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Genotoxic damages in zebrafish submitted to a polymetallic gradient displayed by the Lot River (France)

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ABSTRACT

Genotoxic effects of a polymetallic pollution gradient displayed by the Lot River and one of its tributary have been assessed on zebrafish *Danio rerio*. Three methods were compared: RAPD-PCR, the comet assay, and 8-oxo-7, 8-dihydro-2′-deoxyguanosine (8-oxodG) formation. The fishes were exposed for 14 days to waters collected from three stations: Joanis, a site polluted by cadmium (Cd) and zinc (Zn) (mean concentrations: 15 μg Cd/L and 550 μg Zn/L), Bouillac (mean concentrations: 0.55 μg Cd/L and 80 μg Zn/L), and Boisse-Penchot, a reference station (mean concentrations: <0.05 μg Cd/L and 7 μg Zn/L). The quantitative RAPD-PCR methodology proved to be sensitive enough to unmask metal genotoxicity after 3 and 7 days of exposure to Joanis water and after 14 days to Bouillac water, whereas the comet assay only detected DNA damages at the most contaminated station (Joanis). The 8-oxodG quantification was not sensitive enough to be used in zebrafish under these environmental conditions.

1. Introduction

Cadmium (Cd) is a highly toxic and carcinogenic heavy metal. This metal inhibits the DNA repair system and several enzymes involved in oxidative stress responses (Giaginis et al., 2006; Joseph, 2009). Cd is dispersed in the environment due to its widespread industrial use. In the South-West of France, the Lot River and its small tributary (Riou-Mort) have a polymetallic pollution gradient with Cd as the main toxic metal (Audry et al., 2004). These streams are polluted with cadmium and zinc originating from a century old industrial Zn factory, the Vieille-Montagne. Downstream on the Riou-Mort, just before its confluence with the river Lot, the cadmium concentration in the water varied between 2 and 28 μg/L during a 3-month survey (mean: 15 μg Cd/L). On the Lot River, about 300 m downstream from the confluence with the Riou-Mort (Bouillac sampling station), Cd concentrations varied from 0.05 to 2.5 μg/L (mean: 1 μg Cd/L) (Achard et al., 2004). In laboratory conditions, we exposed zebrafish *Danio rerio* with environmentally relevant doses of Cd and found an induction of genes involved in the DNA repair process (Gonzalez et al., 2006). In order to assess the effectiveness of Cd-induced genotoxic damages on zebrafish we decided to use a modified RAPD-PCR methodology.

RAPD is currently used for intra-populational polymorphism detection, but the technique also permits the detection of genetic alterations after animal contamination with pollutants, even on non clonal species such as macroalgae *Palmaria palmata* (Atienzar et al., 2000), crustacean barnacles *Elminius modestus* (Atienzar et al., 2002), and bivalve mollusks *Mytilus edulis* (Hagger et al., 2005). Most recently, the RAPD methodology was applied to zebrafish to detect the genotoxic potential of chemicals such as cyclophosphamide and dimethoate (Zhiyi and Haowen, 2004). However, this method only relies on agarose gel electrophoresis analysis of the missing or appearing PCR products between DNA from contaminated and control individuals. We have recently refined this methodology by eliminating the electrophoretic analysis and taking advantage of the quantitative facilities of real-time PCR to address the efficiency of hybridization of the RAPD probe to genomic DNA, and to allow comparison of the melting temperature patterns between each amplification

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reaction (Cambier et al., 2010). Zebrafish were exposed for 21 days to two concentrations of cadmium chloride dissolved in the medium (1.9 and 9.6 μg/L, representative concentration present in the Bouillac and Riou-Mort waters, respectively). RAPD-PCR showed an increase in the relative hybridization efficiency of a Cd-discriminative probe on the genomic DNAs coming from fish exposed to both Cd concentrations as compared to the control condition. In addition, the RAPD-PCR melting temperature patterns showed that Cd contamination resulted in the variation of the distribution of PCR products according to their fusion temperature intervals.

A main objective of the present study is to determine whether this RAPD-PCR methodology can also apply to truly environmental situations, despite the numerous parameters that escape the researcher's control. We therefore decided to expose zebrafish for 14 days to different waters from the Lot River and its tributary, the Riou-Mort. Another objective of this study was to compare the sensitivity and efficiency of the RAPD-PCR methodology to those of two classic assays: the comet test (alkaline single cell gel electrophoresis) and the assessment of the oxidative DNA damage as measured by 8-oxo-7, 8-dihydro-2′-deoxyguanosine (8-oxodG) using high pressure liquid chromatography (HPLC) analysis (Halliwell, 1999; Russo et al., 2004). Cd and Zn concentrations were quantified in fish tissues, and a selection of genes involved in DNA repair and response against stress were scrutinized for modification of their expression, in order to correlate with possible observed genotoxic damages.

2. Experimental section

2.1. Description of the study and sampling zone

The study and sampling zone was located in the South-West of France, in the Aveyron department of the Midi-Pyrénées region, on the banks of the Lot River and one of its metal-polluted tributary, the Riou-Mort River. This tributary is polluted by a zinc ore extraction plant located in Viviez, and which is now owned by the Umicore company. Today there is no more zinc extraction, but accumulated existing extracted materials still pollute the Riou-Mort River, mainly with cadmium and zinc. Three sampling stations were selected for our study (a figure of the site can be seen in Achard et al., 2004): (1) the Joanis station was located on the banks of the Riou-Mort station, 2 km upstream from the confluence with the Lot River and 2 km downstream from the Umicore factory. At this sampling site, the dissolved metal forms represented 92% and 89% of the total Cd and Zn, respectively (Andres et al., 2000); (2) the Bouillac station was located on the Lot River banks 200 m downstream from the confluence with the Riou-Mort River. At this sampling site, the dissolved metal forms represented 85% and 75% of the total Cd and Zn, respectively (Andres et al., 2000); (3) the Boisse-Penchot station was the reference site, located 3.5 km upstream from the confluence with the Riou-Mort River. At this sampling site, the Cd concentration was below the detection threshold of 0.05 μg/L.

2.2. Zebrafish care and contamination

The experiment lasted from May 3 to 18, 2010. On departure from Arcachon, fish were put in oxygen-saturated water, obtained by a direct bubbling of oxygen, and kept under a pure oxygen atmosphere for 4 h during the travel to Viviez. On arrival, fish were directly placed in a tank filled with chlorinated-free oxygenated water warmed to 23 °C. Fish were kept in this tank for 24 h and then transferred to experimental tanks. Since zebrafish cannot withstand low temperatures, we kept fish in tanks filled with water sampled from the 3 described sites at a constant temperature. The experiments were performed in a laboratory lent by the ABC factory in Viviez. Adult zebrafish were randomly distributed in three 60-L tanks equipped with a heater to maintain the water temperature at 25 ± 3 °C, and a bubbling device to oxygenate and mix the medium. Fish were fed daily with a quantity of dry food representing 5% of their body weight (Novo Granomix, JBL). The food contained 38% proteins, 6% fat, 4% fibers, 9% ashes, 0.9% phosphorus, 25 IU/g of vitamin A, 0.4 mg/g vitamin C, 3 mg/g vitamin D3, and 0.33 mg/g vitamin E. Cd and Zn concentrations in this diet were measured and were equal to 2.7 ± 0.5 nmol Cd/g and 860 ± 33 nmol Zn/g, respectively. Tank water was renewed by a third of the total volume each day in order to get rid of the materials in suspension, including pieces of fish excrements, and to maintain the metal concentrations. Water renewal was performed by sampling 20 L each day from each of the 3 sites, Boisse-Penchot, Bouillac, and Joanis. To avoid cold shock to fish, these cold waters were warmed to 25 °C before being poured into the tanks. Thirty-five fishes were harvested on days 3, 7, and 14 from the tank with the Boisse-Penchot water; on days 7 and 14 from the tank containing the Bouillac water; and on days 3 and 7 in the tank receiving the Joanis water. We supposed that 7 days of exposure to the Joanis water would be enough to see effects of metal contaminants in fish; whereas, 14 days would be necessary for fish cultured in Bouillac water. Zebrafish were killed within seconds by immersion in melting ice ($T = 0$ °C). This is in agreement with the ethical guidelines displayed and used by the NIH intramural research program (http://oacu.od.nih.gov/ARAC/documents/Zebrafish.pdf). Tissues were kept at −80 °C until used. Fish blood samples were collected from the caudal vein, diluted 100 times in a cryoprotective solution (250 mM sucrose, 40 mM citrate trisodium salt, 5% DMSO, pH adjusted to 7.6 with 1 M citric acid solution) and stored at −80 °C. Before starting the experiment, 3 fish were collected as controls for metal quantification and comet assay.

2.3. Cd and Zn analysis

Sampled tissues (skeletal muscles, digestive tract, gills, brain) were weighed and digested in 3 mL of concentrated nitric acid added to a pressurized medium (borosilicate glass tube) at 95 °C for 3 h. The resulting solutions were diluted to 15 mL with ultrapure water (MilliQ Plust, Millipore) and analyzed by atomic absorption spectrophotometry. The Cd determinations for water and digested tissue samples were performed with an atomic absorption spectrophotometer (M6 Solaar AA, Thermo Elemental) equipped with a graphite tube atomizer (GF95 Graphite Furnace). To avoid interference, the appropriate diluted solutions were analyzed in a tube atomizer with a mixture of Pd and $Mg(NO_3)_2$. The detection limit was 0.05 μg Cd/L. Zinc concentrations for water and digested tissue samples were determined by flame atomic absorption spectrophotometry (Varian AA220FS). The detection limit was 10 μg Zn/L. The analytical methods were simultaneously validated for each sample series by the analysis of standard biological reference materials (TORT-2, lobster hepatopancreas; DOLT-2, dogfish liver; National Research Council of Canada, Ottawa). The results are expressed as average metal concentrations accumulated in tissues (in nmol Cd/g wet weight ± SD, and μmol Zn/g wet weight ± SD) for three samples from each tissue and sites.

2.4. Quantification of genotoxic damages by RAPD-PCR and analysis of the melting temperature curves of random amplified PCR products

After 3, 7, and 14 days of exposure, 10 fish were collected from the tanks, killed and stored at −20 °C until used. Genomic DNA isolation was performed by mincing frozen fish with a scalpel. Then crushed tissues were digested overnight at 50 °C with 10 mL per gram of tissue of DNA extraction buffer: 10 mM Tris pH 8, 100 mM EDTA pH 8, 0.5% SDS, and 200 μg/mL proteinase K (Promega). Then, the DNA solution was treated with 100 μg/mL RNAse A (Quiagen) during 2 h at 37 °C. Genomic DNA was purified by a conventional phenol/chloroform method (Westerfield, 2007).

Primers used for RAPD-PCRs were the decamer oligonucleotides OPB7 (5′-GGTGACGCAG-3′) and OPB11 (5′-GTAGACCCGT-3′), which were obtained from Sigma-Proligo. Real time RAPD-PCRs were done with the Lightcycler apparatus (Roche). Each reaction was completed to 20 μL (reaction volume) in a capillary tube in which 16 ng of genomic DNA were loaded, 2 μL of primer (6 μM), and 16 μL of PCR mix. The thermal program used for this real time RAPD-PCR was one warming step of 10 min at 95 °C followed by 50 amplification cycles at 95 °C for 5 s, 50 °C for 5 s, and 30 s at 72 °C. After this thermal program, the dissociation curves were obtained by following the decrease of SyberGreen fluorescence level during a gradual heating of the PCR products from 60 to 95 °C.

Melting temperature curves analysis was done using the LightCycler Software 3.5 (Roche). For a given RAPD-PCR capillary tube, the melting temperature (T_m) of each PCR product peak was obtained in order to establish a distribution of the frequencies of appearance of peaks having equal T_m among a set of 10 different temperature intervals ranging from 78 to 89 °C. The comparison of distributions between two different exposure conditions designates temperature intervals for which the frequency of PCR products for a known T_m differed.

For each genomic DNA, quantitative analysis relied on the calculated difference in the cycle numbers necessary to enter in the exponential phase of the PCR, C_T, using probes OPB7 and OPB11, respectively. Indeed, OPB7 proved to be a reference probe, whereas OPB11 was a Cd contamination-sensitive probe (Cambier et al., 2010). This difference, $\Delta = C_T(\text{OPB7}) - C_T(\text{OPB11})$, reflects the relative hybridization efficiency between the two probes, which can be calculated from $2^{-\Delta}$. The value of this relative hybridization efficiency was calculated from the mean of ten replicates (the mean $2^{|CT(OPB7)-CT(OPB11)|}$) for each experimental condition.

2.5. Comet assay

Comet assay was performed within two months on blood cells, according to the protocol of Devaux et al. (1997), a slightly modified version of the procedure