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No chronic effects on biochemical biomarkers, feeding and survival of carnolian honeybees (*Apis mellifera carnica)* after exposure to nanosized carbon black and titanium dioxide

Kronična izpostavitev nanomaterialom titanovega dioksida in črnemu ogljiku nima vpliva na biokemijske biomarkerje, prehranjevanje in preživetje kranjske čebele (*Apis mellifera carnica)*

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Abstract: Honeybees (*Apis mellifera*) are important pollinators threatened by environmental pollution, plan protection products and other potential contaminants. Due to an extensive predicted use of engineered nanomaterials (NMs) in agriculture the impact on honeybees should be investigated. We studied the 10-days chronic dietary effect of carbon black (CB) and titanium dioxide (TiO_2) NMs on the antioxidant activities, cholinergic function, feeding behaviour and survival of honeybees. Exposure of honeybees *Apis mellifera carnica* to TiO_2 and CB NMs (1 mg ml^{-1}) did not affect the feeding and survival. No alteration of catalase, acetylcholinesterase and glutathione S-transferase enzymatic activity was noticed in the brain of honeybees, indicating that TiO_2 and CB NMs at the tested exposure dose had no adverse effects on honeybees. Currently predicted environmental concentrations for TiO_2 and CB NMs are significantly lower than the concentration tested in the current study. Based on our findings we conclude that the potential use of TiO_2 and CB NMs in agriculture is currently safe for honeybees at the tested concentration level and presents potential advantages compared to other NMs with known toxic potential.

Keywords: nanopesticide, carbon black nanomaterial, titanium dioxide nanomaterial, acetylcholinesterase, glutathione S-transferase, catalase, feeding behaviour.

Izvleček: Medonosne čebele (*Apis mellifera*) so pomembni opraševalci, ogroženi zaradi onesnaževanja okolja, fitofarmacevtskih sredstev in drugi možnih onesnaževalcev. Zaradi široke predvidene uporabe inženirsko proizvedenih nanomaterialov v poljedelstvu je potrebno raziskati njihov vpliv na medonosne čebele. V tej študiji smo preučili 10-dnevni kronični prehranski učinek nano-črnega ogljika (nČO) in nano-titanovega dioksida (nTiO_2) na prehranjevalno vedenje, antioksidativno aktivnost, na delovanje holinergičnega živčnega sistema in preživetje čebel. Pokazali smo, da nTiO_2 (1 mg ml^{-1}) in nČO (1 mg ml^{-1}) nista vplivala na hranjenje in preživetje kranjskih čebel *Apis mellifera carnica*. Hranjenje z obema vrstama nanomaterialov ni vplivalo na aktivnost treh biomarkerskih encimov katalaze, acetilholinesteraze in glutation S-transferaze v možganih čebel, kar pomeni, da nanomateriali verjetno niso imeli škodljivega učinka na čebele. Trenutno ocenjene napovedane vrednosti okoljskih koncentracij za nTiO_2 in nČO so znatno nižje od teh, ki smo jih uporabili v sedanji študiji. Na podlagi naših ugotovitev sklepamo, da je morebitna uporaba nTiO_2 in nČO NM v kmetijstvu varna za čebele v okviru testiranih koncentracij in predstavlja potencialno prednost v primerjavi z nanomateriali z znanim toksičnim potencialom.

Ključne besede: nanopesticidi, nano-črni ogljik, nano-titanov dioksid, acetilholinesteraza, glutation S-transferaza, katalaza, prehranjevalno vedenje.

Introduction

Honeybees (*Apis mellifera*) are important pollinators and many agricultural crops depend on pollination. Colony collapse disorder (CCD) causes massive deaths in honeybees and significant economic losses (van Engelsdorp et al. 2009). In the recent years it is evident that CCD is caused by the combined action of parasites, pathogens and pesticide stressors (Sánchez-Bayo et al. 2016). Synergistic interactions among the parasitic mite *Varroa*, viral pathogens and pesticides could severely reduce host immune competence potentiating the sensitivity of honeybees to other possible stress agents. Among the stress agents, pesticides are now widely studied while other emerging environmental contaminants, such as nanomaterials (NM) are still highly neglected.

Engineered nanomaterials are extensively used in agriculture to reduce amount of applied plant protection products, minimize nutrient losses in fertilization, and increase yields through an optimized nutrient management (Gogos et al. 2012; Kah and Hofmann 2014). New formulations containing nanomaterials are called “nanopesticides” and “nanofertilisers” (Kah and Hofmann 2014). Up to 3000 of patents and over 100 peer-reviewed publications directly related to nanopesticides have been published until 2011 (Kah and Hofmann 2014), indicating intense research activity in this field.

Nano-sized titanium dioxide (TiO_2 NM) was one of the first nanomaterials commercially available and is used in a wide variety of materials and applications, including self-cleaning surface coatings, light-emitting diodes, solar cells, disinfectant sprays, sporting goods, water-treatment agents, cosmetics and agriculture (IARC/WHO, 2010). These NMs are widely used due to their high stability, anticorrosive and photocatalytic properties. In agriculture, TiO_2 NM is added to pesticide formulation to catalyse the photodegradation of the pesticide organic active ingredient. Examples of such nanoformulations with TiO_2 are: chlorfenapyr (Cao et al. 2005), imidacloprid and avermectin (Guan et al. 2008, 2011). TiO_2 NMs are also interesting in terms of their antimicrobial activity and several studies suggest their suppressing action on bacterial and fungal pathogens growth on crops (Paret et al. 2013 a,b). Until 2013 70 patents including TiO_2 have been registered as nanopesticides (Kah et al. 2013).

Nano-sized carbon black (CB NM) is produced at rates of several million tons per year (Navarro et al. 2008). It is a product of incomplete combustion of fossil fuels and vegetation, and is used in rubber production, as black pigment in printing inks, as electrodes in batteries, and in leather production. In comparison to TiO_2 NM, CB NM is much less explored in terms of potential hazard for the environment. Significantly more information is available for other carbon nanomaterials, such as carbon nanotubes and fullerenes (Jackson et al. 2013).

Insects, including honeybees, are among the least investigated non-target organisms in terms of potential nanomaterial hazard (Garner et al. 2015). Currently only one study on the effect of NM (zinc oxide; ZnO) on honeybees exists (Milivojević et al. 2015). Honeybees (*Apis mellifera*) are potentially exposed to TiO_2 and CB NM due to their foraging behaviour via contact with contaminated plants as well as water droplets. Flying bees, beehives or flowers attracting the bees may come into contact with NM also *via* traffic dust contaminated with TiO_2 and CB NM (Perez et al. 2010).

In the present work, we aimed to investigate the chronic effects of CB and TiO_2 NM on feeding behaviour, survival and stress enzyme activities in the honeybee brain. Three commonly applied biomarkers of exposure and effect were measured: antioxidant enzymes catalase (CAT) and glutathione S-transferase (GST), and neurotoxic biomarker acetylcholinesterase (AChE) (Jemec et al. 2010). Catalase decomposes hydrogen peroxide (Halliwell and Gutteridge 2007). GSTs are a family of detoxification enzymes, which catalyse the conjugation of glutathione with xenobiotics and cytotoxic aldehydes produced during lipid peroxidation. GSTs are considered as both antioxidant and detoxification enzymes (Barata et al. 2005). Acetylcholinesterase plays an established role in cholinergic transmission by hydrolysing the neurotransmitter acetylcholine thereby terminating the synaptic transmission (Kim and Lee 2013). In addition, a number of non-neuronal functions of AChE have also been proposed (Karczmar 2010). A disruption of the honeybees' neuronal cholinergic signalling affects their orientation, olfactory learning and navigation abilities, which results in their failure to return to hives, even potentially leading to CCD (Farooqui 2013). Knowledge on the effect of pollutants on the cholinergic system of honeybee brain is therefore important for understanding the potential environmental hazard of NMs.

Materials and methods

Chemicals

The following chemicals were purchased from Sigma Aldrich (Germany): monobasic and dibasic 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 ethylenediaminetetraacetic acid. BCA Protein Assay Reagent A and Reagent B were purchased from Pierce (US). All chemicals were of the highest commercially available grade, typically >99%. CB nanopowder was provided by PlasmaChem GmbH (Berlin, Germany) and TiO_2 NM was provided by Nanologica (Sweden) in the framework of the EU FP7 NanoValid project.

Preparation and characterisation of NM suspensions

A suspension of TiO_2 NM or CB NM was prepared by adding nano powder to 1.5 M sucrose solution in milli-Q water with sonication (PIO Iskra, Sonnys 2GT; 40 kHz, 2x100 W) of the suspension for 24 h. For better comparison with the effects of ZnO NM on honeybees, published by Milivojević et al. 2015, where the same experimental set-up and tested concentrations were applied, we used final nominal concentration 1 mg mL-1 of NMs in sucrose.

The properties of CB NM were as reported by Mesarič et al. (2013). CB is composed of amorphous, globular primary nanoparticles with diameter of about 20 nm (**Supplementary information Fig.S1A**). The primary sizes of TiO_2 NM were in range between 110 and 170 nm, showing a large size distribution (**Supplementary information Fig.S1B**). The secondary size of NMs in the 1.5 M sucrose solution was not measured since the size of NM aggregates/agglomerates alters in different fluids present in the honeybee digestive system. Therefore, it is important to bear in mind that the data on the size of aggregates in the sucrose solution have no actual correlation with the actual size of NM in the digestive system of honeybees.

Test animals

Adult summer honeybee workers (*Apis mellifera carnica*, Pollman 1879) were randomly collected inside the hive from colonies that were maintained according to the good beekeeping practice at the Biotechnical Faculty, University of Ljubljana, Slovenia. Honeybees were then transferred to wooden cages (9.5 x 4 x 7.5 cm) and supplied *ad libitum* with water and 1.5 M sucrose solution in gravity feeders until all bees were collected. All bees were maintained in cages for 1 h at 27 °C before treatments.

Honeybee exposure to TiO_2 and CB NM via food

A group of 120 bees was divided into 6 groups of 20 animals. Each group of bees (20 specimens) was placed into separate wooden cage. Two cages with a control group were fed with 1.5 M sucrose solution only, two groups of bees received a suspension of TiO_2 NM in 1.5 M sucrose (1 mg mL^{-1} TiO_2) and two groups were fed with a suspension of CB NM in 1.5 M sucrose (1 mg mL^{-1} CB). Each cage received a syringe with tap water. The sucrose solution and water were renewed every 2 days. The cages were placed for 10 days in an incubator at 27 °C and 95% relative humidity during the overall exposure period. The feeding was estimated as the volume of total consumed solutions/suspensions per exposure group after 10 days. Mortality of bees was monitored during 10 days of the experiment.

Brain dissection and homogenization

After 10-days of dietary exposure to NM, the brains were isolated from honeybees according to Carreck et al. (2013). Isolated brains were submersed into a droplet of honeybee Ringer solution, both hypopharyngeal and postcerebral glands were removed. The brains were then stored at -20 °C until analysis. Individual brains were homogenized in 200 mL of 100 mM potassium phosphate buffer (pH 7.4) per sample. Individual homogenates were centrifuged for 15 min at 12,092 *g* and 4 °C, and the supernatants were stored at -20 °C for enzyme analyse.

Enzyme and protein assays

AChE, GST and CAT activity measurements

AChE activity was analysed according to Ellman et al. (1961), GST activity was analysed according to the method of Habig et al. (1974) and CAT activity was determined according to Jemec et al. (2008). All procedures were adapted as described in Milivojević et al. (2015) and Jemec et al. (2008). AChE activity was expressed in nmoles of hydrolyzed acetylthiocholine chloride/min/mg protein (extinction coefficient ε_{405}=13,600 M^{-1} cm^{-1}). GST activity was expressed in nmoles of conjugated GSH/min/mg protein (extinction coefficient ε_{340}=9600 M^{-1} cm^{-1}). The CAT activity was expressed as μmoles of degraded hydrogen peroxide/min/mg protein (extinction coefficient, ε_{240} = 43.6 M^{-1} cm^{-1}).

Protein quantification

Protein concentration in the supernatant of honeybee brain homogenates was analyzed using a BCA™ Protein Assay Kit, a modification of the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA).

Statistical analysis

The significant differences between the control and exposed groups of animals were determined by Kruskal-Wallis analysis and Mann-Whitney U test ($p<0.05$) using OriginPro software.

Results

Feeding and survival

After 10-days of exposure to CB NM the total volume of consumed suspensions of CB NM (1 mg mL^{-1}) in 2 groups of 40 individuals was similar to the total volume of consumed sucrose solution in 2 groups of 40 individuals indicating that CB NM did not affect the feeding when comparing treated and control groups of honeybees (Fig.1). In honeybees (2 groups, n=40) exposed to the TiO_2 NM (1 mg mL^{-1}), we observed a slightly (13.9 %) higher food uptake than in honeybees fed with control sucrose solution (Fig.1), but this increase was not statistically significant. The total volume of consumed suspensions of CB NM, TiO_2 NM and control sucrose solution in 10-days were 15.8 ml (8 mL and 7.8 mL), 18 mL (9 mL and 9 mL) and 16 mL (8 mL and 8 mL) per treatment (and per groups), respectively (Fig.1).

The chronic 10-days oral exposure of honeybees to TiO_2 (1 mg mL^{-1}) and CB NM (1 mg mL^{-1}) did not affect the survival in both treated groups (data not shown). There was no morality in all groups during the 10-days exposure period.

Enzyme activities after exposure to tested substances

Chronic 10-days exposure to TiO_2 NM (1 mg mL^{-1}) or CB NM (1 mg mL^{-1}) did not alter significantly the activities of brain AChE, CAT and GST (Fig.2) (Kruskal-Wallis analysis and

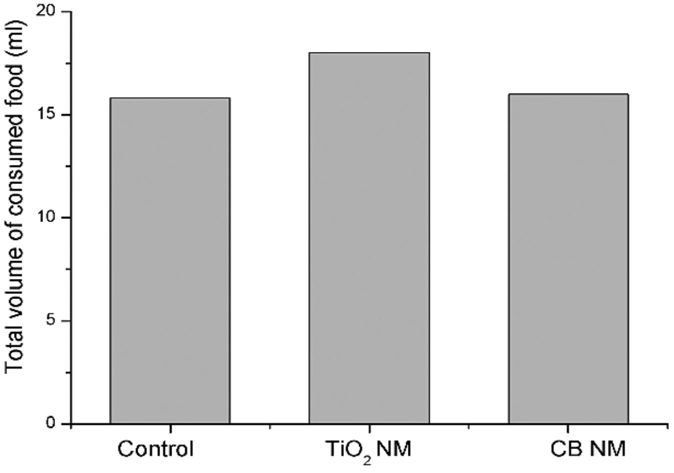

Figure1: The effect of titanium dioxide (TiO_2 NM) and nano carbon black (CB NM) on honeybee feeding. Total volume of consumed food (mL) in bees after 10-days exposure to TiO_2 NMs suspension (1 mg mL^{-1}) and to CB NM suspension (1 mg mL^{-1}) is shown (N=40).

Slika 1: Vpliv nano-črnega ogljika (nČO) in nano-titanovega dioksida ($nTiO_2$) na prehranjevanje medonosne čebele. Prikazana je skupna količina porabljene hrane (mL) pri čebelah po 10-dnevni izpostavljenosti suspenziji $nTiO_2$ (1 mg mL^{-1}) ali nČO (1 mg mL^{-1}) (N = 40).

Mann-Whitney U test ($p<0.05$)). The mean (±SE) activity of AChE in bee brains (Fig. 2A) after chronic oral exposure to TiO_2 NM and CB NM were 2.08±0.16 and 2.08± 0.16 nmol/min/mg protein, respectively, and were similar to control values (1.91±0.15 nmol/min/mg protein). The mean activity of GST in bee brains (Fig. 2B) after chronic oral exposure to TiO_2 NM and CB NM were 4.15±0.13 and 4.81± 0.12 nmol/min/mg protein, respectively, and were similar to the control values (4.59±0.15 nmol/min/mg protein). The mean activities of CAT in bee brains (Fig. 2C) after chronic oral exposure to TiO_2 NMs and CB NMs were 0.64 ± 0.046 and 0.77 ± 0.044 µmol/min/mg protein, respectively, and were similar to control values (0.72 ± 0.048 µmol min/mg protein).

Discussion

The results of the present study show that the TiO_2 (1 mg mL^{-1}) and CB NMs (1 mg mL^{-1}) do not cause any adverse effects, neither sub-lethal nor lethal, on honeybees *Apis mellifera carnica* after a 10-days chronic dietary exposure. No effects on survival, feeding behaviour, antioxidant activities (CAT and GST), and cholinergic enzyme activity (AChE) in the brain of honeybees was observed.

We have previously anticipated that TiO_2 NM and CB NM might not be highly toxic to honeybees, since these two materials have been previously recognised as presumably inert, e.g. having little biological interaction with the test organisms (Bondarenko et al. 2016). Namely, Bondarenko et al. (2016) have used a set of assays to screen seven NMs using 14 different test species and cell lines. The toxicity decreased in the following order: Ag>ZnO>CuO>carbon nanotubes>Au>SiO_2=TiO_2. It is now well established that the toxicity of the NMs is mainly driven by two basic intrinsic properties of NMs: high solubility (e.g. Ag, CuO and ZnO) and high aspect ratio (describes the proportional relationship between its diameter and its length) (e.g. carbon have high aspect ratio). TiO_2 and CB NMs are neither soluble in aqueous media nor have high aspect ratio, which could explain their low toxic potential.

In contrast to soluble NMs, both TiO_2 and CB NMs have high adsorption potential for the body

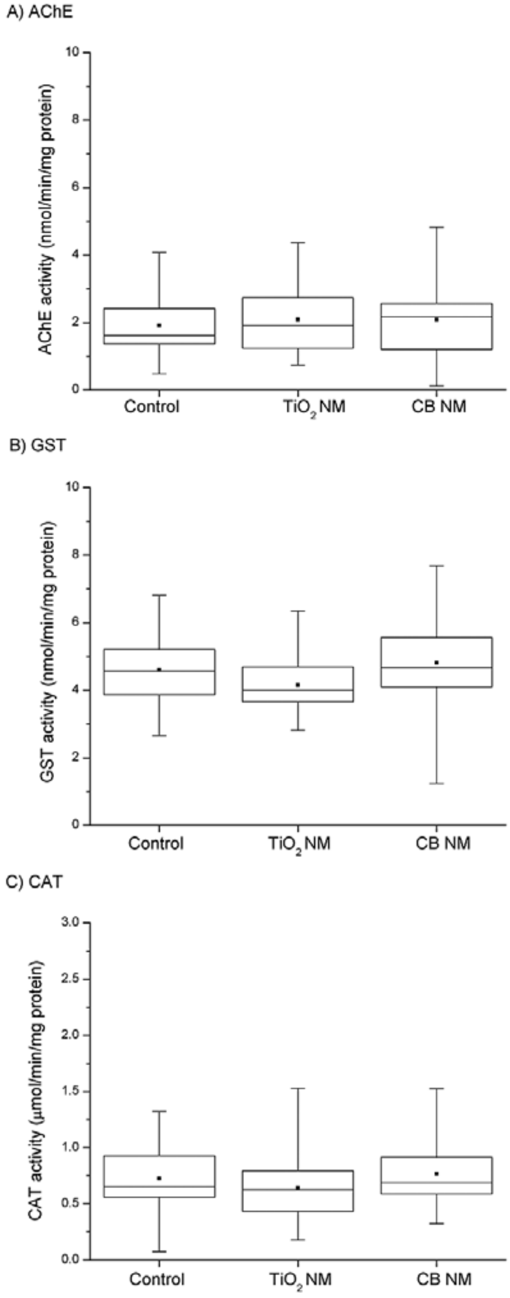

Figure 2: Acetylcholinesterase(A), glutathione-S-transferase (B), and catalase (C) activity in honeybee brains after chronic exposure to TiO_2 NM (1 mg mL^{-1}) and CB NMs (1 mg mL^{-1}) (N=40).

Slika 2: Aktivnost acetilholinesteraze (A), glutation S-transferaze (B) in katalaze v možganih čebel po kronični izpostavitvi $nTiO_2$ (1 mg mL^{-1}) ali nČO (1 mg mL^{-1}) (N=40).

surface which was shown to induce adverse effects on organisms (Xia et al. 2011; Mesarič et al. 2015a, b; Nielsen et al. 2008). CB NM adsorbed on the sperm and embryo of brown algae *Fucus serratus*, to body surface of *A. salina* larvae (Mesarič et al. 2015a) and to sperm of sea urchin (*Paracentrotus lividus*) (Mesarič et al. 2015b), resulted in abnormal development of algae, inhibition of larvae swimming, and reduced fertilisation of sea urchin eggs, respectively. The adsorption of TiO_2 NM on crustaceans *Daphnia magna* (Dabrunz et al. 2011) and algae (Aruoja et al. 2009) had a negative effect on the immobility and growth, respectively. Based on available literature data on TiO_2 and CB NM and their high adsorption potential these NM could adsorb onto the digestive tract surface of honeybees, which may result in feeding disruption. However, this potential effect was not confirmed during 10-days exposure of honeybees to 1 mg mL^{-1} of CB and TiO_2 NMs.

Chronic exposure of honeybees to CB and TiO_2 NMs did not significantly change the feeding, but we observed a slightly higher food uptake in honeybees fed with TiO_2 NM in comparison to honeybees that received only control sucrose solution. Behavioural response such as feeding alteration due to possible stress agents could occur before the alterations in biochemical biomarkers (Hellou 2011). Currently, no data in the literature are available confirming that honeybees are able to sense metals. The effect of different metals on honeybees behaviour was explored only by few studies (Hladun et al. 2012; Burden et al. 2016; Søvik et al. 2015), and only in our last study the effects of metallic NMs was addressed (Milivojević et al. 2015). In our study with tested ZnO NMs we found that chronic exposure did not alter the feeding in honeybees whereas Zn^{2+} salt increased the feeding (Milivojević et al. 2015). Due to potential environmental burden of CB and TiO_2 NMs it would be important to investigate the possible preference/avoidance towards solutions contaminated with these NMs.

Catalase, GST and AChE are among the most commonly applied biochemical biomarkers of toxicant-induced physiological changes in organisms (Jemec et al. 2010). It has been previously shown that 1 mg mL^{-1} of CB NM caused an increase of GST and AChE activities in *Artemia salina* after 48 h exposure (Mesarič et al. 2015a), while no alteration of CAT was reported. CB or TiO_2 NMs (0.005 mg mL^{-1}) increased the activities of CAT and GST in the digestive gland and gills of mussels *Mytilus galloprovincialis* after 24 h (Canesi et al. 2010), while 0.001 mg mL^{-1} of CB NM decreased the AChE activity in gastrulae of sea urchin (*Paracentrotus lividus*) after 24 h (Mesarič et al. 2015b). In the present work, CAT and GST were not altered in the head of honeybees, which indicates that most probably no oxidative stress and detoxification process occurred. Also, the activity of AChE was not changed which is in contrast to the effect of ZnO NM previously reported for honeybees (Milivojević et al. 2015). We can conclude that both TiO_2 and CB NMs have low neurotoxic potential in honeybees. In the study of Milivojević et al. (2015) the alteration of AChE was predominately explained as an effect of released $Zn^{2+}$from ZnO NM. Nevertheless, in the study of Romih et al. (2015), the authors suggested that both dissolved ions and NMs in the subtoxic range could be responsible for the activation of different metabolic pathways in the hepatopancreases of crustacean *Porcellio scaber*. They have found that the ZnO NM induced different metabolic responses from those induced by Zn^{2+} salt. Based on this study it could be also the case with TiO_2 and CB NMs but at the moment no data are available to support this theory. We can conclude that both TiO_2 and CB NMs have low neurotoxic potential in honeybees.

At the moment no data on the realistic environmental concentrations of nano TiO_2 and nano CB are available. Predicted environmental concentration (PEC) for TiO_2 NM was estimated at 107 mg kg^{-1} for wastewater treatment plant sludge, and 21 ng L^{-1} for surface waters (data from 2009) (Gottschalk 2009). TiO_2 NM production market is constantly increasing and these NMs are expected to have higher release into soil, water and air in the future (Keller et al. 2013). The upper quantity of TiO_2 NM estimated to pass through the waste water system in 2010 was nearly 48,000 t/year, with a potential for over 38,000 t/year to be added to the soil, and 32 000 t/year to landfill mainly through application of biosolids, but also small quantities through atmospheric deposition (1600 t/year) (Keller et al. 2013). Carbon nanomaterials are also among those with high expected production, but their emission rates are lower compared to TiO_2

NM (e.g. 10 times lower for carbon nanotubes) (Keller et al. 2013). We were unable to find the PEC values for nano CB, but PEC values have been calculated for non-nano CB based on the data obtained in the period 1999-2010 (Screening Assessment for the Challenge 2013). The estimated PEC from industrial emission (inks and paints industry) was 6.6 mgL^{-1} for river, which is based on a total of 4 336 447 kg of CB used/year.

In conclusion, currently estimated PEC values for TiO_2 and CB NMs are significantly lower than those tested in this study (1 mg mL^{-1}). Future production and release rates of these two NMs are expected to be very high (Keller et al. 2013). Therefore it is reasonable to analyze the potential hazard of high TiO_2 and CB NMs exposure concentrations (up to 1 mg mL^{-1}). The current study reveals that both TiO_2 and CB NM have no adverse effects, neither sub-lethal nor lethal, on honeybees after a 10-days exposure. In this regard, the use of carbon and titanium NMs in agriculture, instead of those with more hazardous potential, could have an advantage.

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Povzetek

Medonosne čebele (*Apis mellifera*) ogrožajo različni dejavniki, med katere lahko štejemo tudi povečano proizvodnjo in uporabo različnih nanomaterialov v kmetijstvu v obliki nano-pesticidov in nano-gnojil. Vpliv kovin na čebele, sploh v obliki nanodelcev, je zelo slabo raziskan. Zato smo se odločili, da preučimo učinke dveh vrst nanomaterialov, ki veljata za dokaj inertna v smislu raztapljanja in interakcije z biološkimi sistemi: nano-črnega ogljika (nČO) in nano-titanovega dioksida ($nTiO_2$). Odrasle čebele delavke kranjske čebele (*Apis mellifera carnica)* smo kronično (10 dni) hranili s suspenzijama $nTiO_2$ (1 mg mL^{-1}, n=40) ali nČO (1 mg mL^{-1}, n=40), v 1.5 M raztopini saharoze. Kontrolna skupina čebel (n=40) je bila hranjena samo z 1.5 M raztopino saharoze. Ugotovili smo, da 10-dnevno hranjenje z $nTiO_2$ ali nČO ni vplivalo na preživetje ali stopnjo prehranjevanja čebel. Prav tako $nTiO_2$ ali nČO nista vplivala na aktivnosti dveh antioksidativnih encimov, katalazo in glutation S-transferazo ter na aktivnost pokazatelja delovanja holinergičnega živčnega sistema encima acetilholinesteraza v možganih čebel. Ti rezultati kažejo na to, da uporabljeni nanomateriali verjetno niso imeli škodljivega učinka na čebele. Trenutno ocenjene predvidene vrednosti okoljskih koncentracij za $nTiO_2$ in nČO so znatno nižje od teh, ki smo jih uporabili v sedanji raziskavi. Sklepamo, da je morebitna uporaba $nTiO_2$ in nČO v kmetijstvu primernejša od tistih pripravkov, ki imajo več škodljivih učinkov na organizme.

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Supplementary information

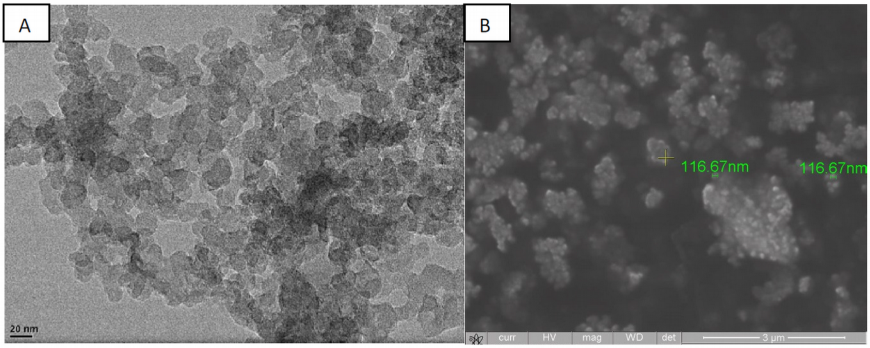

Figure S1: TEM image of CB (left) (JEOL 2100 microscope operated at 200 kV) and SEM image of TiO_2 NM (FEI Quanta 3D200 (right)).

Slika S2: TEM posnetek nano črnega ogljika (levo) (JEOL 2100 mikroskop, 200 kV) in SEM posnetek nanodelcev titanovega dioksida (desno) (FEI Quanta 3D200 (desno)).