Impact of dietary cadmium sulphide nanoparticles on *Danio rerio* zebrafish at very low contamination pressure

Chiraz Ladhar¹, Benjamin Geffroy¹, Sébastien Cambier¹, Mona Treguer-Delapierre², Etienne Durand², Daniel Brèthes⁵, & Jean-Paul Bourdineaud¹

¹University of Bordeaux, CNRS, UMR 5805, Arcachon Marine Station, Place du Dr Peyneau, 33120 Arcachon, France, ²CNRS, UPR 9048, Université de Bordeaux, Institut de Chimie de la Matière Condensée de Bordeaux, 87 avenue du Dr. Albert Schweitzer, 33608 Pessac, Cedex, France and ³CNRS, UMR 5095, Institut de Biochimie et Génétique Cellulaires, Université Victor Segalen-Bordeaux 2, Bordeaux, France

Abstract

To address the impact of cadmium sulphide nanoparticles (CdSNPs) of two different sizes (8 and 50 nm), *Danio rerio* zebrafish were dietary exposed to very low doses: 100 or 40 ng CdSNPs/day/g body weight for 36 or 60 days, respectively. The results obtained using RAPD-PCR genotoxicity test showed genomic alteration since the number of hybridisation sites of the RAPD probes was significantly modified after CdSNPs exposure. In addition, selected stress response genes were either repressed or upregulated in tissues of CdSNPs-exposed fish. Mitochondrial dysfunction was also caused by the presence of CdSNPs in food. Cadmium accumulation in fish tissues (brain and muscles) could only be observed after 60 days of exposure. CdSNPs toxicity was dependent on their size and concentration.

Keywords: cadmium, nanoparticles, zebrafish, genotoxicity, mitochondrial respiration

Introduction

Quantum dots (QDs) based on type II–VI semiconductors, that is, involving elements from columns II and VI of the periodic table are the most frequently used for biomedical imaging. Currently, the most popular and commercially available QDs are those comprising cadmium-tellurium (CdTe) or cadmium-selenium (CdSe) cores (Lim et al. 2003). Both QDs have received considerable attention because they act as long-lasting fluorescent elements. Their spectral characteristic can be adjusted simply by modifying their dimension. The potential toxicity of NPs is due to many factors, including shape, chemical composition and tiny size (Casals et al. 2008; Hardman 2006). Cd-based QDs induce cytotoxicity and oxidative stress in *in vitro* studies (Chan & Shiao 2008; Cho et al. 2007; Choi et al. 2007; Clift et al. 2010; Koeneman et al. 2009; Lee et al. 2009; Male et al. 2008). Injection of Cd/Se/Te-based QDs into mice caused oxidative stress and hepatotoxicity (Lin et al. 2011).

The average estimation of the amount of NPs released into the aquatic environment per year is 65 tons (Blaser et al. 2008). Those NPs come from manufactured goods, effluent and spillage during the shipping and handling of products, or through deliberate disposal (Handy et al. 2008a, b). Therefore, the different components of the environment (sediment, water and organisms) can be harmedly affected (Moore 2006; Wiesner et al. 2006). NPs may contaminate the aquatic environment and exert toxic effects in aquatic species (Farré et al. 2009). For example, exposure of freshwater mussels to Cd-based QDs was immunotoxic and led to oxidative stress in gills and DNA damage after an exposure period of 24 h at very high waterborne doses ranging from 1.6 to 8 mg/l (Gagné et al. 2008a). In fish, nanometals can cause sublethal effects including respiratory toxicity, perturbation of trace elements, inhibition of Na⁺K⁺-ATPase and oxidative stress (Shaw & Handy 2011). In rainbow trout hepatocytes, CdTe-based QDs increased Cd levels in tissues and induced a number of biomarkers such as metallothio- neins (Gagné et al. 2008b). Lower leukocyte density and viability were observed in rainbow trout exposed to 1–6 μg/l CdS/CdTe QDs (Gagné et al. 2010). Hepatic pathology and compromised reproductivity fitness of male sticklebacks are associated with exposure to CdS QDs ranging from 5 to 500 μg/l (Sanders et al. 2008).

Up to now, only a few studies addressed the toxicity of QDs on live aquatic animals and they have been performed at high waterborne doses ranging from 1 to 8000 μg/l, giving contamination pressures ranging between 0.3 and 32 μg/day/g of body weight (Table I). Given the scarcity of published...
data, the contamination pressures were calculated, apart from this study, for just two out of six studies. The authors decided to expose *Danio rerio* zebrafish to QDs through the diet and at very low contamination pressure to mimic what really happens in the natural habitat. The authors looked for impacts of cadmium sulphide nanoparticles (CdSNPs) of two sizes (8 and 50 nm) on mitochondrial activity, DNA damage and genetic response to oxidative and general stress in different organs (muscle, liver, brain and digestive tract), at contamination pressures of 40 and 100 ng NPs/day/g of body weight.

**Materials and methods**

**Elaboration of cadmium sulphide nanoparticles**

The CdSNPs were manufactured by the Institute of Condensed Matter Chemistry of Bordeaux (ICMCB, Bordeaux, France). The CdSNPs were prepared via a colloidal approach in aqueous solution. CdSO$_4$ (99.99%) and sodium polyphosphate were prepared and introduced in a threenecked flask. The pH was adjusted to 9.5 with NaOH. The solution was degassed by bubbling with Ar(g) for 3 h at 80°C. The solution was then bubbled with mixed H$_2$S/Ar (g) for 30 min at 80°C with stirring, at which point it was left under Ar (without heating) for about 3 h to eliminate any excess of H$_2$S. This procedure resulted in a yellow solution consisting of CdSNPs of 8 nm of diameter. Larger spherical CdSNPs were produced by using a seed-mediated route. The CdS seeds (8 nm), produced as described above, were introduced into a 100 ml of water solution containing CdII precursor (10 mM) and sodium polyphosphate (300 μM) at pH 9.5. The solution was degassed with Ar and then H$_2$S to enlarge the seeds diameter. The same procedure was repeated to obtain almost spherical particles of average size of 50 nm diameter. Structural and size characterization of the NPs was investigated by using conventional and high-resolution transmission electron microscopy (TEM and HRTEM). Preliminary TEM and HRTEM observations were performed using a JEOL 2200 FS equipped with a field emission gun, operating at 200 kV. HRTEM micrographs were acquired with a Gatan Ultrascan CCD 2k × 2k. Moreover, in order to be representative and statistically meaningful, many images from several

---

*Table I. A literature survey of QDs doses used in live aquatic organisms.*

<table>
<thead>
<tr>
<th>Exposure route and species</th>
<th>Doses in water or feed, contamination pressures and accumulated metal$^a$</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waterborne, freshwater mussels <em>Elliptio complanata</em></td>
<td>1.6, 4 and 8 mg/L of CdTe for 24 h. Mussels accumulated 6.5, 6.5 and 12.5 μg Cd/g dry weight in the digestive gland after exposure to 1.6, 4 and 8 mg NPs/L, respectively.</td>
<td>Gagné et al. 2008a; Peyrot et al. 2009</td>
</tr>
<tr>
<td>Waterborne, sticklebacks</td>
<td>5, 50 or 500 μg/L CdSNPs for 21 day, making 0.32, 3.2 and 32 μg CdS/day/g body weight$^a$</td>
<td>Sanders et al. 2008</td>
</tr>
<tr>
<td>Waterborne, zebrafish embryo</td>
<td>CdS$<em>{core}$/ZnS$</em>{shell}$ QDs (0.2, 2 or 20 μM Cd equivalents) for 120 h. Larvae accumulated 4 and 15 ng Cd/g wet weight after exposure to 2 and 20 μM QDs, respectively</td>
<td>King-Heiden et al. 2009</td>
</tr>
<tr>
<td>Waterborne, rainbow trout</td>
<td>CdS/CdTe QDs (1, 2 and 6 μg/L) for 96 h, making 1.1, 2.1 and 6.4 μg QDs/day/g body weight, respectively$^a$</td>
<td>Gagné et al. 2010</td>
</tr>
<tr>
<td>Waterborne, <em>Daphnia magna</em></td>
<td>CdSe/ZnSe QDs (1–95 μg/L) for 48 h$^b$</td>
<td>Kim et al. 2010</td>
</tr>
<tr>
<td>Waterborne, polyp <em>Hydra vulgaris</em></td>
<td>CdTe QDs (0.4, 1 and 2 mg QDs/L) for 12 and 24 h. Polyps accumulated 1.6 and 2.3 μg Cd/individual after 12 and 24 h exposure to 1 mg QDs/L</td>
<td>Ambrosone et al. 2012</td>
</tr>
<tr>
<td>Dietary, zebrafish</td>
<td>4 μg CdSNPs/g of feed given at 2 or 0.8% of the body weight per day, making 40 and 100 ng/day/g body weight for 60 or 36 days of exposure, respectively</td>
<td>This study</td>
</tr>
</tbody>
</table>

$^a$To calculate the contamination pressure of waterborne exposed fish, one had to take into account the ventilation rate which was for rainbow trout 740 ml/kg/min (Wood & Munger 1994), making 1.07 L/day/g body weight, and for sticklebacks 72 ventilations/min (Bell et al. 2010), making 64 mL/day/g body weight; $^b$The article gave no data about the accumulated cadmium concentration; CdSe, cadmium-selenium; CdSNPs, cadmium sulphide nanoparticles; CdTe, cadmium-tellurium; QDs, quantum dots.
regions of various samples were recorded and the most characteristic results are presented here.

Fish diet elaboration
The food containing NPs was elaborated by Special Diet Services (Witham, Essex, UK). Thirty millilitres of the solutions prepared above and containing NPs (0.1 mg/ml) were diluted in 15 ml of pure water, and then added to 600 g of fish diet (SDS400, manufactured by Special Diets Services, Witham, Essex, UK; French commercial representation: Dietex, Saint-Gratien, France). According to Special Diets Services, SDS400 diet is made up with shrimp flour, and a macroanalysis gives 60% protein, 14.5% fat, 11.5% ashes and 3% fibres, and the particles size was between 0.5 and 0.8 mm. These diets were dried at 80°C for 15–20 min. At the end of this process, two batches of artificial food containing NPs of 8 and 50 nm each were elaborated and noted respectively CdS1NP and CdS2NP. The genuine SDS400 regimen without NPs was used as control food. The cadmium concentration of each regimen was quantified by inductively coupled plasma/optical emission spectrometry (ICP/OES). As expected, nothing was found in control food, contrarily to the contaminated food where 4.2 and 4.6 mg Cd/g for CdS1NP and CdS2NP, respectively were found. The incorporation of CdSNPs into food did not modify their morphology as analyzed by TEM (Figures 1 and 2). Samples for electron microscopy were prepared by suspending the food reduced in powder in alcohol by ultrasonication and depositing a drop of the suspension on a copper grid. The grid was finally air-dried for 15 min.

Fish treatment
The authors divided their study in two distinct experiments: D. rerio fish were exposed to NPs through diet for 36 and 60 days, in a first and second experiment, respectively. Besides the exposure time, these two experiments differed mainly by the fact that the authors used two different levels of contaminations: the highest dose was delivered for 36 days and the lowest for 60 days.

The 36 days long experiment
One hundred and fifty adult male fishes (body weight: 0.88 ± 0.03 g, wet weight; standard length: 3.63 ± 0.05 cm, n = 9) were equitably and randomly placed in three tanks, containing 50 L of chlorine-free permanently oxygenated water. Males were used in order not to be confused by hormonal bias. Fish from the two first tanks were fed with 20 mg/fish/day of the two kinds of artificial food mentioned above, which corresponds to 84 and 106 ng CdSNPs/fish/day for CdS1NP and CdS2NP diets, respectively. Thus, each fish

Figure 2. TEM micrographs of 8 nm CdS nanoparticles (CdS1NPs) after incorporation within fish food. Micrographs are showing two NPs of 8 nm in bright field (upper left micrograph) and in dark field (upper right micrograph). Bottom panel: elemental analysis by EDAX of the CdS2NP (50 nm) sample under the microscope.
fed with CdS1NP and CdS2NP received 33.6 billions and 292 millions of NPs per day, respectively. Throughout this experiment, temperature was maintained at 22 °C and water was filtered with a pump in a closed system. Tanks were regularly cleaned during the 36 and 60 days trials as fish faecal matter might have been an additional source of NPs for zebrafish.

Fish in the third experimental unit received the pristine food, and constituted control animals. For gene expression analysis and cadmium quantification, samplings were performed after 36 days of exposure. For bioenergetics and genotoxicity assessment (RAPD-PCR), samplings were performed after 30 days of exposure.

The 60 days long experiment
In order to know whether a lower concentration of NPs could have different subcellular effects on the zebrafish, the authors conducted a second experiment in which they contaminated during 60 days two groups of 50 fish with 8 mg/fish/day of contaminated food, giving 33.6 and 42 ng CdSNPs/fish/day for CdS1NP and CdS2NP diets, respectively. This corresponds to 13.4 billions and 116.8 millions of NPs/day for each fish fed CdS1NP and CdS2NP diets, respectively. At day 60 of exposure, gene expression analysis, bioenergetics and genotoxic assessment were performed. A group of 50 fish that received the pristine food were used as control fish. Throughout this experiment, the oxygenated water was maintained at a temperature of 24 °C and filtered with a pump in a closed system.

Zebrafish were killed within seconds by immersion in melting ice and in agreement with the ethical guidelines displayed and used by the NIH intramural research program.

Genotoxicity assessment
The genotoxicity analysis was performed using a modified amplified polymorphism DNA methodology (RAPD) as previously described (Cambier et al. 2010; Orieux et al. 2011). With this method, it is possible to quantify the number of hybridisation sites of the probe and access tiny modifications borne by the genomic DNA.

Ten zebrafish were harvested per condition, killed by cold shock, and their genomic DNA extracted as previously described (Geffroy et al. 2012). Whole fish were used in order to be sure that DNA alterations appearing in some cells of a given tissue could represent hybridisation targets for RAPD probes. RAPD reactions were performed using the following decamer oligonucleotides: OPB7 (5′-GGTGAC GCAG-3′) and OPB11 (5′-GTAGACCGGT-3′), and real-time RAPD-PCRs were done with the LightCycler apparatus (Roche) as previously described (Geffroy et al. 2012).

Melting temperature curves analysis was done using the LightCycler Software 3.5 (Roche). For a given RAPD-PCR capillary, the melting temperature (Tm) of each PCR peak was obtained and listed in a logical table covering 11 different temperature intervals ranging from 74 to 89 °C. The frequency of occurrence of melting peaks for each interval was compared between controls and contaminated fish.

To quantify the number of hybridisation sites per genome unit of a RAPD probe, reference PCR were also made with each genomic DNA using 2 oligonucleotide probes flanking the 5′ and 3′ regions of the fifth exon of the β-actin 1 gene and therefore complementary to the fourth and fifth introns, respectively. These were 5′-CGTTTTGGTTTTATGGTGCAGG-3′ and 5′-GAAAATGGGCAGGTCACTACC-3′ for the upstream and downstream probes, respectively. The threshold cycle obtained with the β-actin probes, Ct(β-actin), is just indicating four hybridisation events per genome unit (two probes on each of two β-actin loci). Therefore, the number of hybridisation sites per genome unit of a RAPD probe, is \[ 2 \times (Ct(\text{β-actin}) - Ct(\text{OPB7}) + 2) \], where \( Ct(\text{OPB7}) \) is the threshold cycle value of a single RAPD-PCR performed with the OPB7 probe. The authors did the same to calculate the number of hybridisation sites for OPB11, swapping \( Ct(\text{OPB7}) \) for \( Ct(\text{OPB11}) \) in the formula.

Gene expression analysis
The authors analyzed the expression of eight genes, which could have been modified by the ingestion of NPs. These genes encode for proteins involved in metal chelation (mt2), mitochondrial metabolism (cox1), DNA repair (rad51), apoptosis (c-jun, p53 and bax) and antioxidant defenses (sod1 and sod2) (Table S1). For this analysis, the authors used the liver or digestive tract, skeletal muscles and brain of five fish of each treatment and sampled after 36 or 60 days of exposure. Each organ was crushed and the total RNAs were extracted from these tissues using the Absolutely RNA RT-PCR Miniprep kit (Stratagene), according to the manufacturer’s instructions. However, in order to eliminate lipids and proteins, the authors added a step of phenol:chloroform:isoamylic alcohol (25:24:1) extraction. At the end of this process, 30 µL, containing the totality of RNA per organ, were collected.

First-strand cDNA was synthesized from 14 µL of total RNA using the Affinity Script Multi Temperature cDNA Synthesis kit (Stratagene) as previously described (Geffroy et al. 2012). The cDNA mixture was conserved at -20 °C until it was used in a real-time PCR reaction.

Real-time PCR reactions were performed in a thermocycler (Stratagene) following the manufacturer’s instructions. Each 25 µL reaction contained 12.5 µL of BrilliantSYBR Green QPCR Master Mix (Stratagene) enabling the monitoring of the PCR amplification, 9.5 µL of ultra pure water, 1 µL of reverse transcribed product template and 1 µL of each primer. All primer pairs were designed in a previous study (Gonzalez et al. 2006) and reported in Table S1 as well as the function of the gene product. Relative quantification of each gene expression level was normalized according to the β-actin gene expression. The choice of the β-actin gene as a reference gene was relevant because its expression did not vary with the different CdSNPs exposure as highlighted by the fact that the mean Ct was the same whatever the exposure type. For instance, the threshold cycle values collected in brain after 60 days of exposure were 28.0 ± 0.5, 28.8 ± 1.0 and 28.7 ± 0.8 for control, CdS1NP and CdS2NP diets, respectively (n = 5, mean ± SEM).

Bioenergetics on skinned muscle fibres and brain homogenate
After 30 or 60 days of exposure, brain and muscles were removed from nine fish of each treatment. The mitochondrial
oxygen consumption was monitored polarographically at 28°C using a Clark oxygen electrode connected to an OXymer (Heito, Paris, France) in a 1 ml thermostatically controlled chamber.

Bundles of muscle fibres were taken off from the end of the tail – red muscle rich in mitochondria – and incubated for 20 min in 5 ml of solution A (0.1 M MES pH 7.0, 20 mM imidazole, 10 mM EGTA, 3 mM MgCl₂, 20 mM taurine, 0.5 mM dithiothreitol) in order to rinse the fibres. Then they were permeabilised for 50 s in solution A containing 50 μl/ml saponin, 5 mM ATP and 20 mM phosphocreatin as described (Cambier et al. 2009) except that the length of treatment with saponin and the washing step were each shortened to 1 min.

The brain of each animal was extracted, and treated as described (Bourdineaud et al. 2013).

Mitochondrial respiration recording were performed as previously described (Cambier et al. 2012).

**Cadmium analysis**

After 36 and 60 days of exposure to 40 ng CdSNPs/day/fish, seven fish per condition were taken off: four of them were crushed and lyophilised for 48 h. The cadmium concentration was measured three times for 10 mg of each fish powder. The other three were dissected and organs (brain, liver and muscles) were weighed (wet weight). Samples were digested with 0.5–1 ml of nitric acid in pressurized borosilicate glass tubes at 100°C for 3 h. After dilution of the digestates with ultrapure water (MilliQ plus), cadmium concentration were measured by electrothermic atomic absorption spectrophotometry (M6 Solaar AA, Therm Elemental) with a detection limit of 0.1 μg/l.

**Statistical analysis**

Significant differences in mitochondrial impact and number of hybridising sites when compared with control were determined with Mann–Whitney U-test or a T-test depending on the data normality using the statistical software “R” (R Development Core and Team, 2009). Differences in frequency of occurrence of PCR products in a defined temperature interval after fusion were revealed by a frequency test using the software Statistica 6.0.

**Results**

**Nanoparticles preparation and incorporation into food**

TEM micrographs of the CdSNPs of different sizes, prior their incorporation in the fish food, are shown in Figure 1. In both cases, the particles formed were uniform and were produced within a narrow size of distribution. The average size of the particles was 8 ± 1 nm (CdS1NP) for the smallest ones, and 50 ± 3 nm for the largest ones (CdS2NP) (n = 300 for each size group). HRTEM for all preparations shows the hexagonal CdS structure (Figure 1, middle micrograph). Both aforementioned NPs were stabilized with sodium polyphosphate at the surface. The presence of the polyphosphate anions created a negative zeta potential of -22.7 ± 3.2 mV. The authors then addressed the question whether cadmium could be dissolved from CdSNPs in solution. After 2 days of rest, the authors could not detect dissolved cadmium from a 16 mg CdSNPs/l colloidal solution containing 8 nm NPs, but after 10 weeks they could detect a concentration of soluble cadmium equal to 2.2 ± 0.7 mg Cd/l (n = 3), therefore indicating a fraction of dissolved cadmium representing 13.7% of the total initial mass of nanoparticulat cadmium. Thus, although ionic cadmium can be freed from CdSNPs, this process is rather being slow given that the authors promptly added freshly synthesized NPs to feed, and that once in feed NPs are no more in solution.

After incorporation of NPs within fish food, their TEM detection was difficult due to their low contrast in the organic environment (Figure 2, upper left micrograph). Only detection in dark field (Figure 2, upper right micrograph) and energy X-ray fluorescence (EDAX) analysis (Figure 2, bottom panel) allowed revealing the presence of the NPs. No clustering of the particles or morphological changes were detected in TEM data. The analysis of dark field micrographs and the ratio of sulphur/cadmium signals from the different preparations following food incorporation indicated no change in crystal structure.

The 36 days long experiment (highest dose)

**Cadmium accumulation**

Cadmium has been quantified in fish organs (brain, liver and skeletal muscle) after 36 days of dietary exposure (Table II). The levels of Cd in brain and muscles of exposed and control fish were below the detection threshold. Only in the liver could the authors measure the Cd burden for two fish exposed to CdS1NP and CdS2NP diets (85 and 35 ng Cd/g, respectively), and for one control fish (9 ng Cd/g), so that statistical computations were impossible to perform.

**Genotoxicity**

RAPD-PCRs were performed on genomics DNAs extracted from 10 individuals of CdSNPs-contaminated and control fish. The number and nature of PCR products obtained depend on the number of hybridisation sites of the primer on the entire genome but also the topographic position of each of these hybridisation sites. Herein, PCR products acquired with either OPB7 or OPB11 were classified.
Table III. Frequency of appearance of melting peaks of various products of RAPD-PCR with primers OPB7 and OPB11 for fish contaminated with CdS1NP, CdS2NP diets, and control fish after 30 days of exposure.

<table>
<thead>
<tr>
<th>Temperature intervals (°C)</th>
<th>OPB7 C</th>
<th>CdS1NP</th>
<th>CdS2NP</th>
<th>OPB7 C</th>
<th>CdS1NP</th>
<th>CdS2NP</th>
</tr>
</thead>
<tbody>
<tr>
<td>I: (74-78)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>I: (78-80)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.8</td>
<td>0.5</td>
</tr>
<tr>
<td>I: (80-81)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>I: (81-82)</td>
<td>1</td>
<td>0.9</td>
<td>0.8</td>
<td>0.7</td>
<td>0.3*</td>
<td>0.2*</td>
</tr>
<tr>
<td>I: (82-83)</td>
<td>0</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
<td>0.7*</td>
<td>0.8*</td>
</tr>
<tr>
<td>I: (83-84)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.1</td>
<td>0.2</td>
<td>0.7*</td>
</tr>
<tr>
<td>I: (84-85)</td>
<td>0.8</td>
<td>0.9</td>
<td>0.8</td>
<td>0.5</td>
<td>0.6</td>
<td>0*</td>
</tr>
<tr>
<td>I: (85-86)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>0.2</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>I: (86-87)</td>
<td>1</td>
<td>0.9</td>
<td>0.8</td>
<td>0.8</td>
<td>1</td>
<td>0.9</td>
</tr>
<tr>
<td>I: (87-88)</td>
<td>0</td>
<td>0.1</td>
<td>0.2</td>
<td>0.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>I: (88-89)</td>
<td>1</td>
<td>0.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Asterisks represent the temperature ranges for which the frequency of occurrence of peaks discriminates significantly contaminated genomic DNA from controls (n = 10, p < 0.05).

according to their $T_{m}$ and the frequency of occurrence of PCR products in each $T_{m}$ interval was noted (Table III). The comparison takes into account 10 fish for each experimental condition, which can overcome all of the genetic variability between individuals, so that differences in frequency of appearance of melting peaks for each temperature range were only due to different treatments.

The OPB7 probe did not display significant differences among melting temperature peaks between contaminated and control fish (Table III). With the OPB11 probe, the frequency of melting temperature peaks differed significantly from controls when fish were given CdS1NP (temperature intervals I4 and I5) and CdS2NP diets (temperature intervals I4, I5, I6 and I7) (Table III).

The use of real-time quantitative RAPD-PCR allowed the calculation of the number of available hybridising sites per genome for OPB7 and OPB11 primers (Table S2). The authors noticed a significant twofold decrease in the number of hybridisation sites to genomic DNAs with both primers (OPB7 and OPB11) for CdS2NPs diet when compared with control DNAs. The CdS1NPs only triggered a non-significant decrease tendency of the number of hybridisation sites.

Gene expression analysis

A panel of genes likely to see their expressions changed by NPs was used. The basal expression level of these genes was normalised according to the β-actin gene expression (Table S3). Then, the differential expression level was calculated by comparing control and contaminated genes’ expression (Table IV).

The size of CdS2NPs influenced the pattern of gene expression in tissues. After 36 days of dietary exposure to small size NPs (CdS1), a global trend of downregulation of the genes investigated was observed in the liver, brain and muscles, encompassing genes involved in mitochondrial metabolism, DNA repair, apoptosis and antioxidant defences. However, there was no difference in the expression level of genes between control and fish contaminated with the larger CdS2NPs (Table IV).

Bioenergetics

In muscles, the only observable impact was an increase of the oxygen consumption ($VO_2$) at the respiratory state 4 when fish were contaminated with the smaller CdS1NPs from 1.2 ± 0.2 up to 2 ± 0.2 natom O/mg.min (Table V). In brain, for both type of contamination, oxygen consumption

Table IV. Differential expression of selected genes in zebrafish exposed to dietary CdSNPs

<table>
<thead>
<tr>
<th>NPs type</th>
<th>mt2</th>
<th>cox1</th>
<th>sod1</th>
<th>sod2</th>
<th>c-jun</th>
<th>p53</th>
<th>bax</th>
<th>rad51</th>
</tr>
</thead>
<tbody>
<tr>
<td>36 days of exposure to 84 and 106 ng CdS1NPs/fish/day for CdS1NP and CdS2NP diets, respectively</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>CdS1</td>
<td>=</td>
<td>1/5</td>
<td>1/6</td>
<td>1/5</td>
<td>1/4</td>
<td>1/5</td>
<td>1/7</td>
</tr>
<tr>
<td></td>
<td>CdS2</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Muscles</td>
<td>CdS1</td>
<td>=</td>
<td>1/5</td>
<td>1/7</td>
<td>1/9</td>
<td>1/14</td>
<td>&lt;d.t.</td>
<td>&lt;d.t.</td>
</tr>
<tr>
<td></td>
<td>CdS2</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Liver</td>
<td>CdS1</td>
<td>=</td>
<td>1/41</td>
<td>1/26</td>
<td>1/59</td>
<td>=</td>
<td>1/92</td>
<td>1/35</td>
</tr>
<tr>
<td></td>
<td>CdS2</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>60 days of exposure to 33.6 and 42 ng CdS1NPs/fish/day for CdS1NP and CdS2NP diets, respectively</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>CdS1</td>
<td>29</td>
<td>=</td>
<td>2</td>
<td>=</td>
<td>2</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td></td>
<td>CdS2</td>
<td>3</td>
<td>=</td>
<td>3</td>
<td>3</td>
<td>10</td>
<td>=</td>
<td>2</td>
</tr>
<tr>
<td>Muscles</td>
<td>CdS1</td>
<td>20</td>
<td>=</td>
<td>5</td>
<td>10</td>
<td>6</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>CdS2</td>
<td>=</td>
<td>1/15</td>
<td>=</td>
<td>0.4</td>
<td>=</td>
<td>=</td>
<td>2</td>
</tr>
<tr>
<td>Liver</td>
<td>CdS1</td>
<td>123</td>
<td>1/4</td>
<td>=</td>
<td>1/7</td>
<td>1/6</td>
<td>=</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>CdS2</td>
<td>1/23</td>
<td>=</td>
<td>1/3</td>
<td>1/23</td>
<td>1/6</td>
<td>=</td>
<td>0.2</td>
</tr>
</tbody>
</table>

*Given are the significant expressions compared with control in three organs: brain, liver and skeletal muscles of zebrafish (n = 5). (=): level of expression not significantly different compared with control. <d.t.: below the detection threshold. This means that a relevant Ct value could be found for control, but that for contaminated fish, Ct recorded were equal to the Ct displayed by the negative control (without added DNA template); CdSNPs, cadmium sulphide nanoparticles.
(VO$_2$) at state 3 and 4 increased significantly compared with control zebrafish brain. The respiratory control (RC) in brain of CdS2NP-contaminated fish was significantly different from control. However, the activity of cytochrome c oxidase (COX) and uncoupled respiration measured in presence of carbonyl cyanide m-chlorophenylhydrazone (CCCP) presented no difference between control and contaminated fish brains (Table V).

### The 60 days long experiment (lowest dose)

#### Cadmium level

Cd has been quantified in fish organs (brain, liver and skeletal muscle) (Table II) after 60 days of dietary exposure. For both kinds of CdSNP, liver accumulated much more than brain and skeletal muscles. The liver Cd burden reached 43- and 45-times that in control fish liver (accumulating only 8±8 ng Cd/g), for CdS1NP and CdS2NP, respectively. In brain, the accumulated Cd was significantly different from that in control brain only in the case of smaller CdNPs, with a twofold increase (reaching 19±3 ng Cd/g). In muscles, no statistical differences could be observed between control and exposed animals.

#### Genotoxicity

After 60 days of dietary exposure to CdSNP, the RAPD-PCR genotoxicity test revealed genomic differences between contaminated and control fish. Indeed, the frequencies of appearance of melting peaks within control DNAs were different from those of fish exposed to CdSNP when using OPB7. This was true for the temperature intervals 14, 15, 19, 110 and 111 for CdS2NP. Using the primer OPB11, the frequency of appearance of melting peaks within control DNAs differed from those of fish exposed to cadmium NPs in temperature interval 11 for fish diet CdS1NP (Table S4).

Both CdSNP diets triggered an increase of the available number of hybridising sites per genome of the OPB11 primer with observed 15- and 2000-fold increases relative to controls for CdS1NP- and CdS2NP-treated animals, respectively. And only the CdS2NP diet increased the number of OPB7 probe’s hybridisation sites with a 10-fold magnification from 0.6±0.1 up to 6.5±0.9 hybridisation sites per genome unit (Table S5).

#### Gene expression analysis

The relative expression of selected genes in tissues from control and contaminated zebrafish after 60 days of dietary exposure to CdSNP is indicated in Table S6. In the digestive tract, genes tended to be repressed (4 over 8 and 5 over 8 in response to CdS1NP and CdS2NP diets, respectively). By contrast, in brain several genes were overexpressed compared with controls (3 over 8 and 5 over 8 in response to CdS1NP and CdS2NP diets, respectively). In muscles, the size effect of CdSNP was noticed since apart coxl 7 genes out of 8 were upregulated in fish contaminated with CdS1NP whereas only 1 of them (rad51) was upregulated and even 2 (coxl and sod2) were repressed in fish contaminated with CdS2NP. Size effect of CdSNP was also clear in digestive tract since, for example, the metallothionein gene (mt2) was induced 123-times in fish contaminated with CdS1NP but repressed 23-times in fish contaminated with CdS2NP (Table IV).

#### Bioenergetics

After 60 days in the presence of small sulphide NPs, the consumption rate of oxygen (VO$_2$) remained unchanged in muscles for both states 3 and 4 (Table V). In brain, VO$_2$ at state 4 decreased significantly compared with that of control from 1.5±0.1 to 0.9±0.1, while VO$_2$ at state 3 were comparable (Table V). No differences were observed in brain and muscles RC and COX activity between control and contaminated fish. A significant 20% stimulation of uncoupled respiration measured in presence of CCCP in brain was observed after exposure to CdS2NP (Table V). By contrast, uncoupled respiration measured in presence of CCCP presented no differences between control and contaminated skeletal muscles for both contamination diets (Table V).

#### Discussion

No mortality was observed throughout both experiments, neither within control fish nor in contaminated fish. This observation is consistent with a recent study involving dietary exposure of rainbow trout (Oncorhynchus mykiss) to TiO$_2$ NPs (21 nm) where the mortality was very low (2%) despite the fact that biochemical disturbance was found in the brain (Ramsden et al. 2009).
Cadmium accumulation

Although after 36 days of exposure, gene expression and mitochondrial perturbations were observed, as well as DNA alterations, cadmium was not detected neither in muscles nor in brain, and this was consistent with a previous study describing that cadmium could not be detected in whole body sections (Sanders et al. 2008). It is plausible that cadmium NPs may exert toxicity at tissue levels below the detection threshold. The same conclusion was found after the dietary administration of gold NPs to zebrafish at a size and doses commensurate with those used in the present study. Gold was not detected either after 36 days or 60 days in tissues (brain, liver and muscles) (Geffroy et al. 2012).

Part of QDs toxicity could be related to the release of free Cd\textsuperscript{2+} in cells (Cho et al. 2007; Gagné et al. 2008b; Male et al. 2008), in mice kidneys (Lin et al. 2009) and in the culture medium of exposed Daphnia magna (Kim et al. 2010). However, in these studies, the observed cytotoxicity could not be explained solely by the measured free Cd\textsuperscript{2+} level. Conversely, toxicity of CdTe QDs on human intestinal cells resulted from the nanosize and was not due to cadmium leaching (Koeneman et al. 2009).

In the present study, it cannot be ruled out that part of the effects observed are due to free Cd\textsuperscript{2+} level, but the fact that CdS1NP and CdS2NP diets triggered different patterns of gene expression response, and mitochondrial and genotoxic impacts, for the same concentrations of total cadmium accumulated within tissues, means that the NPs size does matter, and therefore argues in favour of the toxicity of NPs by themselves. In keeping with this, in liver of trout exposed to either QDs or CdSO\textsubscript{4}, 25 and 9 genes responded specifically to CdS/CdTe QDs and dissolved Cd, respectively, and only three more genes were affected by both forms of Cd (Gagné et al. 2010).

Genotoxicity analysis

Data obtained by RAPD-PCR emphasized the genotoxicity of CdSNPs in zebrafish, and showed modifications of the number of hybridisation sites of two short primers (OPB7 and OPB11) on the genomic DNA. CdTe QDs showed genotoxic effects in human breast adenocarcinoma cells exposed to 5 mg/l for 24 h (Choi et al. 2008), in human umbilical vein endothelial cells exposed to 1, 10 and 50 mg/l for 12 h (Wang et al. 2010), in mussels exposed to 1.6, 4 and 8 mg/l for 24 h (Gagné et al. 2008a) and in mice receiving an oral dose of CdSe QDs (2000 mg/kg body weight, representing 0.2-times the LD\textsubscript{50}) for 7 days (Khalil et al. 2011). It is likely that these effects are due to single mutations of genomic DNA caused directly (NPs binding to DNA) or indirectly (oxidative stress) by NPs (Donaldson et al. 2010; Singh et al. 2009). Indeed, the production of reactive oxygen species (ROS) following CdTe QD contamination has been demonstrated for human breast adenocarcinoma cells exposed to 10 mg/l for 15 h (Lovrič et al. 2005), human neuroblastoma cells exposed to 5 mg/l for 24 h (Choi et al. 2007) and in the gills of mussels exposed to 1.6, 4 and 8 mg/l for 24 h (Gagné et al. 2008a). CdS QDs triggered an oxidative stress in Chinese hamster lung cells exposed at 10–80 mg/l for 24 h (Li et al. 2009), and CdSe/ZnS QDs evoked ROS in human monocytes after 30 min of treatment to 5 nM (Lee et al. 2009).

Gene expression analysis

The main result stemming from the analysis of gene expression is the role of the CdSNPs size. Indeed, small CdSNPs featured a greater influence on gene response than bigger NPs although the same amount of total cadmium accumulated in tissues whatever the CdSNPs size. For instance, after 36 days of exposure to 100 ng CdSNPs/day/g body weight, only CdS1NP triggered a differential gene response and six out of eight genes were repressed in brain and in liver (seven out of eight in muscles).

Another important point is related to muscle: despite low accumulated total cadmium levels (10- and 190-times less than in brain and liver, respectively), seven out of eight genes were upregulated in muscles after 60 days of exposure to CdS1NP. The authors also observed an impact on mitochondrial respiratory chain in this organ at that contamination pressure.

The expression of the metallothionein gene mt2 did not change after 30 days of exposure, whatever the size of NPs, but was greatly overexpressed as compared with control after 60 days in brain, muscles and digestive tract in response to CdS1NP. CdS2NP diet only afforded a threefold overexpression in brain (versus 29-fold in response to CdS1NP) and even a 23-fold repression in the digestive tract. It is not possible to use the expression of metallothionein gene to distinguish the effects of Cd-based NPs and free ionic cadmium since it has been found that both forms of Cd were strong inducers of mt gene expression (Gagné et al. 2010).

Bioenergetics

After 30 days of dietary exposure to 100 ng NPs/day, the respiratory state 3 and state 4 increased in brain of contaminated zebrafish compared with control. This observation holds true for both kinds of CdSNPs and explains the decrease of the RC in brain. A diminished RC is often linked to a leakage of protons into the matrix which can be due to lipid peroxidation of the mitochondrial membrane. For instance, treatment of human neuroblastoma cells with QDs (5 mg/l for 24 h) triggered lipid peroxidation of the mitochondrial membranes, and decreased the mitochondrial membrane potential (Choi et al. 2007). Human breast cancer cells treated with QDs (10 mg/l for 24 h) exhibited significant mitochondrial swelling and rounding up. In the QD-treated cells, the mitochondria were seen mostly in the perinuclear area, whereas the mitochondria of cells in the control experiment had a filamentous structure and were more uniformly distributed throughout the cytoplasm (Cho et al. 2007).

Size effect

Overall, higher genotoxicity of larger QDs was observed as well as greater influence of smaller CdSNPs on gene expression (e.g., gene expression after 36 days is apparent only in zebrafish exposed to small CdSNPs). Authors (Albanese et al. 2012) wrote that “evidence suggests that compartmentalization of
CdTe QDs into different subcellular organelles depends on size and cell type: sub-2.1-nm QDs entered the nucleus, whereas 4.4-nm QDs were found in the cytoplasm (Williams et al. 2009)”. Rats treated through diet with 30 nm iron oxide NPs accumulated this nanomaterial within several organs whereas 2 μm iron oxide microparticles could not cross the intestine barrier and were found excreted in faeces (Singh et al. 2013). Also, when compared with other greater sizes, 30-nm amorphous TiO2 and 15-nm silver NPs induced the highest generation of ROS (Albanese et al. 2012; Carlson et al. 2008; Jiang et al. 2008). But it would be too simple to think that the smaller NPs enter cells or organelles more easily and display the greatest effects. Indeed, in other studies the effect of NP size did not appear to be significant. For instance, the uptake of silver NPs and QDs into macrophages induced the expression of inflammatory mediators such as TNF-α, MIP-2 and IL-1β independent of size (Albanese et al. 2012; Fischer et al. 2010; Carlson et al. 2008). And the cellular uptake of gold NPs by mammalian cells displayed a biphasic pattern: it increased from 10 to 50 nm and then decreased from 50 to 100 nm in size (Chithrani et al. 2006).

### Conclusion

The present study represents up to now the first trial that have evaluated the effect of dietary QDs on a vertebrate organism, and in addition at such low contamination pressures as 40 and 100 ng/day/g body weight. The food used in the present study mimics the dispersion of NPs in aquatic environments and its transfer along the food web at environmentally realistic concentrations. The authors showed that different effects, such as gene expression level modifications, mutations and mitochondrial impairment, occurred for both sizes of CdSNPs. These results highlight a major ecotoxicological concern ultimately questioning the possible impact of NPs on human health. Indeed, NPs ingested through drinking water or food will possibly cross the human intestinal barrier, since it has been shown that QDs could breach the integrity of a layer of human intestinal cells (Koeneman et al. 2009).

### Acknowledgments

The authors would like to thank Bruno Etcheverria for technical assistance.

### Declaration of interest

All the authors of this article declare that no potential conflicts of interest are linked to the work described in this manuscript. They also declare not presenting financial, consulting and personal relationships with other people or organisations that could influence the present work. This work was supported by a grant from the French National Research Agency (number ANR-07-SEST-02301). French academic institutions employ all the authors. No funding source, lawyer or private company has the right to review, comment or approve this manuscript before publication.

### References


Supplementary materials available on online

Supplementary Tables S1–S6.