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NANOTECHNOLOGY, NANOPOLLUTION, NANOTOXICOLOGY AND NANOMEDICINE (NNNN)

Sublethal effects of chronic exposure to CdO or PbO nanoparticles or their binary mixture on the honey bee (*Apis millefera* L.)

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Abstract

Cadmium and lead-based nanotechnologies are increasingly used in agricultural, industrial, and biological processes; however, potential adverse effects of nanomaterials on honey bees had not been assessed. In this study, effects of exposures to sublethal concentrations of PbO and CdO nanoparticles (NPs), either separately or in combination on honey bee (*Apis mellifera*) workers, were assessed. Honey bee workers were orally exposed for 9 days under laboratory conditions to sublethal concentrations (20% of LC_{50}) of CdO (0.01 mg/ml^{-}) and PbO (0.65 mg/ml^{-}) NPs either separately or combined. Effects on survival, feeding rate, activity of acetylcholinesterase (AChE), and expression of selected stress-related detoxifying enzymes were quantified. Survival and feeding rates decreased particularly in bees fed sugar syrup containing CdO NPs or binary mixtures of NPs of both metal oxides. Expressions of genes involved in detoxification of xenobiotics were affected by various combinations. Expression of catalase was 13.6-fold greater in bees consumed sugar syrup diet containing binary mixture of sublethal concentrations of both CdO and PbO NPs than it was in unexposed, control bees. AChE activity in heads of honey bees was inhibited by 3.8-, 3.0-, and 2.8-fold relative to control, respectively, in response to exposure to Cd or/and Pb oxide NPs. This result indicates potential neurotoxic effects of these NPs to honey bees. CdO NPs exhibited greater potency to honey bees. Overall, sublethal concentrations of CdO or/and PbO NPs resulted in detrimental effects on honeybee workers.

Keywords Nanotoxicology · Honeybees · Environmental pollution · Detoxifying enzymes · Metal oxide nanoparticles

Introduction

Metal-based nanotechnologies including metals, metal sulfides, metal oxides, and quantum dots are widely used in various end applications including multiple consumer products that are already in use (Hussain and Mishra 2018). Unique properties of nanoparticles (NPs), including small size (0.1–100 nm), large specific surface area, reactivity, and shape, enable them to enter organisms and be transported into tissues, cells, and even into organelles, in ways that larger particles might not (Kovochich et al. 2007). This has resulted in potential hazards for human health and the environment (Nowack and Bucheli 2007; Handy et al. 2008a; Maurer-Jones et al. 2013).

Unfortunately, information on risks that come with use of NPs is limited (Christian et al. 2008) and their widespread application and inevitable releases into the environment might result in harmful effects on organisms including honey bees (Mueller and Nowack 2008). Bees are the predominant and most important group of managed pollinators worldwide (Potts et al. 2010), with the western honey bee (*Apis mellifera*

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L.) being the most economically valuable pollinator for monoculture crops (Klein et al. 2007). Therefore, there was a need to investigate potential toxic effects of NPs made of oxides of metals to honey bees.

Cadmium (Cd) and lead (Pb) are metals of current toxicological concern and are frequently associated with urbanization and industrial processes (Lei et al. 2010; Al Naggar et al. 2014; Jabłońska-Czapla et al. 2016). Their detrimental effects on physiological, biochemical, and behavioral functions have been documented in animals and humans (Patra et al. 2011; Cao and Ding 2010; Mirčić et al. 2013). These metals have been measured in bees, honey, and other bee products (Al Naggar et al. 2013; Solayman et al. 2016; de Oliveira et al. 2017; El-Sofany et al. 2018). Additionally, lead, iron (Fe), and barium (Ba) were detected in particulate matter (PM) associated with emissions of dust from nearby industrial plants, collected from South West Sardinia, Italy, that was found to be adhering to surfaces of honey bees (Negri et al. 2015). Adverse effects of sublethal concentrations of both Cd and Pb on honey bees have been previously investigated (Collet and Belzunces 2007; Di et al. 2016; Gauthier et al. 2016; Nikolić et al. 2016). However, potential toxicities of their metal oxide NPs on honey bees had yet to be addressed. Effects of some NPs, such as Ag-TiO_2, ZnO-TiO_2, and TiO_2 (Özkan et al. 2015), ZnO (Milivojević et al. 2015), cerium (IV) oxide (CeO_2s) (Kos et al. 2017) had been reported for honey bees.

Currently available consumer products contain nanomaterial (NM) use NPs made of PbO or CdO. For instance, solar cells, gas sensors, transparent electrodes and photodiodes, catalysts, photocatalysts, and optoelectronic devices contain these NMs (Thovhogi et al. 2016). NMs made of these oxides are also used in many biological and medical applications (Heidari and Brown 2015; Savale et al. 2017). PbO-NMs are used in commercial applications such as optical and electrical glasses; in vitreous enamels, glazes, and fine tableware; in lead soaps for varnishes; and as a base material for producing various lead pigments as well as other compounds of commercial interest such as lead arsenate, lead acetate, and sodium plumbite (Blair 1998). Such an abundance of products containing PbO and CdO NMs in use globally raises issues relative to their toxic potentials (Cao and Ding 2010; Shaikh et al. 2015; Dumkova et al. 2016).

Honey bee colonies are exposed to xenobiotics when foragers bring contaminants back to the hive while collecting food. During their wide-ranging foraging activities, these hymenopterans are exposed to pollutants including metals associated with particulates of various sizes, which are present in the atmosphere, soil, vegetation, and water (Lambert et al. 2013; Al Naggar et al. 2014; Negri et al. 2015). Exposure of honey bees to polluting sources could occur through adhesion of particles to the insect body hairs, inhalation of pollutants via spiracles of the tracheal system, or ingestion of contaminated nectar, pollen, and water. Contaminants are brought back to the hives and may also be found into the apiary products, such as honey and wax (Satta et al. 2012; Porrini et al. 2014).

Several mechanisms are known to protect organisms against effects of oxidative damage caused by exposure to metals. There are both primary and secondary antioxidant enzymes, which act directly or indirectly on reactive oxygen species (ROS) molecules. Defense against ROS is provided by three primary antioxidant enzymes that act directly on ROS: superoxide dismutases (SODs), catalase, and peroxidases (Corona and Robinson 2006). Insects have three families of genes that encode antioxidant enzymes that act as peroxidases: TPXs, also known as peroxiredoxins (Radyuk et al. 2001), phospholipid-hydroperoxide GPX homologs with thioredoxin peroxidase activity (GTPX) (Missirlis et al. 2003), and glutathione S-transferases (GSTs) (Tang and Tu 1994; Toba and Aigaki 2000). Therefore, concentrations and activities of antioxidants can be used as biomarkers for adverse health effects of some classes of chemicals (Valavanidis et al. 2006).

Acetylcholinesterase (AChE; EC 3.1.1.7.) that hydrolyzes the neurotransmitter acetylcholine is used as a biomarker for neurotoxicity of anticholinesterase inhibitors, such as organophosphorus and carbamate insecticides as well as other classes of environmental contaminants including complex mixtures of metals, detergents, and other organic pollutants (Carvalho et al. 2013; Boily et al. 2013; Al Naggar 2016). Therefore, measurement of AChE activity is the most frequently used method for estimating the cholinergic function of an organism's nervous system.

Considering the health status of pollinators and the reported toxicity of Cd and Pb as metals and as metal oxide NPs, laboratory bioassays were performed to assess whether exposure to sublethal concentrations of PbO- or CdO-NPs either separately or in combination can exert adverse effects on worker bees. Effects on survival, feeding rate, activity of AChE, and expressions of selected stress-related detoxifying and antioxidative enzymes were quantified.

Materials and methods

Preparation of metal oxide NPs

Cadmium oxide NPs

Cadmium acetate (0.5 M) was dissolved in 100 ml distilled H_2O and ammonia solution added to take the solution to a pH of 8. The white precipitate that formed was allowed to settle overnight. This was then filtered and washed 4 times with distilled H_2O, dried at 100 °C for 6 h, and then homogenized by use of a glass mortar and pestle. The resulting powder was calcined at 400 °C for 2 h (DurgaVijaykarthik et al. 2014).

Lead oxide NPs

Lead oxide nanoparticles were synthesized by placing 60 ml of 1.0 M Pb (lead (II) acetate trihydrate ($Pb(C_2H_3O_2)_2{\cdot}3H_2O$)) aqueous solution that was prepared in de-ionized water and heated to 90 °C. This solution was added to an aqueous solution of 50 ml of 19 M NaOH in a beaker and stirred vigorously. Upon adding lead (II) acetate, the solution initially became cloudy, and then turned to a peach color, and finally to a deep orange red. At this point in the synthesis, stirring was stopped, and the precipitate was allowed to settle. The supernatant was then decanted, filtered on a Buchner funnel, washed with deionized water repeatedly, and dried for overnight in a drying oven at 90 °C. The sample was then removed and lightly crushed in a mortar and pestle (Alagar et al. 2012).

Characterization of synthesized CdO and PbO NPs

Crystalline phases of CdO and PbO NPs were analyzed by X-ray diffraction (XRD) by use of a Siemens D500 X-ray diffractometer. Spectra were recorded using a Phillips PW 1830 instrument operating at 40 kV and 30 mA with Cu Kα 1 radiation. Data were collected for the 2θ range of 10° to 90° with a step of 0.02°. Relative intensities and full-width at half-maximum (FWHM) of synthesized NPs were derived. Size and morphology of both CdO and PbO NPs were also characterized using HR-TEM (high-resolution transmission electron microscopy) using a JEOL 100CX (Jeol Inc., Peabody, MA, USA) operated at 80 kV. Particle sizes were analyzed using Image J software.

Test animals

Experiments were conducted during July–October 2017 with *A. mellifera* workers of the subspecies *carnica*, obtained from hives of the apiary of the experimental farm, city of scientific research and technological applications (SRTA-city) (New Borg El Arab, Alex., Egypt). Brood frames have been selected and nurse bees have collected, taken to the laboratory, and placed in refrigerator at 4 °C for approximately 10 min to slow movement.

Determination of LC_{50}

Five serially diluted concentrations of synthesized NPs (CdO: 0.002, 0.02, 0.2, 2, 20 mg/ml; PbO: 0.6, 1.2, 2.5, 5, 10 mg/ml) were tested against honey bee workers to determine the median lethal concentration (LC_{50}). Adult workers were transferred to ventilated wooden cages (9.5 × 4 × 7.5 cm), with 20–25 bees per cage. Bees were deprived of food for 4 h prior to exposures and thereafter fed suspensions of sucrose CdO NPs or PbO NPs with desired concentration of each metal oxide NPs or control sucrose solution (1.5 M). Access to sucrose solution was through a 50-mm diameter Petri dish placed at the bottom of each cage. The Petri dish was covered with a plastic net to prevent bee drownings. During the experiment, the caged bees were kept in an incubator at 27 °C and 65% relative humidity. Experiments were conducted in triplicate for each concentration. Preliminary results of the LC_{50} bioassay for both CdO and PbO NPs indicated that the data obtained after exposure for 24 h and 48 h failed to fulfill the requirements for determination of LC_{50}. Therefore, mortality was recorded daily for 4 days, and then, cumulative mortality was calculated for each concentration tested. LC_{50} for each metal oxide NPs was estimated by the log-probit model using the LdP Line^R software (Ehabsoft (http://www.ehabsoft.com/ldpline).

Exposure of bee workers to sublethal concentrations of CdO and PbO NPs

Bee workers were chronically exposed to sublethal concentrations (20% LC_{50}) of CdO and PbO NPs separately or combined for 9 days. Mixture of sublethal concentrations of both metal oxides NPs was prepared according to LC_{50} of each metal oxide NP (i.e., 0.4 mg of CdO NP + 26 mg of PbO NP were added to 40 ml of sugar syrup to get 0.01 mg/ml and 0.65 mg/ml for CdO and PbO NPs, respectively). Feeding and survival rates were used to monitor stress levels caused by CdO or/and PbO NPs in food. Numbers of dead bees were recorded daily, and survival plots were compared between experimental groups and expressed as a percent of maximal survival. Feeding rate was calculated by dividing the volume of consumed food per day per animal assuming that mass of food was uniform per bee. Volume of consumed suspension/solution was recorded from Petri dishes feeders daily after survival check. Testing solutions or suspension were kept refrigerated. Feeders were refilled (1.5 ml) daily from original solution or suspension. Before refilling feeders, suspensions of metal oxide NPs were vortexed. One additional cage, designated the evaporation cage, was established for each treatment to correct estimates of diet consumption for losses due to evaporation. Feeders in the evaporation cage were filled with the same tested syrup, to estimate as accurately as possible the evaporative characteristics of the diets being tested. The corrected volume loss of each feeder was then divided by the number of surviving bees in each cage to calculate diet consumption per bee per day.

Each bioassay consisted of three replicate cages per treatment and was repeated on three separate occasions (i.e., blocks in time). On the ninth day, subsamples of surviving bees were collected from each cage to quantify the gene expression (section 2.4) and AChE activity (section 2.5). Heads for quantification of activity of AChE and alimentary canal quantification of gene expression were removed from

bee specimens by dissection on ice, and samples were stored at − 80 °C.

Some surviving bee workers were also dissected, and alimentary canals were removed and used to monitor possible accumulation of both metal oxide NPs in mid gut tissues employing proton-induced X-ray emission (PIXE) spectroscopy. Preparations of honey bee tissues were carried out as described previously by Kuterbach and Walcott (1986). Specimens were investigated using field emission scanning electron microscopy and energy dispersive X-ray spectroscopy (SEM–EDX) (FE-SEM; JEOL JSM-5300 LV EDX; EDS/WDS Oxford Instruments INCA).

Expressions of genes

Real-time quantitative PCR (RT-qPCR) was performed to determine effects of chronic exposure of honey bee workers to sublethal concentrations of both metal oxide NPs separately or combined on expression of four selected genes important in detoxification of xenobiotics by honey bees and antioxidative responses. Total RNA was isolated from alimentary canals of worker bees by use of an RNA extraction kit (Thermo scientific) according to the manufacturer's protocol. Concentrations of RNA were determined by use of a Nanodrop ND-1000 spectrometer (Nanodrop Technologies, Wilmington, DE). Then, first-strand cDNA was synthesized by use of a SensiFAST cDNA synthesis kit (Bioline, Singapore) according to the manufacturer's protocol. A mixture of random hexamer and anchored oligo (dT) 18 primers were used. RT-PCR reactions were performed in a 25-μl reaction volume which contained 12.5 μl SYBER Green PCR master mix, 2 μl primer (50 nmole), 2 μl template cDNA, 8.5 μl deionized water, by use of two-step cycling SensiFAST SYBR Lo-ROX kit (Bioline, Singapore) according to the manufacturer's procedure. Primers were from Hu et al. (2017) (see Supplementary material Table S1).

Beta actin was used as a housekeeping gene. Thermal cycling conditions were: 95 °C for 2 min, followed by 40 cycles 95 °C for 15 s, 52 °C for 30 s, and 72 °C 30 s. In total, qPCR was performed on RNA isolated from three composite samples ($n = 15$) per treatment; one randomly selected from each set of treatments from each of the three bioassays (blocks). Fold changes in abundances of transcripts of detoxification genes of interest were analyzed by use of the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

AChE activity

Bee heads ($n = 25$) from each of the three bioassays (blocks) that survived each exposure scenario were randomly selected, weighed, pooled, and homogenized 0.1 M phosphate buffer, pH 8.0 (polytron homogenizer) for 15 s. Homogenates were centrifuged at 5000 rpm for 20 min at 4 °C (Sigma 3-30K5, Germany). The supernatant was used for quantification of enzyme activities by methods described previously (Ellman et al. 1961) using acetylthiocholine iodide as the substrate. The activity of AChE was calculated as nanomoles of substrate hydrolyzed per mg protein per min. Total protein was determined according to method of Lowry et al. (1951) with bovine serum albumin as standard.

Data analyses

Data were analyzed by use of GraphPad Prism version 5.00 for Windows, (GraphPad Software, La Jolla California USA, www.graphpad.com). Normality of data was assessed by use of the Kolomogrov-Smirnov test, and homogeneity of variance was assessed by use of Levene's test. If necessary, data was log10-transformed to better approximate normality and homogeneity of variance. Effects of treatments on survival of bees were assessed by log-rank paired test, $p < 0.0083$ after Bonferroni correction. Differences in rates of feeding among treatments were done using one-way repeated measure ANOVA with Tukey post hoc test, ($P < 0.05$ for acceptance). Activities of AChE were assessed using one-way ANOVA followed by Tukey's post hoc test. Abundances of transcripts of genes involved in detoxification and antioxidative defense (C cytochrome P450 gene (CYP4G11) and superoxide dismutase (SODH2) and glutathione S-transferase D1 (GstD1) and catalase were assessed using one-way ANOVA followed by Tukey's post hoc test and Kruskal-Wallis ANOVA with Dunn's multiple comparison post hoc test, $P < 0.05$, respectively. An alpha level of 0.05 was used for all tests.

Results

Characterization of synthesized CdO and PbO NPs

XRD analysis

XRD patterns of synthesized CdO and PbO NPs are shown (Fig. 1). Diffraction peaks are absorbed at 2 theta (θ) values. The Scherer equation (Eq. 1) was used to estimate sizes of NPs represented by prominent peaks (Eshaghi et al. 2010) (Eq. 1).

$$d = \frac{0.94\,\lambda}{\beta\cos\theta} \tag{1}$$

where λ is the wavelength ($\lambda = 1.542$ Å) (Cu Kα), β is the full width at half maximum (FWHM) of the line, and θ is the diffraction angle.

Mean crystalline sizes estimated for CdO and PbO NPs were 69.84 and 52.64 nm (Table 1). Very sharp and strong

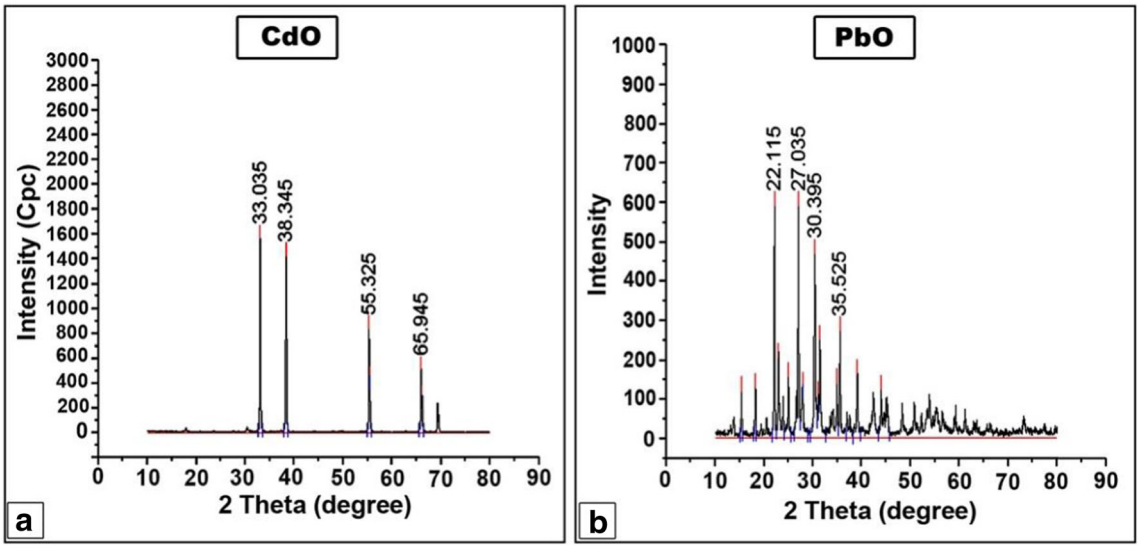

Fig. 1 XRD patterns of **a** CdO nanoparticles prepared at 0.5 M of cadmium acetate and **b** PbO nanoparticles prepared at 0.02 M of lead nitrate at room temperature

peaks indicate crystallization state of prepared CdO and PbO NPs (Fig. 1). Diffraction peaks are clearly seen and perfectly indexed CdO and PbO according to peak position of CdO (Joint Committee for Powder Diffraction Studies (JCPDS) File No. 05-0640) and of PbO (JCPDS 05-0561) (Fig. 1).

HR-TEM analysis

Results of HR-TEM morphological and nanostructural studies of synthesized CdO and PbO NPs are shown (Fig. 2). The HR-TEM micrograph also showed sizes of both NPs to be in the nanometer range.

Determination of LC_{50}

The potency of a poison is a measure of its strength compared to other poisons. Based on survival of honey bee workers to 96 h post-treatment, CdO was more potent than was PbO (Table 2). Based on comparisons of LC_{25} for CdO (LC_{25} = 0.01; LC_{50} = 0.06; LC_{90} = 1.46 mg/ml) and PbO (LC_{25} = 1.18; LC_{50} = 3.27; LC_{90} = 22.5 mg/ml) against honey bee workers, CdO was 118-fold more potent than was PbO, which had a relative potency of 0.008. Slopes of dose-response relationships for the two metal oxide NPs were significantly different, which indicated different responses of bee workers to the two metal oxide NPs.

Proton-induced X-ray emission

Energy-dispersive X-ray (SEM-EDX) spectra of air-dried mid gut tissues of bee workers showed small peaks for CdO (0.8 ± 0.06%) and PbO (1.7 ± 0.4%) NPs in bee workers fed sugar syrup contained sublethal concentrations of CdO and PbO NPs, respectively. Mid gut tissues of bee workers fed sugar syrup containing the mixture of sublethal concentrations of both metal oxide nanoparticles contained traces of both CdO (1.1 ± 0.1%) and PbO (1.9 ± 0.3%) NPs while mid gut tissues of bee workers fed sugar syrup only (control) contained no metal oxide NPs (Supplementary material, Fig. S1).

Table 1 XRD data of CdO and PbO nanoparticles prepared at 0.5 M and 0.02 M, respectively

Metal	d-spacing (Å) observed	Intensity (cps)	FWHM β (deg)	(2Theta)	Grain size (nm)
CdO	2.71	100.00	0.17	33.03	50.93
	2.34	91.25	0.17	38.34	51.70
	1.66	55.26	0.10	55.32	95.64
	1.41	34.97	0.12	65.94	81.11
PbO	4.01	100.00	0.12	22.11	67.12
	3.29	99.24	0.15	27.03	56.91
	2.93	79.61	0.23	30.39	36.17
	2.52	47.41	0.17	35.52	50.38

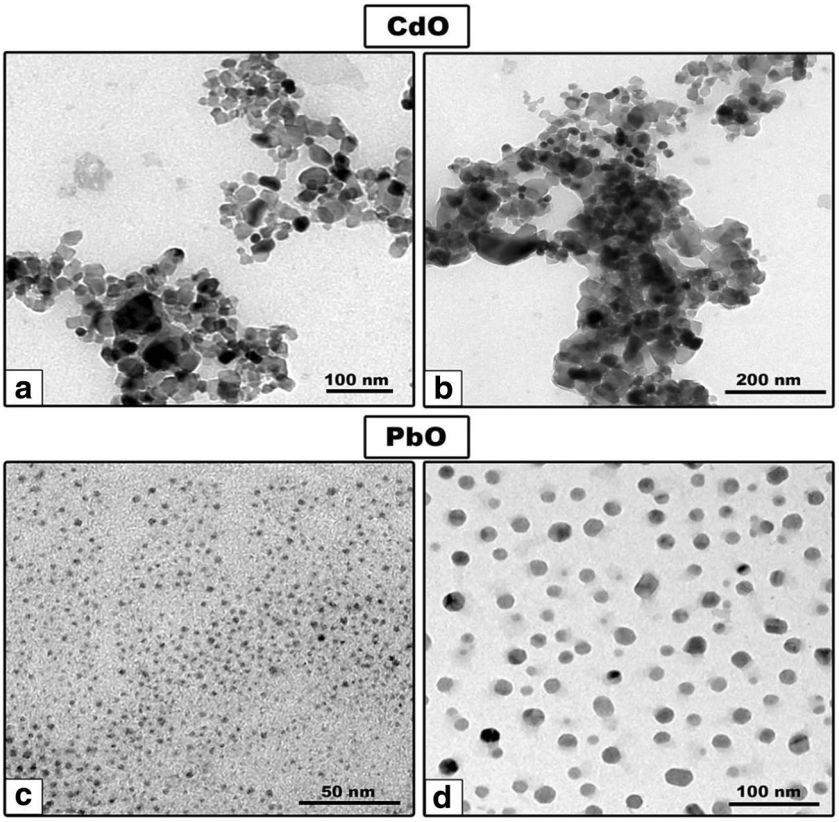

Fig. 2 HRTEM micrograph of synthesized CdO NPs scale bar: **a** 100 nm, **b** 200 nm and PbO NPs scale bar **c** 50 nm and **d** 100 nm

Feeding rate and survival

During chronic exposure of honey bee workers to sublethal concentrations (20% of LC_{50}) of CdO and PbO NPs separately or combined, rates of survival in treated groups decreased significantly compared to the control (log-rank (Mantel-Cox) test: (CdO NPs, $x^2 = 17.92$, $p = 0.0001 < 0.0083$; PbO NPs, $x^2 = 7.12$, $p = 0.007 < 0.0083$; binary mixture, $x^2 = 8.54$, $p = 0.003 < 0.0083$ after Bonferroni correction). Least survival was in bees exposed to CdO NPs, followed by those exposed to PbO NPs and they differed significantly (log-rank test) ($x^2 = 7.30$, $p = 0.006 < 0.0083$ after Bonferroni correction) (Fig. 3a).

By repeated measure ANOVA, metal oxide NPs tested either separately or combined had a significant effect on average daily consumption of syrup per bee ($P < 0.0001$) compared to control. Bees consuming the control diet ingested more syrup than did bees consuming syrup containing CdO NPs and the binary mixture of both metal oxides NPs ($P < 0.05$). Bee workers consumed more syrup containing PbO NPs than did bee workers consuming syrup containing CdO NPs or the binary mixture of both metal oxide NPs ($P < 0.05$) (Fig. 3b). There was no significant difference in mean daily consumption per bee between untreated control bees and those fed PbO NPs (Fig. 3b).

By considering temporal effects and treatment on feeding rate, significant differences were found between treatments and time. This included the interaction term 'treatment X time' ($p < 0.001$) where the 'repellent' nature of the tested metal oxides NPs retarded the start of dietary uptake. Additionally, there was a more than 2-fold decrease in feeding rate starting from day 2 to day 5 in bee workers fed sugar syrup spiked with CdO NPs and the binary mixture of both metal oxide NPs (Supplementary material, Fig. S2).

Table 2 Susceptibility of honey bee workers *(Apis mellifera)* to CdO and PbO NPs after 96-h exposure

Metal	N^a	LC25 (95% CLd[b]) (mg/ml)	LC50 (95% CLd[b]) (mg/ml)	LC90 (95% CLd[b]) (mg/ml)	Slope ± SE[c]
CdO	300	0.01 (0.006–0.02)	0.06 (0.04–0.09)	1.46 (0.78–3.51)	0.94 ± 0.09
PbO	300	1.18 (0.65–1.70)	3.27 (2.35–4.81)	22.50 (12.10–75.55)	1.53 ± 0.27

[a] *N* is the number of bee workers tested

[b] CL is the 95% confidence limits

[c] Slope ± standard error

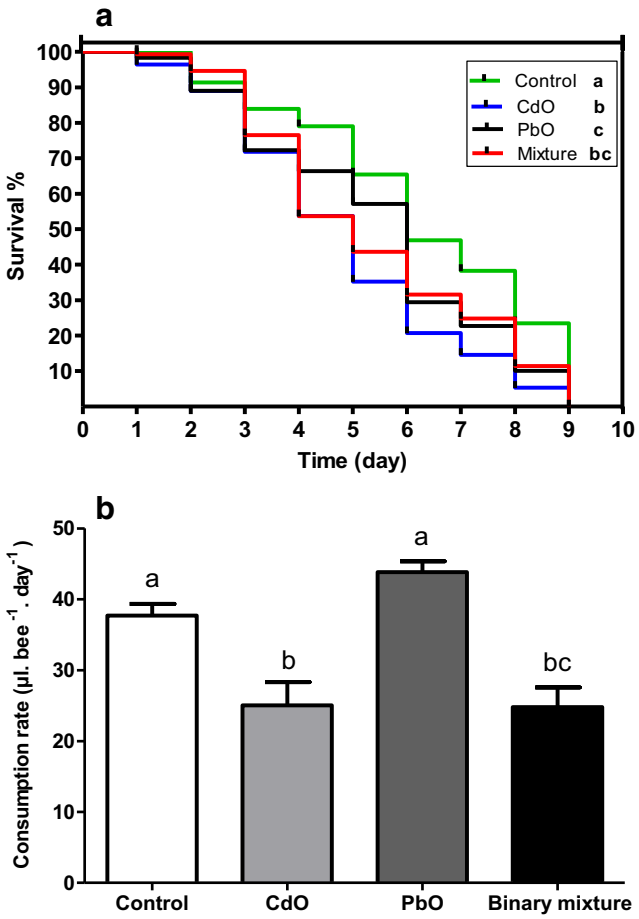

Fig. 3 Effects of CdO or/and PbO NPs on honey bee survival (**a**) and feeding rate (**b**). **a** Kaplan–Meier plot of survival of honey bee workers that chronically exposed to sublethal concentrations (20% of LC_{50}) of: CdO NP suspension (0.01 mg ml^{-1}) or PbO NP suspension (0.6 mg ml^{-1}) or their binary mixture in 1.5 M sucrose or control (1.5 M sucrose). Different lower-case letters indicate statistical differences between treatments (log-rank (Mantel-Cox) paired test, $p < 0.0083$ after Bonferroni correction). **b** Consumption rate (the volume of consumed food per day per bee) (mean ± SD) of honey bee workers that chronically exposed for 9 days to sublethal concentrations of either CdO NPs or PbO NPs or their binary mixture in 1.5 M sucrose or control (1.5 M sucrose). Different letters denote significant differences among treatments (one-way repeated measure ANOVA with Tukey post hoc test, $P < 0.05$)

Expressions of genes

Chronic exposure of honey bee workers to sublethal concentrations of CdO and PbO NPs individually or combined in sugar syrup diets caused significant difference in abundance of transcripts of detoxification and antioxidative defense genes, CYP4G11 ($p < 0.0001$) and SODH2 ($p < 0.0001$) compared to control (Fig. 4). Expression of GstD1 in bees consuming syrup containing PbO NPs was up-regulated 2.2-fold relative to that of the control. In honey bee workers that ingested CdO or PbO NPs or their binary mixture, expressions of CYP4G11 were up-regulated 33-, 9-, and 54-fold relative to control bees consumed diet lacking any metal oxides NPs, respectively. Chronic feeding of honey bee worker diets containing either CdO or PbO NPs caused down-regulation of 5.7- and 1.8-fold for SODH2 expressions relative to bee workers fed control diet, respectively. However, SODH2 is overexpressed by 3.5-fold in bees consumed sugar syrup diet containing the binary mixture of both CdO and PbO NPs than control (Fig. 4).

Abundances of transcripts of catalase in bee workers chronically fed diets of sugar syrup containing sublethal concentrations of both CdO and PbO NPs differed significantly (Catalase; $H = 17.8$, $df = 3$, $P = 0.01$) relative to bee workers fed control diet (sugar syrup only) (Fig. 4). Expression of the antioxidative enzyme catalase was 13.6-fold greater than that in control bees consumed sugar syrup diet containing binary mixture of sublethal concentrations of both CdO and PbO NPs.

AChE activity

Exposure of bee workers to sublethal concentrations of CdO and PbO NPs either separately or combined in sugar syrup caused significant decrease ($p < 0.0001$) in specific activity of AChE in honey bee heads compared to control (Fig. 5). In honey bee workers fed diets containing CdO or PbO NPs or their binary mixture, activity of AChE inhibited by 3.8-, 3.0-, and 2.8-fold relative to control, respectively.

Discussion

Serious ecological consequences and effects on human and animal health have been found to be related to the presence of NPs in biosystems (Handy et al., 2008b). The most risky implications are associated with chronic consumption and inhalation of NPs (Moore 2006). In the present study, CdO NPs were more potent "exert adverse effect" to honey bee workers than were PbO NPs, a result which is consistent with those of previous studies (Gauthier et al. 2016; Nikolić et al. 2016; Di et al. 2016), in which cadmium salts were more potent to bee workers than were lead salts. The LC_{50} of CdO (0.06 mg/ml) was comparable to the LC_{50} of dissolved $CdCl_2$ (0.07 mg/ml) while the LC_{50} of PbO (3.27 mg/ml) NPs were more than that of $PbCl_2$ (0. 34 mg/ml) (Di et al. 2016). It was hypothesized that due to their unique properties, NPs formed from Cd and Pb oxides would be more potent to honey bees than their metal salts. However, these differences might be attributed to differences in sensitivities among subspecies of honey bees used in various studies.

Accumulation of Cd and Pb metal oxide NPs in mid gut tissues of bee workers in the present study might be attributable to unique properties of NPs that enable them to enter organisms and be transported into tissues, cells, and even into

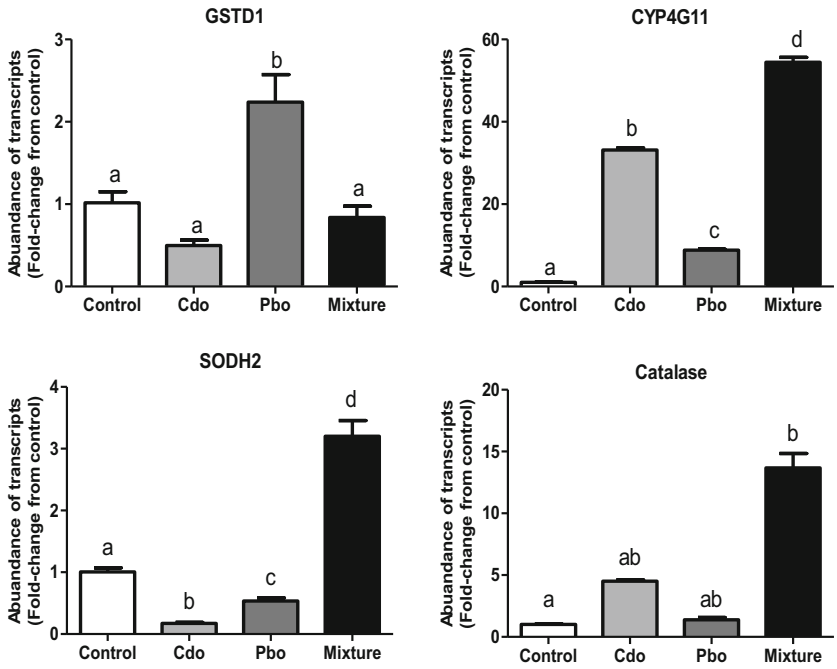

Fig. 4 Fold changes in abundances of transcripts of genes involved in metal oxide NP detoxification and antioxidative defense in honey bee workers chronically exposed (9 days) to sublethal concentrations (20% of LC_{50}) of CdO and PbO separately or combined. Bars represent mean concentrations (±SEM) of three samples. Different letters denote significant differences among treatments (one-way ANOVA with Tukey post hoc test, $P < 0.05$; Kruskal-Wallis ANOVA, with Dunn's multiple comparison post hoc test, $P < 0.05$ for catalase)

organelles, in ways that larger particles might not (Kovochich et al. 2007). After reaching specific cell organelles, the NPs can affect metabolic processes by enhancing production of reactive oxygen species (ROS) and affect the biochemical processes (Miao et al. 2010).

Rates of feeding were greater in the group fed PbO NPs than CdO NPs and the binary mixture-treated groups, but not when compared to the control group. Similar adverse effects for the same soluble salts of these metals on development and survival of honey bees under laboratory and field conditions have been reported previously (Di et al. 2016; Hladun et al. 2016). Honey bees might possess some specific elements of the physiological stress response that coordinate rapid changes in metabolic activity and behavior, such as increased energy mobilization and arousal (Even et al. 2012). This is a possible explanation for the increase in average feeding rate observed in PbO NP group compared to both CdO NPs and the binary mixture-treated groups but not with the control. Milivojević et al. (2015) also reported an increase in feeding rate in bees consumed Zn^{2+} but not with ZnO NPs or the control group.

Alternatively, the lesser rate of feeding and survival of bees exposed to CdO NPs could be attributed to greater toxic potency of CdO NPs. It is possible that ingestion of diets of sugar syrup containing more potent CdO NPs causes a malaise effect in workers, causing them to consume less of the diets (Ayestaran et al. 2010; Hurst et al. 2014). This effect was confirmed by observing and recording these malaise-like' behaviors, particularly of bees fed CdO NPs and the binary mixture (see supplementary material videos). These malaise-like' behaviors included more time spent performing of specific behaviors such as being unable to perform the righting reflex, being curled up, or abdomen dragging as reported in previous studies (Ayestaran et al. 2010; Hurst et al. 2014).

In nature, organisms can be exposed, simultaneously, to mixtures of xenobiotics (metals, pesticides, and toxic gases etc.). Therefore, interactions between xenobiotic substances as well as xenobiotic and animal systems are potentially important (Oesch et al. 2015). Rates of survival and feeding were less in the group treated with the binary mixture of both metal oxide NPs than the control group. It was concluded that there was antagonism between CdO and PbO NPs observed in this study, could have been due to lesser rates of feeding of bees fed the binary mixture or CdO NPs relative to the greater rate

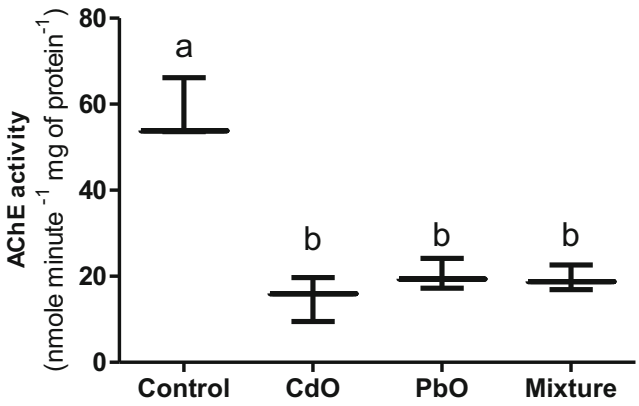

Fig. 5 Effects of exposure to CdO and PbO NPs either separately or combined spiked food for 9 days on AChE activity in the heads of honey bee workers. Symbols on the box plot represent maximum and minimum values (whiskers: ⊤ ⊥), mean values (-). Different letters denote significant differences among treatments (one-way ANOVA with Tukey post hoc test $P < 0.05$)

of feeding observed for bees fed PbO NPs. These results are consistent with those of a previous study where antagonistic effects on luminosity between Cd and Pb on the luminescent bacterium *Photobacterium phosphoreum* T3S were observed (Zeb et al. 2017).

An intrinsic feature of organisms exposed to pollutants is the ability to adapt to certain concentrations of pollutants by turning on a variety of compensatory or detoxification mechanisms or variation in expression and activity of many enzymes. In the current study, expression of GSTD1 in bees consuming the diet containing PbO NPs was significantly greater (2.2-fold) relative to that of the controls, compared to other treated groups. Similarly, greater GST activities were observed in brains of honey bees exposed for 10 days to diets containing ZnO NMs or $ZnCl_2$ (Milivojević et al. 2015). GST is widely distributed in tissues of honey bees, including brains. However, its main activities are in the midgut (Diao et al. 2006).

The "Phase I" cytochrome P450 superfamily of enzymes (CYP450) is generally the first defense employed by the cell to bio-transform organic xenobiotics; however, influence of metal ion on expression of CYP genes has also been reported (Korashy and El-Kadi 2005; Zhang et al. 2015). In the present study, CYP4G11 was over expressed in bee workers exposed to CdO and/or PbO NPs compared to the control. These observations were consistent with those of previous studies where induction of CYP450 genes in honey bees was observed in response to dietary phytochemical and pesticide exposures (Feyereisen 2012; Johnson et al. 2012; Mao et al. 2011; Hu et al. 2017). It was hypothesized that if exposure of bees to CdO and/or PbO NPs reduced the CYP activities, it could make bees more sensitive to effects of organic xenobiotics. However, over-expression of CYP genes in bee workers might be indicative of adaptation of bees to respond to Cd and Pb oxide NPs. To our knowledge, the present study is the first to examine the expression of genes encoding CYP450 enzymes and its possible role in metal-detoxification in honey bees.

Some ingested metals alter biological pathways of the honey bee involved in anti-oxidative responses, there by depressing the immune system and causing a greater rate of bee mortality (Gauthier et al. 2016; Polykretis et al. 2016; Hladun et al. 2016). SOD enzymes dismute superoxide radicals, breakdown hydrogen peroxides and hydroperoxides to harmless molecules (H_2O_2/alcohol and O_2). In the current study, SODH2 and CAT were over-expressed only in bees fed the binary mixture of CdO and PbO NPs compared to control. In contrast, expressions of SODH2 were down-regulated relative to that in control bees when fed CdO or PbO NPs, respectively. These enzymes are functionally linked; SOD catalyzes dismutation of superoxide radical anions producing H_2O_2, which is a substrate for CAT (McCord and Fridovich 1969; Fridovich 1997). Thus, induction of both CAT and SODH2 only in bees fed the binary mixture might be an indication of the decrease in the amount of ROS produced due to antagonism between CdO and PbO NPs, which was sufficient to activate and induce both CAT and SODH2. Inhibition, when bees were exposed to metal oxide NPs individually, might be a result of adverse effects and toxicity of each metal oxide NP, that increased levels of ROS, resulting in lipid peroxidation and oxidative stress (Korsloot et al. 2004; Kakkar and Jaffery 2005; Nikolić et al. 2016). Further studies are required to investigate and validate this hypothesis.

Alterations of AChE activity can influence the process of cholinergic neurotransmission and promote undesirable effects, which have been observed in several neurological disorders (Acker et al. 2011; Boily et al. 2013). Neurotoxic effects due to inhibition of AChE in heads of honey bees after consuming CdO or PbO NPs separately, or in combination, could be due to a direct action of metal oxide NPs on the active site of this enzyme (Richetti et al. 2011). Cd inhibits release of acetylcholine, probably by interfering with calcium metabolism (Desi et al. 1998; Collet and Belzunces 2007) and Pb negatively influenced both the V max and the enzyme-substrate binding affinity (Gupta et al. 2015).

Effects on AChE activity, observed during this study, are consistent with results of studies showing inhibition of cholinesterase activities by carbamate and organophosphate pesticides (Boily et al. 2013), metal ions (Cu^{+2}), and metal oxides NPs (CuO and ZnO) (Gomes et al. 2011; Jun et al. 2013). In contrast, greater activities of AChE in bees after having been fed ZnO NMs or $ZnCl_2$ (Milivojević et al. 2015) and cerium (IV) oxide nanoparticles ($nCeO_2$) (Kos et al. 2017) have been reported. Furthermore, an increase of 46% AChE activity in brains of adult rats was observed after exposure to cadmium (1 mg/kg/day for 14 days) (Carageorgiou et al. 2005). The discrepancies in AChE activity might be attributed to several factors including: sensitivity of the animal or species tested; the dose or the concentration and mode of action of the substance tested; the length or/and time of exposure and the age of the test organism and the exposure conditions.

Conclusions

The results of this study demonstrated, for the first time, that exposure to sublethal concentrations of either CdO or PbO NPs separately or combined caused number of adverse effects on honeybees after chronic (9 days) exposure. Rates of survival decreased in all treatments compared to control, but only more profound in bees fed diets contained CdO NPs. Expression of selected stress-related detoxifying enzyme; CYP4G11 was up-regulated in all test groups in response to metal oxides NPs, while the expressions of the antioxidizing enzymes CAT and SODH2 were up-regulated in only bees consumed sugar syrup diet containing binary mixture of

sublethal concentrations of both CdO and PbO NPs relative to bee workers fed control diet (sugar syrup only). AChE activity in brain of honey bees was inhibited by exposure to Cd or/and Pb oxides NPs, compared to the control. Both Cd and Pb oxides NPs have harmful effects on fitness of the honey bee. CdO NPs are of greater potency on honey bees than PbO NPs. The results offer specific insight into the potential risk of CdO or/and PbO NP release into the environment to honeybees, which may be translated to other ecologically and economically important pollinators.

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Sublethal Effects of Chronic Exposure to CdO or PbO Nanoparticles or Their Binary Mixture on the Honey Bee (*Apis millefera* L.)

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Supplementary material

Table S1. Sequences of primers used for quantification of abundances of transcripts of genes involved in detoxification and antioxidative defense in honey bees (*Apis mellifera*) (**Hu et al., (2017)**.

Gene name	Forward sequence	Reverse sequence
Actin	TTCCCATCTATCGTCGGAAG	CTCTCTTTGATTGGGCTTCG
GSTD1	GCCGCTTCAAAAGAAGTACG	GTGGCGAAAACAAGGATGAT
SODH2	CAGTGCATGGTAGCCTGAGA	ACAGTGCTCCTTCAGCCAAT
Catalase	GTCTTGGCCCAAACAATCTG	CATTCTCTAGGCCCACCAAA
CYP4G11	CAAAATGGTGTTCTCCTTACCG	ATGGCAACCCATCACTGC

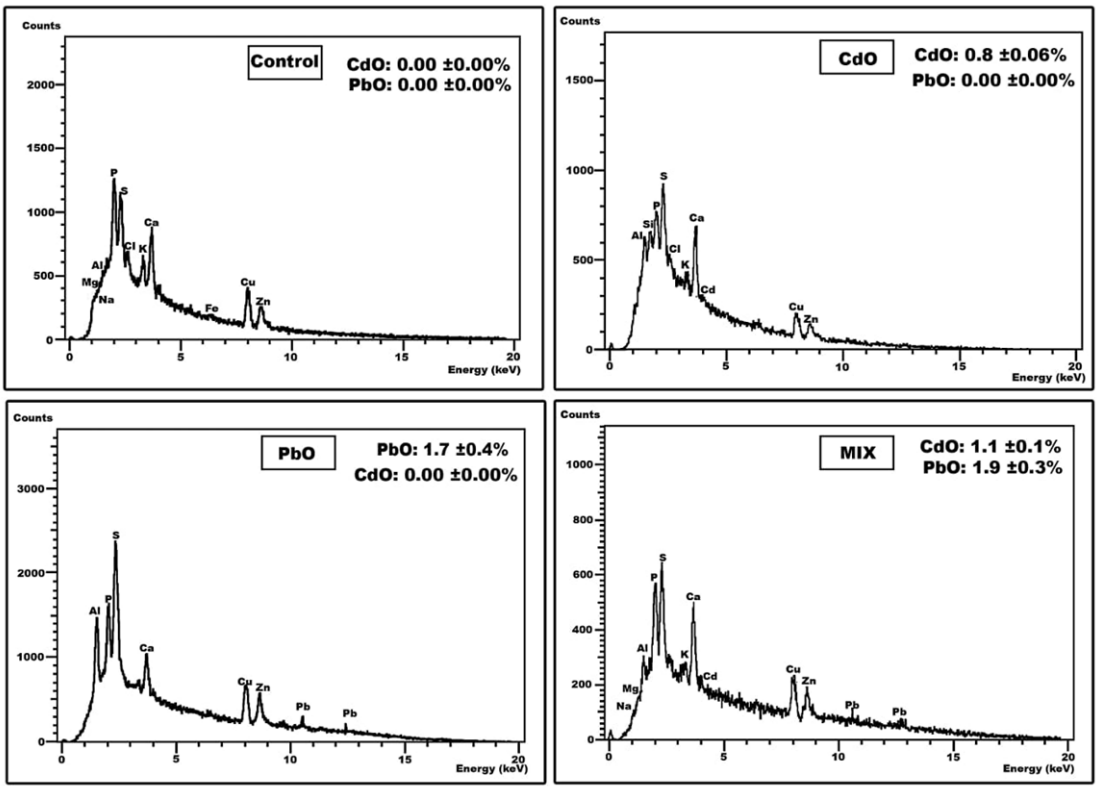

Figure S1. SEM-EDX spectra of air-dried mid gut tissues of honey bee workers chronically exposed for 9 days to; CdO NPs suspension (0.01 mg ml^{-1}) or PbO NPs suspension (0.6 mg ml^{-1}) or their binary mixture in 1.5 M sucrose or control (1.5 M sucrose). Semi-quantitative detection (% of height peak) of metal oxides NPs were expressed (mean ± SD) (n=3).

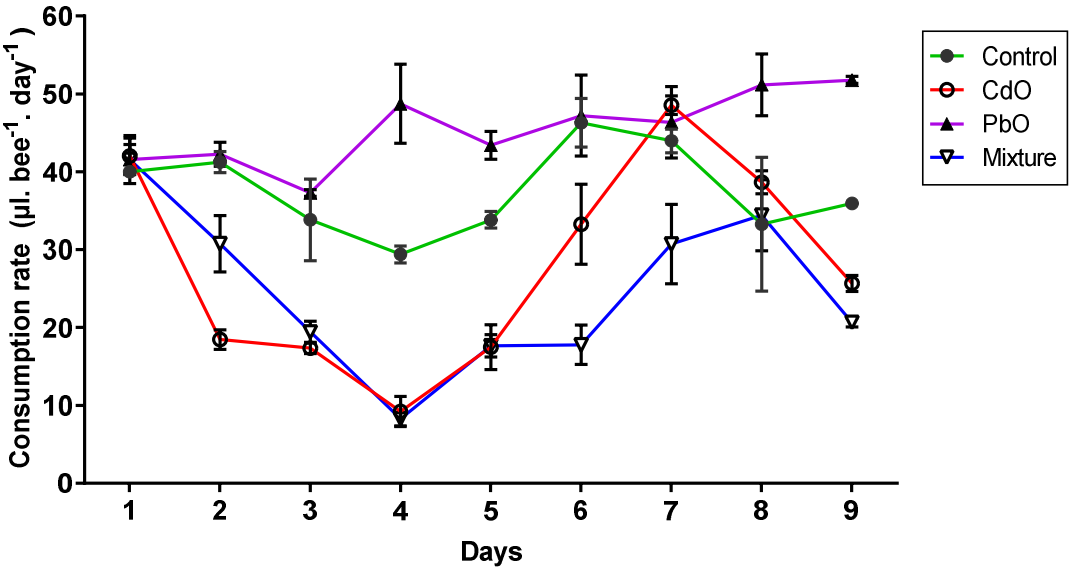

Figure S2. Daily consumption rate (µl. bee^{-1}. day^{-1}) (Mean ± SE) of honey bee workers that chronically exposed for 9 days to sublethal concentrations (20 % Lc_{50}); CdO NPs suspension (0.01 mg ml^{-1}) or PbO NPs suspension (0.6 mg ml^{-1}) or their binary mixture in 1.5 M sucrose or control (1.5 M sucrose). Significant differences were found between treatments and time including the interaction term 'treatment X time' ($p < 0.001$; Two-way ANOVA, $p < 0.05$).