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DNA Alterations Triggered by Environmentally Relevant Polymetallic Concentrations in Marine Clams *Ruditapes philippinarum* and Polychaete Worms *Hediste diversicolor*

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Abstract We exposed marine clams (*Ruditapes philippinarum*) and aquatic worms (*Hediste diversicolor*) to environmentally relevant concentrations of two metal mixtures each containing three divalent metals [(C_1 in μg/L) cadmium (Cd) 1, mercury (Hg) 0.1, and lead (Pb) 4] and [C_2 in μg/L) Cd 17, Hg 1.1, and Pb 55]. Animals collected in the Arcachon Bay were exposed for 8 days in microcosms made up of a mixed biotope consisting of a water column and natural marine sediment both taken up from the Arcachon Bay. Bioaccumulation analysis showed a significant increase of Cd, Hg, and Pb in clams, particularly at C_2 concentration in the water column reaching, in soft body, 2.3 ± 0.3 μg Cd/g, 0.7 ± 0.2 μg Hg/g, and 45 μg Pb/g dry weight (dw). DNA alterations and upregulation of the *cox1* mitochondrial gene were also observed in clam gill after exposure to the metal blend. For worms exposed to the C_2 metal blend, DNA alterations and significant increase of Cd and Hg concentrations were observed reaching 0.5 ± 0.1 μg Cd/g and 2 ± 0.6 μg Hg/g dw.

Among the many contaminants released into Skikda Bay in Algeria, due to the industrial nature of the oil refineries adjacent to the city, are metals. These create significant marine pollution (Kehal et al. 2004; Nafissa et al. 2005) that may affect the aquatic environment through their potential toxicity to various ecosystem inhabitants, including humans, animals, and plants.

Metals may accumulate in aquatic species, such as bivalves, at concentrations several times greater than concentrations in water and sediment due to the ability of these animals to filter large quantities of particles, including contaminants, from seawater, sediment, or food. Consequently, clams *Ruditapes philippinarum*, commonly known as Manila clams, are used as sentinel organisms in monitoring programs assessing anthropogenic pressure such as metal pollution (Ramos-Gómez et al. 2011; Roméo and Gnassia-Barelli 1997; Smaoui-Damak et al. 2009; Wang et al. 2012). Polychaetes comprise an important proportion of the total biomass of deposit-feeding aquatic benthic invertebrates and are key species of the benthic community in coastal and estuarine sediments. The sediment-dwelling ragworm, *Hediste diversicolor*, is known to play a crucial role in the fate of chemicals in estuarine areas as a consequence of its relative tolerance and its influence on metal speciation through bioturbation, particle mixing, and irrigation (Banta and Andersen 2003; Berthet et al. 2003; Mouneyrac et al. 2003). *H. diversicolor* is thus an appropriate test organism for examining the fate and effects of metal in sediment systems. Its range extends from the Baltic Sea and North Sea southward to the Azores and Mediterranean Sea.

Metal genotoxicity has been investigated in bivalve species using various methodologies such as the comet assay, micronucleus assay, 8-oxoguanosine quantification, and random amplified polymorphic DNA (RAPD)-based methodology in clam species *R. decussatus* (Jebali et al. 2007) and *R. philippinarum* (Sacchi et al. 2013) and in mussel species *Mytilus galloprovincialis* (Bolognesi et al.

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1999) and *M. edulis* (Emmanouil et al. 2007; Pruski and Dixon 2002). A drawback of these in situ studies is that they cannot link genotoxic outcomes with precisely identified pollutants especially when a natural environment is polluted with several heavy metals and hydrocarbons (Jebali et al. 2006; Sacchi et al. 2013). Micronucleus assay detected the onset of genotoxicity in *M. galloprovincialis* gill cells at 40 μg/L of divalent copper chloride and 32 μg/L of mercury chloride, but it showed no effect at a Cd chloride concentration as high as 184 μg/L (Bolognesi et al. 1999). Comet assay showed DNA damage in digestive gland of *M. edulis* after 10 days of exposure to 200 μg/L Cd and 10 μg/L hexavalent chromium (Cr) corresponding to accumulated concentrations of 39 ± 11 μg Cd/g wet tissue and 2.7 ± 2.9 μg Cr/g wet tissue (Emmanouil et al. 2007). In contrast, comet assay showed no effect of Cd in the same organism exposed to 200 μg/L for 4 weeks (Pruski and Dixon 2002). Thus, some discrepancies are linked to the genotoxic effects of Cd in mussel species.

In the present study, we assessed the genotoxic potential of polymetallic blends each containing three divalent metals (cadmium [Cd], lead [Pb], and mercury [Hg]). The aim was to assess the impact of environmentally relevant contamination, such as those encountered in the Skikda Bay, on two marine organisms, marine clams (*R. philippinarum*) and aquatic worms (*H. diversicolor*), to two metal mixtures. Animals collected in the Arcachon Bay were exposed for 8 days in microcosms made up of a mixed biotope consisting of a water column and natural marine sediment both taken up from the Arcachon Bay. Metal concentrations corresponded to the minimal and maximal observed in situ in Skikda Bay (Kehal et al. 2004). Metal accumulation was investigated in both animals, worms and clams (soft body and gill), whereas DNA alterations and gene expression modification were performed in clam gill only. Gill was chosen owing to its direct contact with the water: It constitutes the main target organ for metal uptake and accumulation and therefore is more susceptible to present gene expression modifications and DNA alterations.

Materials and Methods

The experiment was performed in the laboratory in fall 2010 using glass aquaria of 12 × 12 × 24 cm containing a bottom layer of 7 cm of sediment and filled with 2 L of seawater (Arcachon bay, France). Water temperature was 16 ± 0.9 °C, salinity 33.5 ± 0.6 ‰, and pH 7.65 ± 0.8. The experimental units (EUs) were permanently aerated by air bubbling in the superficial layers of the water column to produce an oxygen-saturated environment and exposed to a 12 to 12-h light-to-dark regime. The inside aquaria walls were lined with plastic to avoid metal adsorption and contamination. The sediment used was collected in eastern Arcachon bay in Graveyron (N 44° 42′ 21″, W 0° 57′ 12″, France) and was homogenized and sieved through a 2.5-mm mesh. Background metals concentrations were 0.52 ± 0.007, 0.095 ± 0.07, and 1.85 ± 0.20 μg/g (dw) (mean ± SD) for Cd, Hg, and Pb, respectively.

We used two species: a bivalve (*R. philippinarum*) collected in fall 2010 at the entry to Arcachon bay, the Banc d'Arguin (N 44° 39′ 50″, W 1° 9′ 51″, France) [30.6 ± 2.7 mm shell length (mean ± SD)] and a polychaete worm (*H. diversicolor*) collected in fall 2010 from the natural sediment at Graveyron [57.0 ± 3.4 mm length (mean ± SD)]. It was essential to use the same sediment (that of Arcachon bay) as not to disrupt the natural style of life of the animals.

The animals were introduced into the EUs at least 1 week after sediment and water settling to allow physicochemical stabilization and acclimated for 24 h before metal addition. The experimental conditions were as follows: EUs with clams only, EUs with worms only, and EUs without animals. To mimic the contamination levels in Skikda Bay, the water column was contaminated for 8 days with two metal mixtures containing Cd, Hg, and Pb chlorides. The contamination levels were as follows: a control condition C_0, a first nominal contamination level [(C_1 in μg/L) Cd 1, Hg 0.1, and Pb 4] and a second nominal contamination level [(C_2 in μg/L) Cd 17, Hg 1.1, and Pb 55]. C_1 and C_2 mimic the lowest and the highest metal concentrations observed in Skikda Bay, respectively (Kehal et al. 2004). Water analyses were impossible to perform for Cd and Pb because it was necessary to dilute the seawater samples to avoid the Zeeman quenching effect due to sodium chloride. After a 15-fold dilution of water samples, and because Cd and Pb concentrations were low, it appeared that metal concentrations were lower than the threshold of the Solaar spectrophotometer (0.1 μg Cd/L and 20 μg Pb/L). Indeed, to assay Cd and Pb in water samples, it was necessary to obtain salinity below or equal to 2.2 ‰ in water sample (original salinity was 33 ‰). Therefore, water contamination was based on two additions of solutions containing $CdCl_2$ and $PbCl_2$ at times 0 and 48 h. At time 0, 200 μL (C_1) or 3.4 mL (C_2) of a 10 mg/L $CdCl_2$ solution and 0.8 μL (C_1) or 11 μL (C_2) of a 10 g/L $PbCl_2$ solution were added to aquaria to give the desired C_1 and C_2 contamination levels. At time 48 h, double the time 0 volumes were added, thereby doubling the contamination levels.

Hg contamination was based on daily additions of a 150 mg/L $HgCl_2$ solution. Each day Hg concentration was analyzed, and the amount of $HgCl_2$ (ultrapure $HgCl_2$, Merck, Darmstadt, Germany) added was adjusted to compensate for the decrease in metal concentration during the

24-h cycles. Water additions were performed carefully every day to compensate for losses due to evaporation of water. No daily change of water compartments was performed so as not to resuspend sediment particles and cause water column contamination.

After 8 days of exposure, worms and clams were removed from microcosms and dissected. Soft body and gills were collected from each clam and small body sections from each worm for metal quantification, genotoxicity, and gene expression assessments. All samples were conserved at −80 °C until analyses.

Tissues were digested in 1–3 mL of nitric acid (depending on dry-tissue weight) at 100 °C for 3 h. The liquid underwent 6-fold dilution with ultrapure water (MiliQ, Bedford, MA, USA). Cd and Pb concentrations were determined by electrothermic atomic absorption spectrophotometry (AAS) with Zeeman correction using a graphite furnace (M6 Solaar AA spectrometer; Thermoptec, Mulgrave, Australia). Quantification of Hg for water and dried tissues was performed by flameless AAS (AMA 254; Altec, Prague, Czech Republic) with an estimated detection limit of 0.01 µg Hg/L. The analytical methods were simultaneously validated for each sample series by analyzing standard biological reference materials (Tort-2 Lobster Hepatopancreas and Dolt-4 Dogfish Liver from the National Research Council of Canada, Ottawa, Canada). Values were consistently within the certified ranges [(Tort-2 in µg/g) Cd 26.7 ± 0.6, Pb 0.35 ± 0.13, and Hg 0.27 ± 0.06; (Dolt-4 in µg/g) Cd 24.3 ± 0.8, Pb 0.16 ± 0.04, and Hg 2.58 ± 0.22].

Genotoxic effects of metal mixtures were assessed using a RAPD-based methodology (Cambier et al. 2010; Geffroy et al. 2012; Orieux et al. 2011). Genomic DNA isolation was performed by mincing frozen clam and worm tissues with a scalpel. For each condition, 1n clams and 10 worms were sampled. Crushed tissues were digested overnight at 37 °C with DNA extraction buffer at 7 mL per gram of tissue made up with 10 mM Tris pH 8, 100 mM ethylene diamine tetraacetic acid (EDTA) (pH 8), 0.5 % sodium dodecyl sulfate, and 100 µg/mL proteinase K (Promega, Madison, WI, USA). After digestion, 2.5 mL saturated NaCl (6 M) was added and followed by centrifugation. RNase (10 mg/mL) was added to the supernatant at 20 µg/mL, and after 15 min of incubation at 37 °C, the DNA was precipitated with 2 volumes of 100 % absolute ethanol and removed by spooling. The DNA was rinsed with 70 % ethanol, dried, and resuspended in TE buffer (10 mM Tris pH 8, 0.1 mM EDTA).

Selected primers for RAPD–polymerase chain reaction (PCR) were obtained from Sigma-Proligo (St.Louis, MO, USA) and were the decamer oligonucleotides OPB7 (5′-GGTGACGCAG-3′) and OPB11 (5′-GTAGACCCGT-3′) for clam DNA and OPB7 and OPA9 (5′-GGGTAACGCC-3′) for worm DNA. Real-time RAPD-PCRs were performed with the Lightcycler apparatus (Roche, Basel, Switzerland) as described (Lerebours et al. 2013). For each clam, using OPB7, OPB11, and ribosomal 28S probes (forward 5′GCTGCTCCATAAGTCG-3′ and reverse 5′-TGAACTATGTCTGAGTAGGG-3′), the difference—Δ = Ct(28S)—Ct(OPB7 or OPB11—reflects the difference of hybridization efficiencies between the RAPD probe and 28S probe. It can be calculated from $2^{\Delta+1}$, which corresponds to the ratio of the number of hybridization sites of the OPB7 or OPB11 probe relative to the number of copies of the 28S gene. The same calculation was performed for worm genomic DNA using OPB7, OPA9, and ribosomal 16S probes (forward 5′-GTCCGCATTGGCC-TACC-3′ and reverse 5′-GTTCGGTTGGGGCGAC-3′).

Melting-temperature curve analysis was performed using LightCycler Software 3.5 (Roche). For a given RAPD-PCR capillary tube, the melting temperature (*T*m) of each PCR product peak was obtained to establish a frequency distribution of peak appearance at equal *T*m for a set of ten different temperature intervals ranging from 78 to 89 °C. The comparison of distributions between two different exposure conditions indicates temperature intervals for which the frequency of PCR products for a known *T*m differed.

After 8 days, clams were dissected and sampled tissues (gill) kept frozen in RNA-later (Qiagen, Limburg, Netherlands) at −80 °C until used. The expression of five genes was analyzed using five clams for each condition. Samples were crushed, and total RNA was extracted using the Absolutely RNA RT-PCR Miniprep kit (Stratagene, Santa Clara, CA, USA), according to the manufacturer's instructions. However, to eliminate the maximum of lipids and proteins, we added a step of phenol chloroform–isoamylic alcohol (25:24:1 ratio) extraction. At the end of this process, 30 µL containing total RNA were collected. First-strand cDNA was synthesized from 14 µL of total RNA using the Affinity ScriptMulti Temperature cDNA Synthesis kit (Stratagene) according to the manufacturer's instructions. Real-time PCR reactions were performed in a thermocycler (Stratagene) according to the manufacturer's recommendations. All primer pairs were designed with the Lightcycler probe designer software (forward and reverse primer, respectively): *cox1* = GTACCCTCCGTTGTCGT CA and CCTGTTACTCCTAAACACCAAGC; *cytb* = TT GATAGAGACGGGGATGT and ATACCACTCAGGCT GGA; *cat* = CTGAGGCTACAGACAGATG and GTTGC CCTGGGCGATG; *sod* = GATAATGTTGATCATGCTG GACC and GTCTACATCAGCGTGAACGCAA; and 16S RNA = AGAAGACCCTGTCGAG and TTACGGCTGT TATCCCT. Relative quantification of each gene expression level was normalized according to β-actin gene expression. The choice of the β-actin gene (forward and reverse primers were CGCACTTCCTCACGCCATCAT and GCAGCCGTCTCCATTTCTTGT, respectively) as a

reference was relevant because its expression did not vary on exposure to different metal blend concentrations since the mean Ct remained constant under different conditions (C_0 25.92 ± 1.0, C_1 24.81 ± 3.2, and C 24.53 ± 2.8).

Results

Cd, Pb, and Hg accumulations were quantified in soft body and gill for clam and in parts of whole body for worm. Results in soft body of clam showed a significant accumulation of the three metals, particularly at the highest concentration (C_2) with 5.5-, 3.5-, and 20-fold increases compared with controls, respectively (2.3 ± 0.3 µg Cd/g, 0.7 ± 0.2 µg Hg/g, and 45 µg Pb/g dw) (Fig. 1a through 1c). In gill, there were insignificant increases in Cd but significant accumulation of Hg with 7.5- and 25-fold increases compared with controls at lower and higher exposures, respectively (0.6 ± 0.2 and 2 ± 0.4 µg Hg/g dw) (Fig. 1b), and in Pb with 2.7- and 14-fold increases compared with controls at lower and higher exposures, respectively (1.63 ± 0.07 and 8.6 ± 0.4 µg Pb/g dw) (Fig. 1c). In worm tissue, no differential accumulation was observed at the lower metal exposure (Fig. 1a through 1c). At greater exposure, Pb accumulation showed an insignificant increasing trend, and there were significant Cd and Hg accumulations with 5.5- and 10-fold increases compared with controls, respectively (0.5 ± 0.1 µg Cd/g and 2 ± 0.6 µg Hg/g dw).

RAPD-PCR on genomic DNA extracted from gills of 10 individual clams showed a significant 10-fold decrease of OPB7 probe hybridization sites in clam DNA at C_2 exposure (Table 1) indicating metal-induced modifications in DNA composition. When comparing the frequency distribution of the OPB7-obtained PCR product *T*m among the temperature intervals, distribution increased in C_2-contaminated clam genomic DNA compared with controls at 83 °C to 84 °C: Only DNA from 5 of 11 control clams *versus* DNA from 10 of 11 C_2-contaminated clams presented a melting peak in this interval ($p < 0.05$) (Table 2).

For exposed worms, there was a 2.5-fold increase in the number of OPA9 probe hybridization sites in genomic DNA after C_1 exposure (Table 1). Differences in the distribution frequency of PCR product *T*m were observed. The OPB7 probe distinguished C_2-exposed DNA from both C_1-exposed and control DNA for the temperature intervals 80–81 °C and 81–82 °C, and the OPA9 probe distinguished C_1- and C_2-exposed worms from control animals for the temperature intervals 88 to 89 °C and 85 to 86 °C, respectively (Table 2).

Relative gene expression in gill from exposed clams was compared with that from control clams. There was a

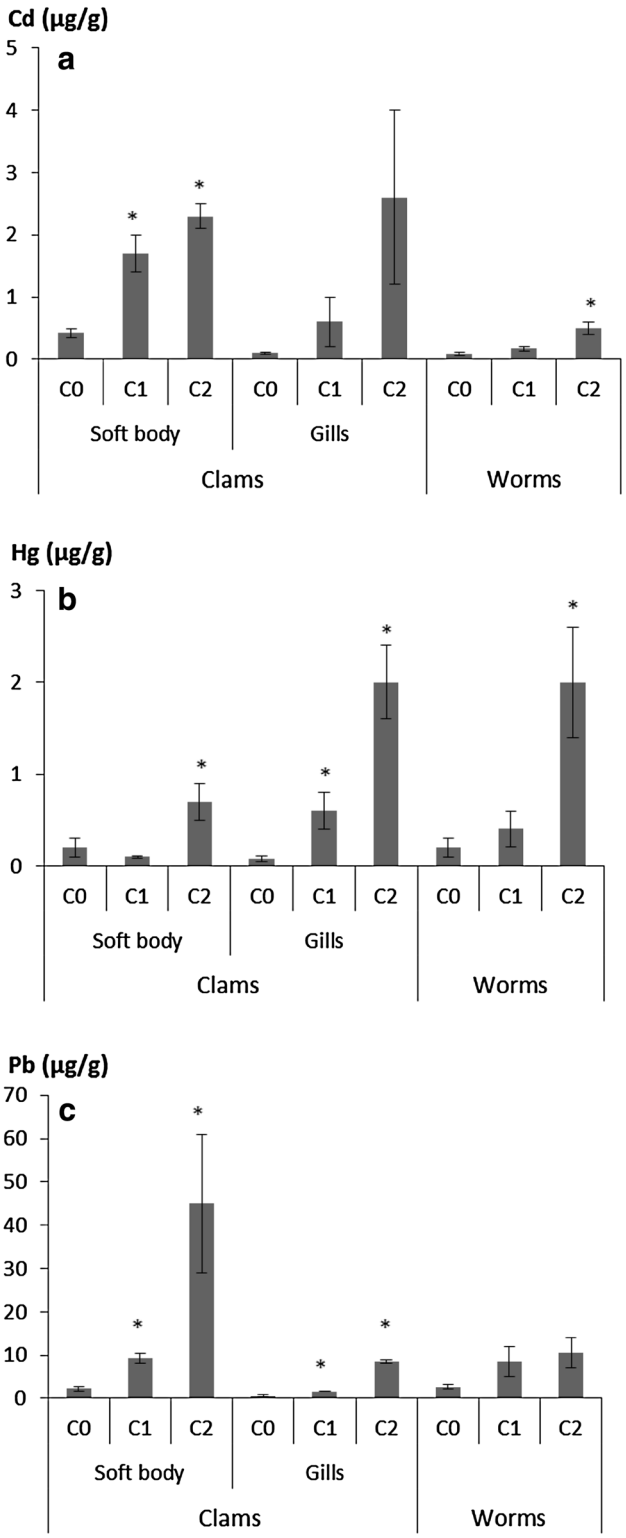

Fig. 1 Metal bioaccumulation in soft body and gill of clams and worms. **a** Cd, **b** Hg, and **c** Pb concentrations in (µg/g dw) as mean ± SE ($n = 3$). *Statistically significant values compared with control (C_0) as assessed by Mann–Whitney *U* test, $p < 0.05$

Table 1 Relative number of hybridization sites per genome unit of RAPD probes on genomic DNA from control and contaminated clams and worms

Experimental conditions	Clams		Worms	
Metallic blend	OPB7	OPB11	OPB7	OPA9
C_0	0.02 ± 0.007	(2.1 ± 0.5) 10^{-4}	3.3 ± 0.9	0.04 ± 0.008
C_1	0.02 ± 0.005	(39 ± 10) 10^{-4}	4.3 ± 1.1	0.1 ± 0.01*
C_2	0.002 ± 0.0006*	(0.36 ± 0.09) 10^{-4}	4.5 ± 2.4	0.03 ± 0.01

Results are mean ± SEM ($n = 11$ for clams and $n = 10$ for worms). The relative number of hybridization sites of a RAPD probe is defined as the ratio of the total hybridization sites of the RAPD probe over 16S (for clams) or 28S (for worms) ribosomal gene copies number

* Significant differences compared with unexposed controls as assessed by Mann–Whitney U test, $p < 0.05$

Table 2 Frequency according to temperature intervals (C°) to which belong the T_m values of PCR products obtained with probes on genomic DNAs from clams and worms

Animal	C_0	C_1	C_2
Clams (°C)	OPB7		
83–84	0.45	0.5	0.91[a]
Worms (°C)	OPB7		
80–81	0.5	0.7	0.1[a]
81–82	0	0.2	0.7[a]
	OPA9		
85–86	0.4	0.7	0[a]
88–89	0	0.8[a]	0.2

[a] Metal blend concentration for which the frequency of occurrences of peaks belonging to the indicated temperature intervals discriminates significantly contaminated genomic DNA from controls ($n = 11$ for clams and 10 for worms) as assessed by Mann–Whitney U test, $p < 0.05$

significant 50-fold induction of *cox1* gene expression in C_2-exposed animals. The expression of *cytb* and mitochondrial ribosomal 16S RNA genes were upregulated 10 and 20 times, respectively (Table 3).

Discussion

The present work showed that exposure of *R. philippinarum* and *H. diversicolor* to metal blends led to the accumulation of Cd, Pb, and Hg and triggered genotoxic effects on both animals. We performed combined exposures because single exposures to metals have already been described for *R. philippinarum* and *H. diversicolor*. When *R. philippinarum* was exposed to 15 µg/L of $CdCl_2$, 400 ng/g (dw, dry weight) of Cd was quantified in gills after 7 days (Paul-Pont et al. 2010). Exposure of *R. philippinarum* to 700 µg/L of $PbCl_2$ during 7 days led to accumulation of Pb reaching 170 and 160 µg/g (dw) in soft body and gill, respectively (Blasco and Puppo 1999). In *R. philippinarum* collected from natural sites containing 0.65 ng/L of total Hg (0.35 ng/L of total dissolved Hg), body burden of Hg reached 47 ng/g dw 16.6 ng/g MeHg dw) (Pan and Wang 2011). Several studies assessed metal (Cd, Pb, and Hg) bioaccumulation in *H. diversicolor*. When *H. diversicolor* was exposed to 10 µM of $CdCl_2$, 4.5 µg Cd/g was recorded in this worm after 7 days (Lianzhen et al. 2012). In *H. diversicolor* collected from Restronguet estuary sediment containing 336 µg/g of Pb dw, Pb body burden reached 89 µg/g dw (Rainbow et al. 2011). In worms collected from Mondego estuary sediment containing 5.3 µg/g of Hg dw, Hg reached 0.1 µg/g (wet weight) (Cardoso et al. 2009). It should be stressed that the metal burden of the marine sediment we collected in Arcachon is far lower than the previously described metal concentrations with 180 times less Pb than in Restronguet estuary and 56 times less Hg than in Chegado estuary. In the present study, differences of Cd and Hg accumulations were observed between clam soft body and gill because gill accumulated two times more Hg than soft body mainly at greater concentration. However, soft body accumulated

Table 3 Relative expression of selected genes in gill from control and contaminated clams after 8 days of metal exposure

Blend	*cox1*	*cytb*	*sod*	*cat*	16S RNA
C_0	4.7 ± 1.3	(8 ± 5) × 10^{-2}	2.3 ± 1.4	0.2 ± 0.1	0.002 ± 0.001
C_1	15.3 ± 6.8	(81 ± 52) × 10^{-2}*	13.6 ± 8.5	0.5 ± 0.1	0.005 ± 0.004
C_2	237 ± 125*	(650 ± 509) × 10^{-2}	20.1 ± 12.9	0.2 ± 0.05	0.04 ± 0.01*

Differential expression of a gene in a tissue is the ratio of its relative gene expression in gill clam in the indicated condition compared with that of control clams. Clam exposure was performed in presence of worms

* Statistically significant differential expression (mean ± SEM, $n = 5$) as assessed by Mann–Whitney U test, $p < 0.05$

5-fold more Pb than gill. This difference could be due to the ability of clams to segregate Pb by forming granules as a way of detoxification. This has been shown in the earthworm *Aporrectodea caliginosa* collected from a nonpolluted soil containing 35 mg/kg dw of Pb. More than two-thirds of Pb accumulated in *A. caliginosa* was mainly found in the granular fraction, in which Pb was bound to cysteine residues coming from the degradation of metallothioneins (Vijver et al. 2006). Exposure of the freshwater bivalve *Hyridella australis* to Pb-spiked sediment also showed a difference of Pb accumulation between tissues due to granules formation. Labial palps accumulated 2.6-fold more Pb than gill reaching 13 μg Pb/g dw after exposure for 28 days to sediment spiked with 419 μg Pb/g. The tissue accumulating the most Pb was hepatopancreas, and 75 % of this metal was sequestered into the granule fraction (Marasinghe Wadige et al. 2014). A reversed pattern of accumulation has been described in that clam species exposed for 7 days to much greater Pb concentrations (350 μg/L), which led to a 2.5 increased burden of Pb in gills compared with soft body (Blasco and Puppo 1999). At greater concentrations of Pb (700 μg/L), the difference vanished. For Cd and Cu, there were no differences in accumulated metal between soft body and gils at 200 or 600 μg Cd/L and 10 or 20 μg copper (Cu)/L (Blasco and Puppo 1999). These discrepancies are likely due to the high metal concentration used in that study compared with the present work.

In the present study, worms accumulated 5 times less Cd and 4.5 times less Pb than clams, whereas worms accumulated 3 times more Hg than clam soft body. This could be due to the animals' lifestyles and exposure route. In fact, *R. philippinarum* and *H. diversicolor* are different in terms of habitat, behavior, and feeding. *H. diversicolor* lives buried in sediment and displays a bioturbation activity called "biodiffusion" and as such regularly irrigates its burrow with polluted water (Nielsen et al. 1995; Scaps 2002). *R. philippinarum* is a deposit filter feeding that lives buried partially in the sediment and filters the water compartment. Accumulation results obtained in *H. diversicolor* are similar to those observed in tubificidae worms exposed to Cd from the overlying water column (20 μg Cd/L) with an observed burden of 12 μg Cd/g dw after 21 days (Ciutat et al. 2005b). This efficient bioaccumulation was shown to result from bioturbation, thus increasing the transfer of Cd from the water column to the sediment with calculated rates after 21 days of 616 ± 46 and 237 ± 44 ng Cd/cm^2/d in the presence and absence of worms, respectively (Ciutat et al. 2005a). Sediment can also be the predominant source of Cd accumulated by polychaete worms (Kalman et al. 2010).

Our results showed deoxyribonucleic acid, DNA alterations caused by metal blends. In fact, genotoxicity was correlated with metals burden in animals. In the present work, using quantitative RAPD we looked at the creation or elimination of hybridization sites of probes on genomic DNAs of contaminated organisms compared with controls along with modifications of PCR products' melting temperature, T*m* profiles, which are effective parameters in detecting small changes in DNA sequences (Cambier et al. 2010; Lerebours et al. 2013). Indeed OPB7, OBP11, and OPA9 probes highlighted DNA alterations in *R. philippinarum* and *H. diversicolor* caused by metal contamination. It should be noted that clams and worms were collected in situ so they were genetically different. This genetic diversity is reflected by the fact that the frequency of PCR products for each melting temperature class is for many of the classes different from 1 and 0 (situation expected in the case of a clonal species). Metal genotoxicity has been shown in *Scrobicularia plana* and the polychaete *H. diversicolor* exposed to 10 μg/L of ionic Cu for 21 days (Buffet et al. 2013), and *H. diversicolor* exposed to a sediment spiked with 50 mg/kg of ionic silver (Ag) for 10 days showed accumulation of 10 ± 5 μg Ag/g dw (Cong et al. 2011). In the present study, the concentrations of metal resulting in DNA damage were in the same range (a few micrograms per gram of dry weight). DNA damage was shown in worms by the increase of OPA9 probe hybridization sites after exposure to the C_1 polymetallic blend but not the C_2 blend. This is reminiscent of the detection of genotoxicity in *M. galloprovincialis* gill cells after exposure to 40 but not 80 μg/L of divalent Cu chloride (Bolognesi et al. 1999). Comet assay showed DNA damage in digestive gland of *M. edulis* after 10 days of exposure to 200 μg/L of Cd and 10 μg/L of hexavalent Cr corresponding to accumulated concentrations of 39 ± 11 μg Cd/g wet tissue and 2.7 ± 2.9 μg Cr/g wet tissue (Emmanouil et al. 2007). In contrast, comet assay showed no effect of Cd in the same organism exposed to 200 μg/L for 4 weeks (Pruski and Dixon 2002).

However, because the detected number of hybridization sites is a balance between created and lost sites, the absence of difference between control and exposed animals does not necessarily indicate the absence of DNA alterations. Indeed, the analysis of the frequency of PCR products showed significant differences for C_2-exposed worms compared with controls.

Because CoxI is a subunit 1 of cytochrome *c* oxidase, which performs the critical function of transferring electrons from cytochrome *c* to oxygen and hence contributing to adenosine triphosphate generation during respiration (Malatesta et al. 1995), mitochondrial respiration was likely impacted in C_2-exposed animals. Overexpression of the *cox1* gene has been shown in a pyrethroid insecticide-resistant strain of *Blatella germanica* (German cockroach) (Pridgeon and Liu 2003), in *Danio rerio* (zebrafish) fed

diets contaminated by methyl Hg (Gonzalez et al. 2005), and in freshwater and marine bivalves exposed to Cd (Achard-Joris et al. 2006). Because CoxI is considered as the rate-limiting step for mitochondrial respiration (Villani and Attardi 2000), *cox1* gene overexpression could be a compensatory mechanism to restore decreased mitochondrial activity and efficiently consume oxygen, thus limiting Cd-induced damage in the cell. The proposal of such an adaptive mechanism is strengthened by the parallel overexpression of the *cytb* gene encoding the main subunit of respiratory chain complex III. Last, the overexpression of the 16S ribosomal RNA gene indicates that the number of mitochondria increased, which fits well with a compensatory response.

To conclude, animals exposed in the laboratory to environmentally relevant polymetallic concentrations displayed DNA alterations and modified patterns of gene expression. The effects of the metal mixture were observed at the greatest concentration after 8 days of exposure, thus showing a rapid effect on animal molecular biology. In their natural habitat, animals are facing these levels of metal pollution for many months at a time; thus, the real impact might well be worse than observed in these microcosms.

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