to characterise the suspension of ZnO tested. Both parameters were measured prior the experiment. Ringer solution for insects, case modified (Vergoz et al., 2007) was prepared by mixing 10 volumes of base solution (128 mM sodium chloride, 1.7 mM sodium bicarbonate, 4.7 mM potassium chloride, 1.9 mM calcium chloride, 13.8 mM glucose) and 1 volume of 150 mM phosphate buffer pH 7.0.

2.3. Honey bee exposure to ZnO or Zn^{2+} via food

In a 10-day chronic exposure experiment, the 244 bees received Zn^{2+} solution, ZnO NMs suspension or only sucrose solution *via* gravity feeders. The feeders were placed at the top of the cages and were extended almost to the bottom. The bees were divided into 6 groups. Each group of bees was placed into separate wooden cage (9.5 × 4 × 7.5 cm). Two cages with a control group of 88 bees altogether were fed with 1.5 M sucrose solution, two groups of altogether 84 bees received a suspension of sucrose ZnO NMs (0.8 mg Zn mL^{-1}), and two groups with altogether 72 bees were fed with a sucrose Zn^{2+} solution containing 0.48 mg Zn mL^{-1}. The cages were placed in an incubator at 27 °C and 95% relative humidity.

2.4. Feeding rate and survival

Feeding rate and survival were used to monitor the stress level induced by ZnO NMs or by $ZnCl_2$ in the food. The number of dead bees was counted daily. These data were used to calculate survival

Table 1
Characteristics of tested Zn-containing solutions and suspensions. Characteristics (zeta-potential, conductance) of sucrose suspension of ZnO NMs (0.8 mg Zn mL^{-1}) and Zn^{2+} (0.48 mg Zn mL^{-1}) solution.

Treatments	Concentration (mg Zn mL^{-1})	Zeta potential ζ (mV)	Standard deviation σ	Conductance (μS)
Control	0	/	/	30–40
Zn NMs suspension	0.8	−5.70	1.35	51
$ZnCl_2$ solution	0.48	/	/	842

during the 10 day exposure period. Survival plots were compared between experimental groups and expressed in percent of maximal survival. The feeding rate was calculated by dividing the volume of consumed food per day per animal. Volume of consumed suspension/solution was recorded from the gravity feeders (5 mL graded syringe) daily. The gravity feeders were refilled on daily bases from original solution or suspension. Before refilling the syringe, the ZnO suspension was vortexed.

2.5. Brain dissection and homogenisation

After the exposure period honey bees were decapitated and brains were isolated by a modification (Carreck et al., 2013) of the method reported by Dade (1962). After isolation, individual brains were weighted and submersed into a droplet of honey bee Ringer solution. The brains were then stored at −E20 °C. Thawed brains were homogenised individually using a glass stick in 200 μL of 100 mM potassium phosphate buffer (pH 7.4) per sample. Homogenates were centrifuged for 15 min at 12 092 g and 4 °C, and finally, the supernatants were stored at −20 °C.

2.6. Enzyme activity assays

Protocols for measuring AChE and GST activities were performed as described by Jemec et al. (2007). Enzyme activities were assessed only in bees that survived the treatment with ZnO NMs or Zn^{2+}.

2.6.1. AChE activity analyses

AChE activity was analysed by the method of Ellman et al. (1961), using a VIS microplate reader (Anthos, UK). The reaction mixture was prepared in 100 mM potassium phosphate buffer (pH 7.4) containing acetylthiocholine chloride and 5,5′ dithiobis-2-nitrobenzoic acid at final concentrations of 1 mM and 0.5 mM, respectively. Protein supernatant (10 μL) was added to 190 μL of the reaction mixture to start the reaction, which was followed spectrophotometrically at 405 nm and 25 °C for 5 min. A blank reaction was performed by replacing the protein supernatant with 10 μL of 100 mM potassium phosphate buffer (pH 7.4). Specific AChE activity was expressed in nmoles of hydrolysed acetylcholine chloride min^{-1} mg^{-1} protein, while the tissue AChE activity was expressed in nmoles of hydrolysed acetylcholine chloride min^{-1} mg^{-1} tissue (extinction coefficient, ε_{405} = 13 600 M^{-1} cm^{-1}).

2.6.2. GST activity analyses

GST activity was analysed by the method described by Habig et al. (1974) using a VIS microplate reader (Anthos, UK). 1-Chloro-2,4-dinitrobenzene was dissolved in ethanol to obtain a 50 mM solution, which was afterwards diluted with 100 mM potassium phosphate buffer (pH 6.5) to a final concentration of 4 mM. This solution (50 μL) was mixed with 100 μL of 2 mM reduced glutathione in 100 mM potassium phosphate buffer (pH 6.5), and with 50 μL of the protein supernatant to start the reaction. A blank reaction was performed by replacing the protein supernatant with 50 μL of 100 mM potassium phosphate buffer (pH 7.4). The final concentration of ethanol in the mixture was 2%, a concentration at which the activity of GST was not inhibited (Jemec et al., 2007). The reaction was followed spectrophotometrically at 340 nm and 25 °C for 3 min. Specific GST activity was expressed in nmoles of conjugated GSH min^{-1} mg^{-1} protein, while the tissue AChE activity was expressed as nmoles of conjugated GSH min^{-1} mg^{-1} tissue (extinction coefficient, ε_{340} = 9600 M^{-1} cm^{-1}).

Protein concentration in supernatants obtained from individual homogenised bee brains was analysed with a BCA™ Protein Assay Kit, which uses a modification of the bicinchoninic acid protein assay (Pierce, Rockford, IL, US). The results were expressed either as specific or as tissue activities, obtained by normalising the enzyme activities to the quantity of proteins or tissue (honey bee brains), respectively. Tissue activity reflects the actual variation of the protein in the tissue (Badiou-Bénéteau et al., 2012; Boily et al., 2013).

2.7. Statistical analysis

For statistical analysis, a one way ANOVA test (95% confidence limits, Bonferroni *post hoc* test) was used to compare variation and means of enzyme activity and protein content between control and test groups. Data were calculated using OriginPro software (OriginPro 8, OriginLab Corporation, US). Difference in feeding rate between experimental groups and treatments were done using Kruskal–Wallis and Wilcoxon test (OriginPro 8, OriginLab Corporation, US). Survival analysis were performed using OASIS online tool: http://sbi.postech.ac.kr/oasis/introduction/ as described by Yang et al. (2011). The restricted mean lifespan (50% lethal time, LT50) and standard error in experimental groups and Log rank test between groups are reported.

3. Results

3.1. Characteristics of tested solutions and suspensions

The zeta-potential and conductance of the ZnO NMs suspension and Zn^{2+} solution (Table 1) were measured to evaluate solution/suspension properties. Electromobility measurements of ZnO NMs suspension show a low negative zeta-potential (−5.7 mV), which classifies it as unstable and rapidly coagulating (Hanaor et al., 2012). On the other hand, Zn^{2+} solution is not a colloidal system and therefore has no zeta-potential. The conductance of a suspension of ZnO NMs is only slightly different comparing to control sucrose solution, while the conductance of the Zn^{2+} solution is 16.8 and 24-fold higher than the ZnO suspension and the control solution, respectively. Low conductance measured for ZnO NMs suspension means low ion dissociation from the aggregate surface. This data explain low dissolution of ZnO NMs in the suspension.

3.2. Feeding rate and survival

During the chronic exposure to ZnO NMs or Zn^{2+}, the survival rate in both treated groups decreased significantly compared to the control (Log rank test: [ZnO Nms] χ^2 = 10.20, p = 0.0014; [Zn^{2+}] χ^2 = 67.83, p < 0.001; Fig. 1A). The 19.5% decrease in the sur-

vival of the group of honey bees treated with ZnO NMs was observed at the end of the experiment. In the group of honey bees treated with Zn^{2+} a considerable decline in the survival was observed earlier; only 50.9% survived after five days. At the end of the experiment 67.2% of bees treated with Zn^{2+} died. The restricted mean lifespan (LT50, based on survival analysis) was 9.63 ± 0.16 days for control bees, 9.06 ± 0.22 days for bees treated with Zn^{2+}, and 6.93 days for bees treated with ZnO NMs. The LT50 values for controls and ZnO NMs-treated bees should be taken with reserve since the survival in both groups was higher than 50% (75% and 93%, respectively). In Zn^{2+} – treated group, 31% of bees survived, which enabled the right estimation of the median life span.

The feeding rate increased only in the group treated with Zn^{2+} starting after the fourth day of the experiment, although transient decline was also observed between the fifth and eight day. Feeding rate in both control and ZnO NMs treated group in general decreased daily. Feeding rate pattern in Zn^{2+} treated group was significantly different from other two groups (Kruskal–Wallis, $H = 7.1$, $df = 2$, $p = 0.029$) (Fig. 1B). There were no significant difference between two repetitions in each experimental group, either for the survival or feeding rates (Wilcoxon test, $p < 0.05$). Although the bees treated chronically with Zn^{2+} and ZnO NMs displayed signs of overall lower motor ability (visual observation), the bees were not impaired in a way that they were not able to move and eat. This impairment was observed mainly at the last (10th) day of the experiment in the bees treated chronically with either Zn^{2+} or ZnO NMs, however the higher rate of food consumption was detected even before.

3.3. Total protein content and enzyme activities after exposure to tested substances

A significant decline of brain total protein content (Fig. 2A) was detected in bee brains after the chronic treatment of bees with ZnO NMs (average concentration = 0.55 mg mL^{-1}) or Zn^{2+} (average concentration = 0.5 mg mL^{-1}), compared to the average concentration of proteins in bee brains of control samples, which was 0.84 mg mL^{-1}. Similarly, the weight of whole honey bee brains (Fig. 2B) significantly decreased after exposure to ZnO NMs (average weight = 2 mg) or Zn^{2+} (average weight = 1.7 mg), in comparison to the control (2.7 mg) (ANOVA, $p < 0.05$).

Chronic exposure to ZnO NMs or Zn^{2+} caused a significant increase (ANOVA, $p < 0.05$) in the activity of both AChE and GST. The specific activity of AChE in bee brains (Fig. 3A) increased more in groups treated with Zn^{2+} solution, with an average value of 5.5 nmol product min^{-1} mg^{-1} protein compared to 3.16 and 2.0 nmol product min^{-1} mg^{-1} protein in groups treated with ZnO NMs and in control samples, respectively. Compared to 0.46 nmol product min^{-1} mg^{-1} protein in control samples, GST specific activity levels (Fig. 3B) were significantly elevated, and this elevation was more pronounced in groups treated with Zn^{2+} in comparison to the groups treated with ZnO NMs (1.9 and 1.7 nmol product min^{-1} mg^{-1} protein, respectively). An increase in the activity of both enzymes in treated groups was obtained also by measuring their tissue activities (Fig. 3C and D). The tissue AChE activity of control groups (0.5 nmol min^{-1} mg^{-1} tissue), was found to be significantly lower (ANOVA, $p < 0.05$) than the tissue AChE activities in groups treated with ZnO NMs or Zn^{2+} (1.3 and

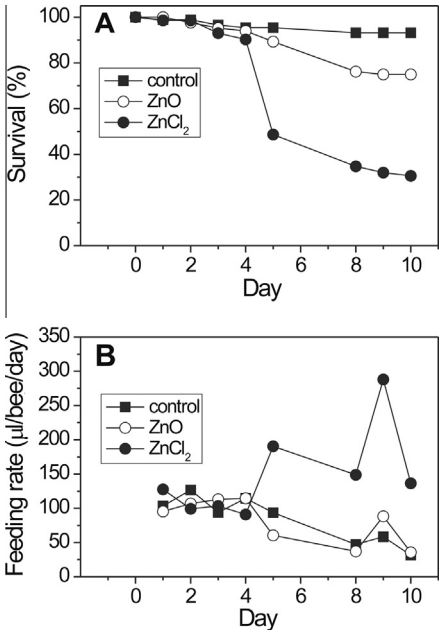

Fig. 1. Effects of ZnO NMs or Zn^{2+} on honey bee survival (A) and feeding rate (B). (A) Survival curves of honey bees chronically exposed to ZnO NMs suspension (0.8 mg Zn mL^{-1}) or Zn^{2+} solution (0.48 mg Zn mL^{-1}) in 1.5 M sucrose as compared to control (1.5 M sucrose). The lowest survival was in Zn^{2+} group and differed significantly (Log-Rank Test) from ZnO NMs group ($\chi^2 = 32.73$, $p < 0.001$) and from control ($\chi^2 = 67.83$, $p < 0.001$). ZnO NMs group had lower survival then the control group ($\chi^2 = 10.20$, $p = 0.0014$). (B) Feeding rate (the volume of consumed food per day per animal) in honey bees after chronic exposure to ZnO NMs (ZnO, 0.8 mg Zn mL^{-1}) or $ZnCl_2$ (0.48 mg Zn mL^{-1}) in 1.5 M sucrose as compared to control (1.5 M sucrose). There was a significant difference between feeding rate patterns of experimental groups (Kruskal–Wallis $H = 7.1$, $df = 2$, $p = 0.029$). Feeding rate pattern of $ZnCl_2$-treated group was significantly different from the control (Wilcoxon test: $z = 2.31$, $p = 0.010$) and ZnO NM (Wilcoxon test: $z = 2.36$, $p = 0.009$).

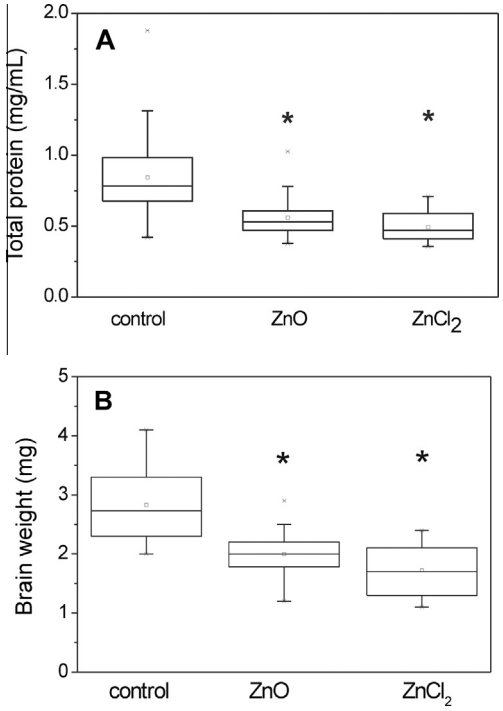

Fig. 2. Effects of ZnO NMs or Zn^{2+} on total protein content (A) and on weight of honey bee brains (B). Total protein content was obtained from aqueous extracts of homogenised isolated bee brain tissue after chronic exposure to 1.5 M sucrose suspension of ZnO NMs or solution of Zn^{2+}. The control group received only 1.5 M sucrose solution. The number of bees per treatment was 88 in controls, 84 in bees treated with ZnO NMs (0.8 mg Zn mL^{-1}), and 72 in bees treated with $ZnCl_2$ (0.48 mg Zn mL^{-1}). * – Statistically important difference compared to control (one way ANOVA, Bonferroni *post hoc* test, $p < 0.05$).

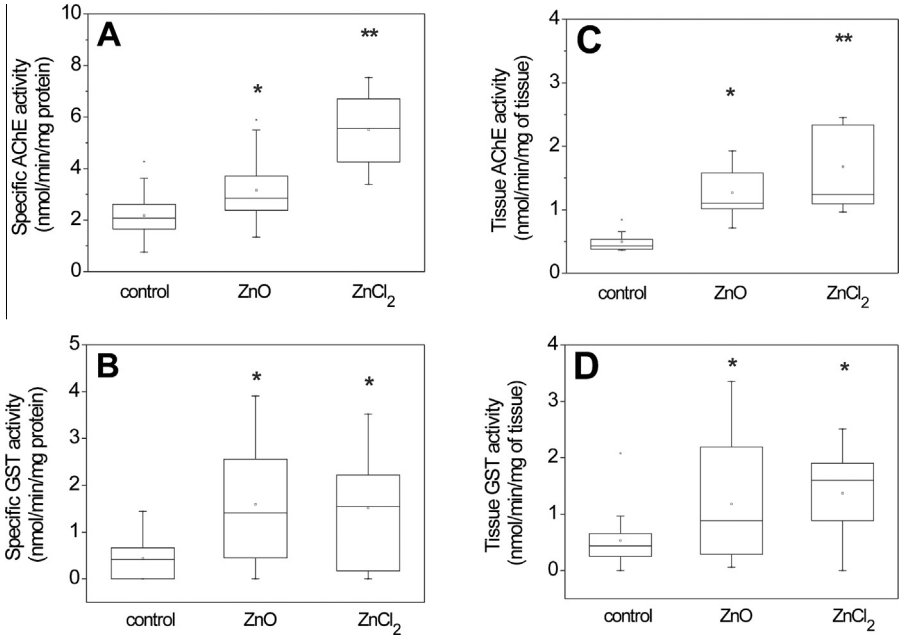

Fig. 3. Effects of ZnO NMs or Zn^{2+} on acetylcholinesterase and glutathione-*S*-transferase activity in honey bee brains. Specific activities of AChE (A) and GST (B) in aqueous extracts of homogenised isolated bee brain tissue after chronic exposure to 1.5 M sucrose suspensions of ZnO NMs or solutions of Zn^{2+}. The number of bees per treatment was 88 in controls, 84 in bees treated with ZnO NMs (0.8 mg Zn mL^{-1}), and 72 in bees treated with $ZnCl_2$ (0.48 mg Zn mL^{-1}). In the right panels, the activities of AChE (C) and GST (D) are expressed as tissue activities. * – Statistically important difference comparing to control (one way ANOVA, Bonferroni *post hoc* test, $p < 0.05$); ** – statistically important difference comparing to control and to group ZnO NMs (ANOVA, Bonferroni *post hoc* test, $p < 0.05$).

1.7 nmol min^{-1} mg^{-1} tissue, respectively). Similarly, the tissue GST activity in controls (0.5 nmol min^{-1} mg^{-1} tissue) was significantly lower than that measured in groups treated with ZnO NMs or Zn^{2+} (1.2 and 1.4 nmol min^{-1} mg^{-1} tissue, respectively). Overall, ZnO treatment induced a 1.6-fold in specific, and a 2.6-fold increase in tissue AChE activity, while the exposure to Zn^{2+} resulted in 2.8 or 3.4-fold increase in specific and tissue activities, respectively. The increases in tissue and specific GST activities were comparable.

4. Discussion

In this study, we provide experimental evidence of neurotoxicity of consumed zinc oxide nanomaterials (ZnO NMs) or Zn ions present in $ZnCl_2$ solution on honey bees. Chronic, 10-day exposure of bees to ZnO NMs (0.8 mg Zn mL^{-1}) or $ZnCl_2$ (0.48 mg Zn mL^{-1}) induced a decrease in survival, a loss of brain weight and protein content, and a significant elevation in AChE and GST activities. AChE and GST activities were higher and survival lower in the case of exposure to $ZnCl_2$.

4.1. Increased feeding rate in $ZnCl_2$ treated honeybees

We observed increased feeding rate in the group treated with Zn^{2+} but not with ZnO NMs or control group. Zn^{2+} – treated group of bees also displayed the lowest survival rate, indicating intoxication. It was purposed that honey bees may possess some specific elements of the physiological stress response that coordinate rapid changes in metabolic activity and behaviour, such as increased energy mobilisation and arousal (Even et al., 2012). The link between specific physiological mechanisms underlying the metabolic and behaviour changes is probably neurotransmitter octopamine which is far more abundant in forager's brains (Harris and Woodring, 1992). Foragers appear to be the colony members most exposed to stressors: foraging is energetically demanding, and exposes honey bees to more unpleasant environments (xenobiotics, predators) than working in the hive. Octopamine is supposed to help foragers to mobilise energy more easily or rapidly and may also make foragers more sensitive to hunger, which could motivate them to gather food (Even et al., 2012). Taken together, the increased feeding rate after the exposure to Zn^{2+} in our experiment could be the physiological adaptation due to relatively high stress. The other plausible explanation is that the increase of feeding rate is the consequence of metabolic rate elevation due to energy demanding mechanisms required to directly combat deleterious effects of metals, as seen from experiments on crayfish (Rowe, 1998; Rowe et al., 2001).

4.2. Compensatory adaptation: elevated activity of AChE

The increased activity of AChE in bees after having been fed with ZnO NMs or $ZnCl_2$ can be explained as a compensatory adaptation to the neurotoxic potential of these substances. Increased AChE activity has been also recorded in many other vertebrate and invertebrate organisms, in addition to bees, as a response to different types of stress. Badiou et al. (2008) provided evidence that the exposure of bees to the pyrethroid ester insecticide deltamethrin results in elevated activity of AChE, which they explained by an increase in the acetylcholine release, leading to regulatory overcompensation by an increase of AChE in the honey bee cholinergic system. In an alternative explanation, deltamethrin was hypothesised to trigger the increase of the AChE soluble form through alterations of the cellular membrane, initiating *de novo* synthesis of the enzyme to replenish the AChE removed from the surface of cellular membranes. Boily et al. (2013) reported a dual response of AChE in bees after exposure to selected biocides. They observed elevated activity of AChE after a 14-day exposure of bees to neonicotinoids, but reduced activity after exposure of bees to the herbicide glyphosate, [N-(phosphonomethyl)glycine]. Williamson et al. (2013) suggested that the increase in AChE may be an adaptive mechanism with which bees counteract the inhibition of AChE by organophosphorus and carbamate pesticides, while Farooqui

(2013) discussed potential links among pesticides, learning and memory, and colony collapse disorder.

In our study, the elevation of AChE activity obtained after feeding the bees with ZnO NMs or $ZnCl_2$ for 10 days could be explained as the result of interplay between several mechanisms. According to the literature reports regarding the metal- and pesticide-induced alterations in AChE activity in invertebrates and vertebrates, the possible mechanisms underlying the elevation of AChE activity in bees can be explained as follows: (i) Zn^{2+} ions or ZnO NMs affect the AChE by metal-induced modifications of lipid membranes (Kumar, 1998), leading consequentially to conformational changes of membrane-embedded AChE, and the replacement of deformed AChEs with newly synthesised ones (Badiou et al., 2008); (ii) membranes affected by Zn^{2+} or ZnO NMs could lead to altered ion homeostasis including Ca^{2+} homeostasis, causing continuous release of acetylcholine and consequential increase in levels of AChE (Konoha et al., 2006), or (iii) Zn^{2+} or ZnO NMs could directly interact with acetylcholine or AChE (Frasco et al., 2005), inducing higher production of AChE. Finally, the observed increase in AChE activity after both treatments was higher when this activity was expressed as a tissue activity, indicating that also other proteins could be modulated by Zn^{2+} or ZnO NMs (Badiou-Bénéteau et al., 2012).

AChE in honey bees was shown to exist in two major forms. A major amount (93–97%) of the enzyme accounts for an amphiphilic (membrane-bound) form, while the minor part is represented by a hydrophilic one (Belzunces et al., 1988; Belzunces and Debras, 1997). In contrast to the membrane-bound AChE, which is mainly expressed in the central nervous system and is responsible for the synaptic transmission, soluble AChE can be found both in central and in the peripheral nervous tissue, as well in non-neuronal tissues, and seems to act as a bio-scavenger playing a protective role against xenobiotics (Kim et al., 2012). In our work, a detergent-free buffer was used for protein extraction from honey bee brains, so our results account mostly for the activity of the soluble enzyme, which is also reflected in rather low measured enzyme activities compared to other studies (e.g. Badiou-Bénéteau et al., 2012). To better understand and explain the alterations of AChE activity after exposure of honey bees to ZnO or Zn^{2+}, the experiments obtained in this study will be upgraded in the future by measuring the whole AChE activity obtained after extraction with detergents.

4.3. GST activity after ZnO NMs exposure or Zn^{2+} exposure

We demonstrated an elevation of GST activities after the exposure of bees to ZnO NMs or $ZnCl_2$ for 10 days. However, Claudianos et al. (2006) reported a deficit in detoxification enzymes in honey bees, compared to other insects. In honey bees, there are only about half as many glutathione-*S*-transferases (GSTs), carboxyl/cholinesterases, and P450 monooxygenases. The experiments in which the detoxifying activity of different enzymes in honey bees was studied confirmed the major role of P450 monooxygenases which are more or less constantly elevated by chemicals occurring naturally in the hive environment, and in the nectar, pollen and propolis (Johnson et al., 2012). Furthermore, only omega, theta and zeta classes of GST are represented as playing key roles in metabolic processes in the honey bees, while delta and epsilon classes of GST, which are directly implicated in insecticide detoxification, are absent (Claudianos et al., 2006). Our finding of the relatively minor role of GST in detoxification in honey bees is supported by reports from other authors, showing that the GST inhibitor diethyl maleate only moderately increases the toxicity of some pesticides (Iwasa et al., 2004; Johnson et al., 2010). The elevation of brain GST activities after the exposure of bees to ZnO NMs or $ZnCl_2$ probably reflects the increase of specific brain metabolism, such as catalysis of tyrosine [BM1] and phenylalanine degradation or the removal of S-thiol adducts from proteins (Claudianos et al., 2006).

In comparison with other studies on GST activity in honey bees (e.g. Badiou-Bénéteau et al., 2012), a rather low GST activity measured here reflects the fact that only bee brains were used in our study. In fact, although GST is widely distributed in different honey bee organs including the brains, its main location are midgut cytosolic fractions (Diao et al., 2006).

4.4. Protein brain content and brain weights after exposure to ZnO NMs or Zn^{2+}

In contrast to the elevation of AChE and GST tissue activity in bee brain, a significant decline of brain total protein content, and in total weight of isolated brains, was detected in both ZnO NMs- and $ZnCl_2$-exposed bees, when compared to controls. Similar results were obtained by Jemec et al. (2008), where a decrease of protein content in daphnids was observed after a chronic exposure to Cd and Cr (VI) together with elevation of AChE and GST activity. Jemec et al. suggested that this could be a reflection of energy allocation of exposed organisms. In other words, a toxic stress induced by metals switches on a variety of compensatory and stress response mechanisms which are energy-demanding. This may be reflected in total amount of proteins, lipids or carbohydrates. Our results indicate that in spite of metabolic disruption, both ZnO NMs and Zn^{2+} trigger specific brain mechanisms for the elevation of AChE and GST activity as a compensatory response of bees to toxic compounds. Effect of feeding on total protein content must also be considered when performing similar studies. In fact, as suggested by Bounias et al. (1982), who studied the hemolymph protein content after a prolonged feeding of honey bees with 2 M sucrose solution as a sole source of food, this protein content decreases rapidly during the first 5–7 days and after becomes constant, which is explained as an effect of nutritional protein deficiency.

4.5. Effect of ZnO NMs versus $ZnCl_2$

In our study, the effect of ZnO NMs was significantly lower than that of $ZnCl_2$. Since the response to ZnO NMs or $ZnCl_2$ leads to qualitatively similar but quantitatively different effects, we conclude that these effects are caused primarily by Zn^{2+} ions. The conductance study which was performed with the aim of assessing the amount of ions produced from particles indicates that in the suspension of ZnO NMs there is a very low concentration of dissolved ions. This however does not exclude the implications of ZnO NMs-derived ions causing adverse effects to the organism, as they may dissolve once present in the digestive system. For example, Golobič et al. (2012) found that up to 99% of the copper from consumed copper NMs appeared as ions dissolved in the isopod's digestive system.

In this study, the concentration of Zn in ZnO NMs was 0.8 mg mL^{-1} which corresponds to 1 mg mL^{-1} of particles. The average estimated environmental concentrations for ZnO NMs in the form of nanoparticles in Europe are 10 μg L^{-1} in freshwater, 0.093 μg kg^{-1} in soil, and <0.0005 μg m^{-3} in air (Gottschalk et al., 2009), and the measured concentrations of Zn in different polluted media are 0.002–50 mg L^{-1} in water (NAS, 1977), <5–2000 mg kg^{-1} in soil (Schacklette and Boerngen, 1984) and < 1 μg m^{-3} in air (Lloyd and Showak, 1984). Monitoring of bee products in Slovenia (Šešerko et al., 2008) showed that bees could get in contact with Zn^{2+} in concentrations higher than 200 mg kg^{-1} in propolis during hive maintenance. Hive compartments are often closed by steel zinced wire mesh windows and separated by steel zinced queen excluders, which are propolised by bees. In addition, use of formic and oxalic acid preparations against Varroa mites inside of the

hives increase oxidation of hive metal parts and release of metal oxides and particles into hive environment (Janko Božič, personal observation from beekeeping practice). Another source of zinc originates from the paint pigment. Taking into these concentrations, our results highly support recommendations of beekeeping extension services to remove all still zincked materials from hives not only to reduce contamination of hive products but, based on our findings, also potential risk of Zn^{2+} intoxication.

5. Conclusions

We observed a significant effect of ZnO NMs on the neuronal system of bees. Both ZnO NMs (1 mg mL^{-1}, corresponding to 0.8 mg Zn mL^{-1}) and $ZnCl_2$ (1 mg mL^{-1}, corresponding to 0.48 mg Zn mL^{-1}) in the diet cause metabolic impairment (decrease in total protein brain level), decrease in survival, and elevation of AChE and GST activities. In addition, we observed increased feeding rate in the group treated with Zn^{2+} but not with ZnO NMs or control group. Consistent with literature data, we rationalise the elevated activity of AChE as a compensatory adaptation to ZnO NMs or Zn^{2+}. We suggest that dissolved Zn^{2+} ions are primarily responsible for the observed changes, although the effect of ZnO NMs as nanoparticles could not be entirely discounted. We conclude that zinc ions have a potential to contribute to colony survival.

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References

Abdel Rasoul, M.A.A., Eid, S.A.K., Marei, I.K.G., 2013. Impacts of multiple applications with biofly (*Beauveria bassiana*) and spintor® (spinosad) on honey bee (*Apis mellifera*) larvae. J. Plant. Prot. Path. Mansoura Univ. 2, 805–827.

Ates, M., Daniels, J., Asran, Z., Farah, I.O., Rivera, H.F., 2013. Comparative evaluation of impact of Zn and ZnO nanoparticles on brine shrimp (*Artemia salina*) larvae: effects of particle size and solubility on toxicity. Environ. Sci.: Process. Impacts 15, 225–233.

Badiou, A., Meled, M., Belzunces, L.P., 2008. Honeybee *Apis mellifera* acetylcholinesterase – a biomarker to detect deltamethrin exposure. Ecotoxicol. Environ. Saf. 69, 246–253.

Badiou-Bénéteau, A., Carvalho, S.M., Brunet, J.L., Carvalho, G.A., Buleté, A., Giroud, B., Belzunces, L.P., 2012. Development of biomarkers of exposure to xenobiotics in the honeybee *Apis mellifera*: application to the systemic insecticide thiamethoxam. Ecotoxicol. Environ. Saf. 82, 22–31.

Badiou-Beneteau, A., Benneveau, A., Geret, F., Delatte, H., Becker, N., Brunet, J.L., Reynaud, B., Reynaud, B., Belzunces, L.P., 2013. Honeybee biomarkers as promising tools to monitor environmental quality. Environ. Int. 60, 31–41.

Bainy, A.C.D., Medeiros, M.H.G., Di Mascio, P., Almeida, E.A., 2006. In vivo effects of metals on the acetylcholinesterase activity of the *Perna perna* mussel's digestive gland. Biotemas 19, 35–39.

Belzunces, L.P., Debras, J.-F., 1997. Comparison of properties of native and lytic forms of acetylcholinesterase from *Apis mellifera*. Arch. Insect Biochem. Physiol. 34, 143–157.

Belzunces, L.P., Toutant, J.P., Bounias, M., 1988. Acetylcholinesterase from *Apis mellifera* head. Evidence for amphiphilic and hydrophilic forms characterized by Triton X-114 phase separation. Biochem. J. 255, 463–470.

Boily, M., Sarrasin, B., DeBlois, C., Aras, P., Chagnon, M., 2013. Acetylcholinesterase in honey bees (*Apis mellifera*) exposed to neonicotinoids, atrazine and glyphosate: laboratory and field experiments. Environ. Sci. Pollut. Res. 20, 5603–5614.

Bounias, M., Morgan, M.R.J., Joliet, C., 1982. Action du chloramphénicol sur l'évolution du spectre protéique de l'hémolymphe d'abeilles nourries sur sirops de saccharose et de tréhalose. Apidologie 13, 115–126.

Carageorgiou, H., Zarros, A., Tsakiris, S., 2003. Selegiline long-term effects on brain acetylcholinesterase, (Na+, K+)-ATPase activities, antioxidant status and learning performance of aged rats. Pharmacol. Res. 48, 245–251.

Carreck, N.L., Andree, M., Brent, C.S., Cox-Foster, D., Dade, H.A., Ellis, J.D., Hatjina, F., van Englesdorp, D., 2013. Standard methods for Apis mellifera anatomy and dissection. The Coloss Beebook, Volume I: Standard methods for *Apis mellifera* research. J. Apicult. Res. 52, 40.

Carvalho, S.M., Belzunces, L.P., Carvalho, G.A., Brunet, J.L., Badiou-Beneteau, A., 2013. Enzymatic biomarkers as tools to assess environmental quality: a case study of exposure of the honey bee *Apis mellifera* to insecticides. Environ. Toxicol. Chem. 32, 2117–2124.

Chang, Y.-N., Zhang, M., Xia, L., Zhang, J., Xing, G., 2012. The toxic effects and mechanisms of CuO and ZnO. Nanoparticles Mater. 5, 2850–2871.

Claudianos, C., Ranson, H., Johnson, R.M., Biswas, S., Shuler, M.A., Berenbaum, M.R., Feyereisen, R., Oakeshott, J.G., 2006. A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. Insect Mol. Biol. 15, 615–636.

Dade, H.A., 1962. Anatomy and Dissection of the Honeybee, fifth ed. IBRA, Cardif.

Diao, Q., Yuan, K., Liang, P., Gao, X., 2006. Tissue distribution and properties of glutathione *S*-transferases in *Apis ceranacerana* Fabricius and *Apis mellifera* ligustica Spinola. J. Apicult. Res. 45, 145–152.

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.

Enayati, A.A., Ranson, H., Hemingway, J., 2005. Insect glutathione transferases and insecticide resistance. Insect Mol. Biol. 14, 3–8.

Even, N., Devaud, J.-M., Barron, A., 2012. General stress responses in the honey bee. Insects 3, 1271–1298.

Farooqui, T., 2013. A potential link among biogenic amines-based pesticides, learning and memory, and colony collapse disorder: a unique hypothesis. Neurochem. Int. 62, 122–136.

Frasco, M.F., Fournier, D., Carvalho, F., Guilhermino, L., 2005. Do metals inhibit acetylcholinesterase (AChE)? Implementation of assay conditions for the use of AChE activity as a biomarker of metal toxicity. Biomarkers 10, 360–375.

Golobič, M., Jemec, A., Drobne, D., Romih, T., Kasemets, K., Kahru, A., 2012. Upon exposure to Cu nanoparticles, accumulation of copper in the isopod *Porcellio scaber* is due to the dissolved Cu ions inside the digestive tract. Environ. Sci. Technol. 46, 12112–12119.

Gonçalves, J.F., Nicoloso, F.T., da Costa, P., Farias, J.G., Carvalho, F.B., da Rosa, M.M., Gutierres, J.M., Abdalla, F.H., Pereira, J.S., Dias, G.R., Barbosa, N.B., Dressler, V.L., Rubin, M.A., Morsch, V.M., Schetinger, M.R., 2012. Behavior and brain enzymatic changes after long-term intoxication with cadmium salt or contaminated potatoes. Food Chem. Toxicol. 50, 3709–3718.

Gottschalk, F., Sonderer, T., Scholz, R.W., Nowack, B., 2009. Modeled environmental concentrations of engineered nanomaterials (TiO_2, ZnO, Ag, CNT, fullerenes) for different regions. Sci. Technol. 43, 9216–9222.

Grossman, E., 2013; <http://e360.yale.edu/feature/declining_bee_populations_pose_a_threat_to_global_agriculture/2645/>, Yale Environment 360 website.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione *S*-transferases, the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.

Hanaor, D., Michelazzi, M., Leonelli, C., Sorrella, C.C., 2012. The effects of carboxylic acids on the aqueous dispersion and electrophoretic deposition of ZrO_2. J. Eur. Ceram. Soc. 32, 235–244.

Harris, J.W., Woodring, J., 1992. Effects of stress, age, season, and source colony on levels of octopamine, dopamine and serotonin in the honey bee (*Apis mellifera* L.) brain. J. Insect Physiol. 38, 29–35.

Iwasa, T., Motoyama, N., Ambrose, J.T., Roe, M.R., 2004. Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. Crop. Prot. 23, 371–378.

Jemec, A., Drobne, D., Tišler, T., Trebše, P., Roš, M., Sepčić, K., 2007. The applicability of acetylcholinesterase and glutathione *S*-transferase in *Daphnia magna* toxicity test. Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol. 144, 303–309.

Jemec, A., Tišler, T., Drobne, D., Sepčić, K., Jamnik, P., Roš, M., 2008. Biochemical biomarkers in chronically metal-stressed daphnids. Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol. 147, 61–68.

Johnson, R.M., Ellis, M.D., Mullin, C.A., Frazier, M., 2010. Pesticides and honey bee toxicity. Apidologie 41, 312–332.

Johnson, R.M., Mao, W., Pollock, H.S., Niu, G., Schuler, M.A., Berenbaum, M.R., 2012. Ecologically appropriate xenobiotics induce cytochrome P450s in *Apis mellifera*. PLoS One 7, e31051.

Kaizer, R.R., Corrêa, M.C., Spanevello, R.M., Morsch, V.M., Mazzanti, C.M., Gonçalves, J.F., Schetinger, M.R., 2005. Acetylcholinesterase activation and enhanced lipid peroxidation after long-term exposure to low levels of aluminum on different mouse brain regions. J. Inorg. Biochem. 99, 1865–1870.

Kim, Y.H., Cha, D.J., Jung, J.W., Kwon, H.W., Lee, S.H., 2012. Molecular and kinetic properties of two acetylcholinesterases from the western honey bee, *Apis mellifera*. PLoS One 7, e48838.

Konoha, K., Sadakane, Y., Kawahara, M., 2006. Zinc neurotoxicity and its role in neurodegenerative diseases. J. Health Sci. 52, 1–8.

Kumar, S., 1998. Biphasic effect of aluminium on cholinergic enzyme of rat brain. Neurosci. Lett. 248, 121–123.
Kumar, S., 1999. Aluminium-induced biphasic effect. Med. Hypotheses 52, 557–559.
Lloyd, T.B., Showak, W., 1984. Zinc and zinc alloys, third ed.. In: Grayson, M. (Ed.), Kirk-Othmer Encyclopedia of Chemical Technology, vol. 24 John Wiley and Sons, New York, pp. 835–836.
Ma, H., Williams, P.L., Diamond, S.A., 2013. Ecotoxicity of manufactured ZnO nanoparticles – a review. Environ. Pollut. 172, 76–85.
McNaught, A.D., Wilkinson, A., 1997. In: Compendium of Chemical Terminology, (the "Gold Book"), second ed. Blackwell Scientific Publications, Oxford, UK.
Miller, R.J., Lenihan, H.S., Muller, E.B., Tseng, N., Hanna, S.K., Keller, A.A., 2010. Impacts of metal oxide nanoparticles on marine phytoplankton. Environ. Sci. Technol. 144, 7329–7334.
Mueller, N.C., Nowack, B., 2008. Exposure modeling of engineered nanoparticles in the environment. Environ. Sci. Technol. 42, 4447–4453.
Muyssen, B.T.A., De Schamphelaere, K.A.C., Janssen, C.R., 2006. Mechanisms of chronic waterborne Zn toxicity in *Daphnia magna*. Aquat. Toxicol. 77, 393–401.
NAS, 1977. Zinc. National Academy of Sciences, Washington DC.
Nowack, B., Ranville, J.F., Diamond, S., Gallego-Urrea, J.A., Metcalfe, C., Rose, J., Horne, N., Koelmans, A.A., Klaine, S.J., 2012. Potential scenarios for nanomaterial release and subsequent alteration in the environment. Environ. Toxicol. Chem. 31, 50–59.
Papadopoulos, A.I., Polemitou, I., Laifi, P., Yiangoua, A., Tananakib, C., 2004. Glutathione *S*-transferase in the insect *Apis mellifera macedonica* – kinetic characteristics and effect of stress on the expression of GST isoenzymes in the adult worker bee. Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol. 139, 93–97.
Romani, R., Isani, G., De Santis, A., Giovannini, E., Rosi, G., 2005. Effects of chlorpyrifos on the catalytic efficiency and expression level of acetylcholinesterases in the bivalve mollusk *Scapharca inaequivalvis*. Environ. Toxicol. Chem. 24, 2879–2886.
Rowe, C.L., 1998. Elevated standard metabolic rate in a freshwater shrimp (*Palaemonetes paludosus*) exposed to trace element-rich coal combustion waste. Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol. 121 (4), 299–304.
Rowe, C.L., Hopkinsb, W.A., Zehnderb, C., Congdon, J.D., 2001. Metabolic costs incurred by crayfish (*Procambarus acutus*) in a trace element-polluted habitat: further evidence of similar responses among diverse taxonomic groups. Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol. 129 (3), 275–283.
Schacklette, H.T., Boerngen, J.G., 1984. Element concentrations in soils and other surficial materials of the conterminous United States. U.S. Geological Survey Professional Paper, 1270. Alexandria, VA 22304: Library of Congress Cataloging in Publication Data, pp. 105.
Šešerko, M., Pokorny, B., Zaluberšek, M., Glinšek, A., Manja Potočnik, 2008. Raziskave polutantov v čebeljih pridelkih. Report, ERICo Velenje, Velenje, Slovenia.
Thany, S.H., Tricoire-Leignel, H., Lapied, B., 2010. Identification of cholinergic synaptic transmission in the insect nervous system. Adv. Exp. Med. Biol. 683, 1–10.
van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13 (2), 57–149.
van Engelsdorp, D., Evans, J.D., Saegerman, C., Mullin, C., Haubruge, E., Nguyen, B.K., Frazier, M., Frazier, J., Cox-Foster, D., Chen, Y., Underwood, R., Tarpy, D.R., Pettis, J.S., 2009. Colony collapse disorder: a descriptive study. PLoS One 4, e6481.
Vergoz, V., Roussel, E., Sandoz, J.C., Giurfa, M., 2007. Aversive learning in honeybees revealed by the olfactory conditioning of the sting extension reflex. PLoS One 2, e288.
Wilczek, G., Babczyńska, A., Migula, P., Wencelis, B., 2003. Activity of esterases as biomarkers of metal exposure in spiders from the metal pollution gradient. Pol. J. Environ. Stud. 12, 765–771.
Williamson, S.M., Moffat, C., Gomersall, M.A.E., Saranzewa, N., Connolly, C.N., Wright, G.A., 2013. Exposure to acetylcholinesterase inhibitors alters the physiology and motor function of honeybees. Front. Physiol. 4, 13.
Yang, J.S., Nam, H.J., Seo, M., Han, S.K., Choi, Y., Nam, H.G., Lee, S.J., Kim, S., 2011. OASIS: online application for the survival analysis of lifespan assays performed in aging research. PLoS One 6 (8), e23525.
Zheng, Y., Li, R., 2009. *In vitro* and *in vivo* studies biocompatibility of ZnO nanoparticles. Int. J. Mod. Phys. B 23, 1566–1571.
Zhu, Y., Apostoluk, A., Masenelli, B., Melinon, P., 2013. Zinc oxide nanoparticles as luminescent down-shifting layer for solar cells. SPIE Newsroom.