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Partial Inventory of ABCB and ABCC Transporter Genes Responding to Cadmium and Zinc Contamination in Zebrafish *Danio Rerio*

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Abstract

ABC transporters belonging to the subfamilies ABCB (MDR/TAP) and ABCC (CFTR/MRP) are likely to play a role in the detoxification of metallic pollutants. We made an inventory of the transcriptional response of 10 *abcb* and 9 *abcc* gene members in zebrafish *Danio rerio* exposed for 7 days to ionic cadmium (89 nM), zinc (7.3 µM), or a blend of both metals. These concentrations correspond to those found in a polluted tributary of the Lot River, France. The general trend was that cadmium is rather an up-regulator (but for high accumulation factors only) whereas zinc is rather a repressor. In muscles the expression pattern of ABC genes in response to metals appeared unpredictable since there was no relationship between differential expression and metal accumulation. Although no increase of zinc burden was observed in fish muscles and gills exposed to zinc, that metal repressed 9 and 4 ABC genes in muscles and gills, respectively. Despite a 3-fold increase in zinc burden in brain, it triggered the down-regulation of 3 ABC genes. Also, despite an accumulation factor of 7 in muscles, cadmium repressed 2 ABC genes in muscles. However, in gills and liver cadmium exposure caused the up-regulation of 4 and 6 ABC genes linked to accumulation factors of 33 and 25, respectively. Beside MDR- and MRP-transporter encoding genes, metals up regulated other genes encoding zebrafish homologues of TAP2 (*abcb*3 and *abcb3L1*), ATM1 (*abcb*7), M-ABC1 (*abcb*8), TAPL (*abcb*9), SUR1 (*abcc*8) and SUR2 (*abcc*9) transporters.

Keywords: *Danio rerio*; Cadmium; Zinc; Bioaccumulation; Gene expression; ABC transporters

Introduction

ATP-binding cassette (ABC) transporters are membrane proteins that belong to a superfamily of representatives in all phyla from prokaryotes to humans. ABC transporters are divided into seven subfamilies, from A to G, based on structural arrangement and phylogenetic analysis [1]. Zebrafish also have an ABCH1 transporter [2]. These efflux pumps have important functions in the transport of a wide variety of compounds across biological membranes, including phospholipids, ions, peptides, steroids, polysaccharides, amino acids, organic anions, bile acids, drugs and other xenobiotics. In tumour cells, the failure of chemotherapy is linked to the overexpression of ABC transporter proteins belonging to the B (MDR1) and C (MRP1 and MRP2) subfamilies [3].

Mammalian ABCC1/MRP1 confers resistance to several toxicants including heavy metals and metalloids, such as arsenite [4,5], antimony [5], cadmium [4,6], platinum [6], mercury [7], and zinc [6]. ABCC2/MRP2 plays important roles in the detoxification of heavy metals, including mercury [7,8], arsenite [9,10] and platinum [11,12]. ABCC5/MRP5 catalyzes efflux of GSH S-conjugates [13,14] and is involved in the detoxification of heavy metals such as platinum [15] and cadmium [16]. Human MDR1 protein and bacterial homologues such as OmrA [17] and LmrA [18] could protect a bacterial mutant hypersensitive to xenobiotics by pumping out cadmium, mercury and zinc conjugated to glutathione [19,20].

In aquatic organisms, the presence of ABC transporters involved in multi-xenobiotic resistance (MXR) has been demonstrated [21,22]. The MXR mechanism is induced by several metals such as tributyltin in oysters [23], and cadmium, copper, mercury, uranium and zinc in freshwater clams [24-26]. In fish several ABCB and ABCC transporters [27-32], along with ABCG2/BCRP [33], have been reported. The *abcb1* gene is absent in the zebrafish genome. Zebrafish ABCB4 and ABCB5 are structurally similar to mammalian ABCB1, and it has been shown that ABCB4, but not ABCB5, transporter conferred resistance of embryos to ABCB1 substrates [28]. In sea urchin embryos ABCC transporters antagonize bio concentration of inorganic mercury [34]. In addition, several ABC genes belonging to the B and C subfamilies proved to be up regulated in fish and fish cell lines in response to metals, but it should be mentioned that fish or cells were exposed to very high concentrations of metals or through unnatural routes. For instance, the up-regulation of *abcb1* and *abcb2* gene members has been recorded in the icefish *Trematomus bernacchii* 7 days after an injection of 2 µg/g of Cd(II) [35], that of *abcb1*, *abcc1*, *abcc2*, *abcc3* and *abcc4* gene members in PLHC-1 fish cell line exposed to 1 µM Hg(II), As(III), Cr(IV) and Cd(II) [36], that of *abcc5* in zebrafish embryos exposed to micromolar amounts of As(III), Cd(II), Hg(II) and Pb(II) [37], that of *abcc2* and *abcc4* in zebrafish ZF4 cells exposed to micromolar amounts of Cd(II) [38], and that of *abcc2* in adult zebrafish exposed up to 1 µM Hg(II) and Pb(II) [39]. Therefore, we decided to perform an experiment with

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zebrafish, in order to see whether ABC gene members of the B and C subfamilies could respond to cadmium and zinc contaminations at environmentally realistic concentrations.

Animals, Materials and Methods

Zebrafish care and contamination

All procedures were conducted in accordance with the laws and regulations controlling experiments/procedures with live animals in France. Fish husbandry and exposure were carried out in the marine station Lab (EA), Arcachon, France. We exposed zebrafish to 10.0 and 478 µg/l of cadmium and zinc, corresponding to 89 nM and 7.3 µM, respectively. Such cadmium and zinc concentrations were selected because they are typical of what one can find in a polluted environment. Indeed, besides the present work, we are studying a polymetallic pollution in the Lot River and its small tributary (Riou-Mort) in the South-West of France. These streams are polluted by cadmium and zinc originating from an old factory that has produced Zn for over a century, on the Vieille-Montagne industrial site. 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 after a 3 month survey (mean: 15 µg Cd/L) and that of zinc varied between 200 and 2200 µg/l (mean: 900 µg Zn/L) [24]. We have previously shown that this polymetallic pollution resulted in a genotoxic outcome when zebrafish were exposed to the water of the Lot and Riou-Mort Rivers [40]. Additionally, we have, in the past, recorded the variations of expression of several biomarker genes in response to 2 different concentrations of cadmium (17 and 86 nM) at 2 sampling time points (7 and 21 days of exposure). We found that the gene expressions varied between the 2 time points for the same Cd exposure concentration. In gills, the highest response was observed after 7 days of exposure to 86 nM whereas almost all biomarker genes returned to the basal level of expression after 21 days [41]. That is why we decided in the present work to select one concentration (89 nM of Cd and 7.3 µM of Zn) and one time point (7 days).

A total of 56 adult male fish (average weight: 0.88 ± 0.03 g, wet wt; standard length: 3.63 ± 0.05 cm) were randomly distributed into eight tanks containing 60 L of chlorine-free, permanently oxygenated water. Female fish were excluded to avoid interferences due to reproduction processes. Two replicate tanks per condition were considered.

During the whole experiment, the temperature was maintained at 23.5 ± 0.1 °C. Tank bottoms were cleaned daily to eliminate fish's faeces and remains of food. Fish were fed daily with a quantity of dry food representing 5% of their body weight (Novo Granomix, JBL). The food contained 38% protein, 6% fat, 4% fibre, 9% ash, 0.9% phosphorus, 25 IU.g^{-1} of vitamin A, 0.4 mg.g^{-1} vitamin C, 3 mg.g^{-1} vitamin D3, and 0.33 mg.g^{-1} vitamin E. Cd and Zn concentrations in this diet were measured and were equal to 2.7 ± 0.5 nmol Cd.g^{-1} and 860 ± 33 nmol Zn.g^{-1}. The contamination of the water was based on a continuous addition, using peristaltic pumps, of aqueous solutions of Cd or Zn (ultra-pure $CdCl_2$ and $ZnCl_2$, Merck) directly mixed in the incoming water stream (in tanks presenting a permanent water renewal). This allowed to compensate for the decrease in metal concentrations over the 24-h cycles, which were measured daily by analysing 10 ml water samples.

Tanks were submitted to a permanent water renewal with peristaltic pumps. In tank 1a and 1b, fish were cultured in uncontaminated water and constituted control animals. In tank 2a and 2b, fish were exposed for 7 days to Cd (mean over 7 days: 10.0 ± 2.0 µg Cd.l^{-1}; 89 ± 18 nM). In tank 3a and 3b, fish were exposed for 7 days to Zn (mean over 7 days: 478 ± 14 µg Zn.l^{-1}; 7.3 ± 0.2 µM). In tank 4a and 4b, fish were exposed for 7 days to both Cd and Zn (mean over 7 days: 8.5 ± 1.2 µg Cd.l^{-1} giving 76 ± 11 nM, and 444 ± 16 µg Zn.l^{-1} giving 6.8 ± 0.2 µM). After 7 days, 14 fish per condition were randomly collected through replicate tanks for metal bioaccumulation (3 for Cd analysis, 3 for Zn analysis and 2 additional ones in reserve) and gene expression analysis (5 fish and an additional one in reserve). They 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). Fish were dissected on ice. On each fish, brain, liver, gills, and skeletal muscle were independently harvested. Tissues intended for metal quantification were kept at -80°C until use. Tissues intended for gene expression analysis were kept in RNA-later (Qiagen) at -80°C until use.

Cd and Zn analysis

The Cd determinations for water and digested tissue samples were performed with an atomic absorption spectrophotometer (M6 Solar AA, Thermo Elemental) equipped with a graphite tube atomizer (GF95 Graphite Furnace) as already described [40]. The detection limit was 0.05 µg Cd.l^{-1}. Zinc concentrations for water and digested tissue samples were determined by flame atomic absorption spectrophotometry (Varian AA220FS) as already described [40]. The detection limit was 10 µg Zn.l^{-1}. The results are expressed as average metal concentrations accumulated in tissues (in nmol Cd.g^{-1} wet weight ± SD, and µmol Zn.g^{-1} wet weight ± SD) for three samples from each tissue and tank.

Selection of ABCB and ABCC transporter genes for gene expression analysis

In the zebrafish's Ensembl program from the Sanger Institute (http://www.ensembl.org/Danio_rerio/Info/Index), when looking for the MDR1 gene homologue, the ENSDARG00000021787 gene is highlighted, but this gene is described to encode an ABCB5 member. From this precise page, one can look for the homologous genes containing the same ABC transporter domain IPR001140. Its primary sequence is highly conserved, displaying a typical phosphate-binding loop (Walker A), and a magnesium binding site (Walker B). Besides these two regions, three other conserved motifs are present in the ABC cassette: the switch region that contains a histidine loop, the signature motif (LSGGQ) specific to the ABC transporter, and the Q-motif (between Walker A and the signature). The Walker A, Walker B, Q-loop and switch region form the nucleotide binding site [42]. 30 genes are appearing in the list of genes possessing that IPR001140 domain: 12 and 16 are encoding ABCB and ABCC transporters, respectively, and two encode uncharacterized proteins (Table 1).

However, when we began this study, there were only 20 genes listed encoding one uncharacterized protein, ten ABCB and nine ABCC transporter members. Among these, one transporter that appeared in the list, with the gene accession number ENSDARG00000070821 and described to be an ABCB11 member, has been deleted from the current, updated list. Therefore, in the present study we present the gene expression variations of those remaining 19 *abcb* and *abcc* genes, which appear in Table S1 in normal characters whereas the transporter genes that were added to the list after we began this study are underlined. The primer couples used corresponding to these 19 genes are listed in Table S2. For gills, the expression of *abcb4*, *abcb11b* and *abcc7* members were excluded from the analysis because their basal expressions were found to be too weak. For brain and liver, the excluded genes were those from *abcb4*, *abcb7*, *abcb10*, *abcb11b*, *abcc6a*, *abcc7* and *abcc12* members.

	Cadmium levels (nmol/g fresh weight)				Zinc levels (mmol/g fresh weight)			
Exposure	Muscles	Liver	Gills	Brain	Muscles	Liver	Gills	Brain
Control	0.05 ± 0.01	0.42 ± 0.24	0.29 ± 0.05	0.12 ± 0.05	0.76 ± 0.20	0.31 ± 0.10	1.1 ± 0.16	0.16 ± 0.07
Cd	0.36 ± 0.07*	10.4 ± 7.2*	9.5 ± 3.3*	0.82 ± 0.27*	0.56 ± 0.22	1.3 ± 1.6	0.94 ± 0.17	0.55 ± 0.14
Zn	0.05 ± 0.01	0.26 ± 0.08	0.37 ± 0.17	0.12 ± 0.07	0.64 ± 0.15	0.95 ± 0.43*	1.87 ± 1.12	0.48 ± 0.09*
Cd + Zn	0.43 ± 0.15*	6.4 ± 6.5*	36 ± 30*	1.3 ± 1.7	0.46 ± 0.09	0.57 ± 0.28	1.04 ± 0.23	0.44 ± 0.08*

[a]Mean ± SD (n = 3). Asterisks indicate significant differential metal accumulation compared to control as given by the Mann-Whitney U test ($p < 0.05$).

Table 1: Metal accumulation within tissues after 7 days of exposure[a].

Gene expression analysis

Total RNAs were extracted from 40 mg of tissue using the Absolutely RNA RT-PCR Miniprep kit (Stratagene), according to the manufacturer's instructions. The elution volume was 30 µl and the concentration of RNA was quantified using a nanodrop spectrometer (Epoch, Biotek). RNA purity was checked and met the following requirements: A_{260}/A_{280}>1.7 and A_{260}/A_{230}>1.5. The integrity of the 18 and 28S ribosomal bands was checked on a 1% agarose-formaldehyde gel. First-strand cDNA was synthesized from 5 µg total RNAs using the AffinityScript Multiple Temperature cDNA Synthesis kit (Stratagene) using 3 µl of random primers (0.1 µg/µl), 1 µl of AffinityScript Multiple Temperature RT, 2 µl of 10x AffinityScript RT buffer, dNTP (25 mM each) and RNase free water in a final volume of 20 µl. The retro-transcription was performed by incubating the reactions for 60 min at 42°C. Specific primer pairs for ABC genes were determined using the LightCycler probe design software (Ver 1.0; Roche) and matched the coding sequence of the target genes. The GenBank accession numbers and the corresponding primer pairs are summarized in Table S2. The corresponding ENSEMBL gene names have already been described for zebrafish [43]. Real-time qPCR reactions were performed using a Mx3000P QPCR System (Stratagene, Agilent). Each 25 µl reaction contained 1 µl of reverse-transcribed product template, 12.5 µl of 2x SYBR Green QPCR Master mix (Stratagene, Agilent), 2 µl the gene-specific primer pairs (at a concentration of 300 nM each) and 9.5 µl of H_2O. The programme used was: one cycle at 95°C for 10 min and then 50 amplification cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min.

Relative quantification of each gene expression level was normalized to the *b-actin* gene expression and calculated by the following formula: $2^{(Ct(bactin) - Ct(gene))}$, where Ct(gene) is the threshold cycle value of a given gene. For each gene expression level, the mean value and the associated standard error mean (n=5) were determined. The differential gene expressions - representing the induction factors - were obtained by comparing each mean value observed in the exposed condition with that of the control condition, and are thus calculated as the ratio of the exposed relative expression to that of the control relative expression ($2^{(Ct(bactin) - Ct(gene))}$ under exposure divided by $2^{(Ct(bactin) - Ct(gene))}$ under control condition). The reaction specificity was determined for each reaction from the dissociation curve of the PCR product. This dissociation curve was obtained by following the SybrGreen fluorescence level during a gradual heating of the PCR products from 60 to 95°C. The *b-actin* gene was chosen as the reference gene because of its stability over time and treatment in the experiment, as highlighted by the fact that the mean Ct values were the same whatever the exposure type. Indeed, the Ct collected after 7 days of exposure were (for control, Cd, Zn and Cd plus Zn exposures, respectively; n=5, mean ± SD): Brain: 22.3 ± 0.4, 21.2 ± 0.5, 21.7 ± 0.4, 21.9 ± 0.4; Liver: 17.9 ± 0.6, 18.5 ± 0.6, 17.5 ± 0.5, 19.0 ± 1.1; Muscles: 25.2 ± 0.9, 23.2 ± 1.0, 25.5 ± 0.8, 26.0 ± 0.7; Gills: 18.5 ± 1.0, 18.9 ± 0.7, 18.6 ± 0.9, 18.9 ± 0.8. All qPCR experiments were performed according to the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines [44]. A MIQE checklist has been inserted in Supplementary materials.

Statistics

Significant differences in gene expression levels in gill, liver, muscle and brain between control and contaminated fish were determined using the nonparametric Mann–Whitney U-test ($p < 0.05$) (Statistica 5.1).

Results

Cadmium and zinc accumulation in fish organs

During the 7 days encompassing the experiment, the physicochemical constants of the water in tanks were recorded each day. The pH and temperature were held constant. Metal concentration fluctuations were maintained within narrow limits so that the average Cd and Zn exposures during the week reached 10.0 ± 2.0 µg/l and 478 ± 14 µg/l, respectively (Table S3).

During the time course of the experiment, we did not observe mortality among fish. Zn and Cd concentrations were quantified in muscles, liver, gills and brain (Table 1). In gills and muscles from animals exposed to Zn, no significant increase in Zn content could be observed as compared to control tissues. In liver and brain 3-fold increases were recorded after 7 days of Zn exposure, corresponding to high bioaccumulation factors of 130 and 66, respectively (the bioaccumulation factor was calculated as the ratio of the metal concentration in a tissue to that in the water column). In the case of Cd exposures significant 7-, 25-, 33-, and 7-fold increases in Cd content were noticed in muscles, liver, gills, and brain, respectively, as compared to control yielding high bioaccumulation factors reaching 4, 117, 107, and 9 in the corresponding tissues. Therefore, liver and gills were by far the greatest Cd accumulator tissues.

Gene expression analysis

At the time we began this study 19 ABC transporter genes belonging to the subfamilies B and C were recorded. Ten belong to the B subfamily and are composed of the ABCB3, ABCB3L1, ABCB4, ABCB5, ABCB7, ABCB8, ABCB9, ABCB10, ABCB11a and ABCB11b members. Nine others belong to the C subfamily and are composed of the ABCC1, ABCC2, ABCC4, ABCC5, ABCC6a, ABCC7, ABCC8, ABCC9 and ABCC12 members. Among the 19 genes analyzed, two (*abcb5* and *abcb11a*) presented so low basal and post metallic challenge expressions that we could not record their relative expressions (their Ct were above that of the negative control PCR containing in the reaction medium the gene-selective primers without added DNA).

In muscles, a surprising expression pattern was observed (Tables S4 and 2). Although this tissue did not accumulate more zinc as compared to control, eight ABC genes were down regulated in response to zinc and to the combined metallic exposure (*abcb3*, *abcb3l1*, *abcb10*, *abcc1*, *abcc2*, *abcc4*, *abcc5* and *abcc8*), and two were up regulated in

Gene[b]	Control	Cd	Zn	Cd + Zn
abcb3	1.3 ± 0.4	1.0 ± 0.2	0.05 ± 0.01*	0.024 ± 0.005*
abcb3l1	1.1 ± 0.6	0.76 ± 0.14	0.10 ± 0.03*	0.09 ± 0.02*
abcb4	ND[c]	0.03 ± 0.01	0.017 ± 0.005	0.008 ± 0.002
abcb7	0.0013 ± 0.0011	0.003 ± 0.001	0.07 ± 0.03*	0.035 ± 0.015*
abcb8	0.0016 ± 0.0006	0.002 ± 0.001	0.007 ± 0.002*	0.008 ± 0.003*
abcb9	0.019 ± 0.009	0.004 ± 0.002	0.011 ± 0.003	0.008 ± 0.002
abcb10	0.38 ± 0.21	0.3 ± 0.1	0.012 ± 0.002*	0.013 ± 0.003*
abcb11b	0.02 ± 0.02	0.04 ± 0.02	0.008 ± 0.004	0.00046 ± 0.00009*
abcc1	1.4 ± 0.6	2.8 ± 1.1	0.11 ± 0.01*	0.08 ± 0.02*
abcc2	1.7 ± 0.4	0.53 ± 0.16*	0.020 ± 0.007*	0.007 ± 0.003*
abcc4	0.32 ± 0.16	0.18 ± 0.03	0.0201 ± 0.006*	0.014 ± 0.005*
abcc5	0.89 ± 0.20	0.27 ± 0.08*	0.10 ± 0.01*	0.086 ± 0.002*
abcc6a	ND	0.011 ± 0.003	0.006 ± 0.001	0.0024 ± 0.0006
abcc7	ND	0.3 ± 0.1	0.005 ± 0.001	ND
abcc8	0.11 ± 0.06	0.05 ± 0.01	0.0019 ± 0.0006*	0.003 ± 0.001*
abcc9	4.2 ± 2.8	0.7 ± 0.5	1.7 ± 0.7	0.8 ± 0.1
abcc12	ND	0.004 ± 0.001	0.0025 ± 0.0007	0.0010 ± 0.0004

[a]Relative gene expressions (mean ± SEM, $n = 5$). *bactin1* was the reference gene
[b]The asterisk indicates a significant differential gene expression in zebrafish muscles, as determined with the Mann-Whitney *U*-test, *$p < 0.05$
[c]ND: not detected. No Ct could be recorded or a Ct equal or above that of the negative control without DNA.

Table 2: *abcb* and *abcc* gene relative expressions in muscles after 7 days of metal exposure[a].

response to zinc and to the combined metallic exposure (*abcb7* and *abcb8* presented 56- and 4-fold increased expressions as compared to control). Four additional genes were up regulated (*abcb4*, *abcc6a*, *abcc7* and *abcc12*): indeed their basal expression could not be quantified (Ct above that of the negative control) but after a metallic challenge, Ct were recorded. The cadmium challenge brought also surprising results, since despite a 7-fold increase in muscles Cd burden compared to control, the *abcc2* and *abcc5* genes were down regulated.

In gills, like in muscles, a surprising expression pattern was observed (Tables S5 and 3). Although this tissue did not accumulate more zinc than control, four ABC genes were down regulated in response to zinc (*abcb3*, *abcc2*, *abcc4*, and *abcc5*), and one was up regulated in response to zinc and to the combined metallic exposure (*abcb8* presented 296-fold and 585-fold increased expressions as compared to control after a zinc alone or a combined metallic exposure, respectively). The cadmium challenge, contrarily to zinc, triggered an expected pattern since in response to a 33-fold increase in Cd burden, four members were strongly up regulated (*abcb8*, *abcc1*, *abcc4* and *abcc8*).

In brain, despite a 3-fold increase in Zn burden, the response to zinc was the down-regulation of *abcb3*, and upon a combined metal challenge the down-regulation of *abcc5* (Tables S6 and 4). Cadmium, contrarily to zinc, triggered an expected pattern, since in response to a 7-fold increase in Cd burden, two ABC genes were up regulated (*abcb9* and *abcb3l1*).

In liver, no differential expressions were observed after a zinc exposure despite a 3-fold increase in Zn burden (Tables S7 and 5). However the 25-fold increase in Cd burden after exposure to cadmium or to the combined metals triggered, as expected, the up-regulation of several ABC B and C member genes (*abcb3*, *abcb9*, *abcc1*, *abcc5*, *abcc8* and *abcc9*).

Discussion

It should be stressed that we exposed zebrafish to very low, environmentally relevant contamination pressures as compared to most of the other studies dealing with fish exposed to 5 to 400-times higher metal concentrations.

For instance, and after just a quick literary review of the most recent literature:

- 5 µM Cd^{2+} and 340 µM Zn^{2+} on zebrafish embryos [45].
- 8.9-35.6 µM of Cd^{2+} on adult zebrafish and embryos [46].
- 5 µM Cd^{2+} on adult zebrafish [47].
- 3 to 5 µM Cd^{2+} on zebrafish embryos [48].
- Up to 33.6 µM Zn^{2+} on zebrafish embryos [49].

In the present study we only used 0.089 µM of Cd^{2+} and 7.4 µM of Zn^{2+}. This should be born in mind when comparing our results of ABC genes expression with those of other studies in which high levels of toxicants or metals have been used. In the present work, no increase in the accumulation of Zn was detected in muscles, liver and gills of

Gene[b]	Control	Cd	Zn	Cd + Zn
abcb3	0.13 ± 0.03	0.59 ± 0.25	0.0095 ± 0.0021*	0.04 ± 0.01*
abcb3l1	0.15 ± 0.04	0.070 ± 0.026	0.072 ± 0.014	0.057 ± 0.026
abcb7	ND[c]	ND	(72 ± 31).10^{-4}	ND
abcb8	(2.6 ± 1.5).10^{-5}	(43 ± 14).10^{-5}*	(770 ± 240).10^{-5}*	(1520 ± 420).10^{-5}*
abcb9	0.0019 ± 0.0009	0.0025 ± 0.001	0.0010 ± 0.0003	0.0017 ± 0.0009
abcb10	(86 ± 44).10^{-4}	(340 ± 150).10^{-4}	(87 ± 27).10^{-4}	(170 ± 50).10^{-4}
abcc1	0.008 ± 0.001	0.091 ± 0.025*	0.013 ± 0.005	0.014 ± 0.006
abcc2	0.054 ± 0.017	0.25 ± 0.10	27.10^{-4} ± 6.10-4*	0.027 ± 0.008
abcc4	0.019 ± 0.002	0.13 ± 0.03*	0.0053 ± 0.0008*	0.010 ± 0.001*
abcc5	0.17 ± 0.04	0.15 ± 0.03	0.032 ± 0.009*	0.040 ± 0.021*
abcc6a	(89 ± 57).10^{-4}	(95 ± 28).10^{-4}	(9.4 ± 1.3).10^{-4}	(4.1 ± 0.7).10^{-4}
abcc8	(79 ± 44).10^{-5}	(593 ± 118).10^{-5}*	(5.2 ± 1.2).10^{-5}	(49 ± 21).10^{-5}
abcc9	0.019 ± 0.006	0.03 ± 0.01	0.012 ± 0.004	0.05 ± 0.02
abcc12	ND	ND	ND	(1.4 ± 0.4).10^{-4}

[a]Relative gene expressions (mean ± SEM, $n = 5$). *bactin1* was the reference gene
[b]The asterisk indicates a significant differential gene expression in zebrafish gills, as determined with the Mann-Whitney *U*-test, *$p < 0.05$
[c]ND: not detected. No Ct could be recorded or a Ct equal or above that of the negative control without DNA.

Table 3: *abcb* and *abcc* gene relative expressions in gills after 7 days of metal exposure[a].

Gene[b]	Control	Cd	Zn	Cd + Zn
abcb3	0.79 ± 0.18	0.5 ± 0.2	0.21 ± 0.07*	0.31 ± 0.07*
abcb3l1	0.135 ± 0.005	0.49 ± 0.16*	0.24 ± 0.11	0.41 ± 0.24
abcb8	(21 ± 4).10^{-4}	(20 ± 6).10^{-4}	(14 ± 10).10^{-4}	(41 ± 15).10^{-4}
abcb9	0.0025 ± 0.0005	(366 ± 4).10^{-5}*	ND[c]	0.006 ± 0.006
abcc1	0.31 ± 0.015	0.35 ± 0.14	0.21 ± 0.07	0.37 ± 0.07
abcc2	0.72 ± 0.11	1.2 ± 0.2	0.7 ± 0.3	1.0 ± 0.3
abcc4	0.45 ± 0.15	0.4 ± 0.1	0.17 ± 0.11	0.45 ± 0.13
abcc5	(16 ± 3).10^{-5}	(6 ± 4).10^{-5}	(26 ± 26).10^{-5}	(1.8 ± 0.6).10^{-5}*
abcc8	0.35 ± 0.03	0.40 ± 0.04	0.3 ± 0.1	0.49 ± 0.16
abcc9	0.12 ± 0.02	0.07 ± 0.02	0.11 ± 0.01	0.1 ± 0.1

[a]Relative gene expressions (mean ± SEM, $n = 5$). *bactin1* was the reference gene
[b]The asterisk indicates a significant differential gene expression in zebrafish brain, as determined with the Mann-Whitney *U*-test, *$p < 0.05$
[c]ND: not detected. No Ct could be recorded or a Ct equal or above that of the negative control without DNA.

Table 4: *abcb* and *abcc* gene relative expressions in brain after 7 days of metal exposure[a].

Gene[b]	Control	Cd	Zn	Cd + Zn
abcb3	0.011 ± 0.004	0.045 ± 0.011*	0.010 ± 0.003	0.03 ± 0.01
abcb3l1	0.09 ± 0.03	0.14 ± 0.03	0.14 ± 0.05	0.14 ± 0.03
abcb8	(17 ± 7).10^{-4}	(38 ± 9).10^{-4}	(16 ± 3).10^{-4}	(39 ± 9).10^{-4}
abcb9	(8 ± 2).10^{-4}	(23 ± 10).10^{-4}	(15 ± 3).10^{-4}	(22 ± 4).10^{-4}*
abcc1	0.003 ± 0.001	0.014 ± 0.003*	0.004 ± 0.002	0.016 ± 0.005*
abcc2	0.09 ± 0.05	0.25 ± 0.06	0.10 ± 0.03	0.20 ± 0.06
abcc4	0.014 ± 0.009	0.04 ± 0.01	0.009 ± 0.004	0.03 ± 0.01
abcc5	0.013 ± 0.006	0.062 ± 0.017*	0.014 ± 0.005	0.041 ± 0.008*
abcc8	(11 ± 4).10^{-5}	(16 ± 4).10^{-5}	(7.5 ± 2).10^{-5}	(28 ± 6).10^{-5}*
abcc9	0.004 ± 0.001	0.014 ± 0.003*	0.013 ± 0.006	0.034 ± 0.012*

[a]Relative gene expressions (mean ± SEM, n = 5). *bactin1* was the reference gene.
[b]The asterisk indicates a significant differential gene expression in zebrafish liver, as determined with the Mann-Whitney U-test, *p < 0.05.

Table 5: *abcb* and *abcc* gene relative expressions in liver after 7 days of metal exposure[a].

fish exposed for 7 days to both Cd and Zn, compared to control fish. This absence of differential Zn bioaccumulation was also observed in zebrafish exposed for 7 days to water from the polluted Riou-Mort River, presenting similar concentrations of Zn and Cd [40]. In contrast, the present results revealed an 8-, 15- and 124-fold increased accumulation of Cd compared to control fish in muscles, liver and gills of fish exposed for 7 days to both Cd and Zn. For fish exposed for 7 days to the water of the Riou-Mort River only gills presented a 10-fold increased accumulation of Cd compared to control fish [40]. This emphasizes the existence of strong homeostatic mechanisms to regulate Zn accumulation inside fish tissues, and also the importance of water properties (ions, particulate matter) on Cd bioavailability.

All the ABC genes transcriptional results are synthesized in a single table in which the metals triggering a differential regulation either up or down are recorded for each of the four tissues tested and for 13 of the most expressed ABCB and C transporter genes analyzed (Table 6). This table underscores the unexpected pattern of expression in muscles. Although no more zinc was accumulated in muscles of exposed fish than in control fish, nine genes over 13 members of the ABC, B and C subfamilies were down-regulated in response to this metal whereas *abcb7* and *abcb8* genes were up regulated. In addition, despite the 7-fold increased Cd burden in muscles, Cd triggered the down-regulation of two members (*abcc2* and *abcc5* also repressed by Zn). The same unexpected pattern appeared in response to Zn in gills in which in absence of a significant increase of Zn, 4 ABC, B and C members were down regulated whereas the *abcb8* gene was up regulated. In the brain the 3-fold increase in Zn burden was accompanied by a down-regulation of three ABC, B and C members. Therefore, it appears that zinc is rather a metal triggering the down-regulation of ABC, B and C member genes even when the tissue Zn burden is null or increased. The only genes for which zinc triggered an up-regulation were *abcb7* and *abcb8*, and so strongly (56-fold for *abcb7* in muscles and 296-fold for *abcb8* in gills) that this suggests the possibility that such an over expression allows to protect mitochondria against metals since the related transporters are embedded within the mitochondrial inner membrane [50-52]. Apart in muscles, the response to cadmium was expected, with the up-regulation of 2, 4 and 6 ABCB and ABCC member genes in brain, gills and liver, respectively. These are *abcb3* (liver), *abcb3l1* (brain), *abcb8* (gills), *abcb9* (brain and liver), *abcc1* (gills and liver), *abcc4* (gills), *abcc5* (liver), *abcc8* (gills and liver) and *abcc9* (liver). In the literature, the reported *abcb* and *abcc* member genes that had been reported to be up regulated by divalent toxic metals in fish and fish cell lines were *abcb1*, *abcc1*, *abcc2*, *abcc3*, *abcc4* and *abcc5* [35-38,53], in other words the MDR1 and MRP1-5 transporters-encoding genes. The over expression of *abcc1* and *abcc5* genes in zebrafish embryos allowed to improve survival against As(III), Cd(II) and Hg(II) [53], and that of *abcc2* allowed to improve survival against Cd(II), Hg(II) and Pb(II) [39]. Therefore it is very surprising to notice in muscles the down-regulation of *abcc1*, *abcc2* and *abcc5* genes triggered by zinc and that of *abcc2* and *abcc5* in response to cadmium.

Here we show that besides *abcb1*, *abcb2* and *abcc1*, *abcc2*, *abcc3*, *abcc4*, and *abcc5* other genes can be up regulated by zinc and cadmium and they are encoding zebrafish homologues of TAP2 (ABCB3 and ABCB3L1), ATM1 (ABCB7), M-ABC1 (ABCB8), TAPL (ABCB9), SUR1 (ABCC8) and SUR2 (ABCC9) transporters. TAP2 is the second subunit of the TAP transporter involved in the translocation into the endoplasmic reticulum lumen of peptides coming from the previous digestion of antigens by the proteasome [54]. TAPL belongs to the TAP family due to its high sequence homology to TAP1 and TAP2. TAPL forms a homo dimer localized in lysosomes with a minor fraction in the ER. It functions as an ATP-dependent peptide transporter [55]. Although TAP and TAPL are involved in the immune response, under stressful life conditions damaged or denatured proteins are targeted to the proteasome and the resulting peptides cannot be distinguished from genuine antigenic peptides. Hence, TAP and TAPL may transport molecular complexes made up by the binding of cadmium to thiol-containing peptides. ATM1 and M-ABC1 are mitochondrial transporters located in the inner membrane [51,52]. ATM1 plays a role in iron homeostasis since a deletion of ATM1 results in the accumulation of up to a 30-fold excess of mitochondrial iron, loss of mitochondrial cytochromes and abnormalities of cytosolic iron metabolism [51]. Therefore, ATM1 and M-ABC1 transporters may protect mitochondria against an excessive cadmium and zinc burden. SUR1 and SUR2 are two major types of sulfonylurea receptor that make a complex with the inwardly rectifying potassium channel (Kir6) to form ATP-sensitive potassium channels. Intracellular levels of adenine nucleotides regulate the channel; with ATP inhibiting and MgADP activating channel activity [56]. However, it has been suggested that the sea urchin ABCB9/SUR2 homologue is more likely involved in MRP-like efflux than SUR-like activity [57]. And in the same species

	Muscles		Gills		Brain		Liver	
Genes[a]	Down[b]	Up[c]	Down	Up	Down	Up	Down	Up
abcb3	Zn[d]		Zn[d]		Zn[d]			Cd
abcb3l1	Zn[d]					Cd		
abcb7		Zn[d]						
abcb8		Zn[d]		Cd, Zn[d]				
abcb9					Zn	Cd		Cd + Zn
abcb10	Zn[d]							
abcb11b	Cd + Zn							
abcc1	Zn[d]			Cd				Cd[d]
abcc2	Cd, Zn[d]		Zn					
abcc4	Zn[d]		Zn[d]	Cd				
abcc5	Cd, Zn[d]		Zn[d]		Cd + Zn			Cd[d]
abcc8	Zn[d]			Cd				Cd + Zn
abcc9								Cd[d]

[a]Genes *abcb4*, *abcc6a*, *abcc7*, and *abcc12* showed no differential expression in response to metals.
[b]Down regulation of gene as compared to control.
[c]Up regulation of gene as compared to control.
[d]The same regulation was also observed in the case of the combined metal exposure.

Table 6: ABCB and ABCC transporter genes for which expression is influenced by metals after 7 days of exposure.

toxicants such as oxybenzone but also organic and inorganic metallic compounds like tributyltin and mercury chloride, triggered the increase in mRNA levels of *abcb1* and *abcc9* genes [58].

Several *abcb* and *abcc* genes were not or were so weakly expressed that no Ct could be recorded. These are *abcb4*, *abcb5*, *abcb11a*, *abcc6a*, *abcc12* and *abcc7*. Maybe these genes are expressed at other developmental stages than during adult life. Indeed, many *abcb* and *abcc* genes displayed different temporal gene expression profiles during the development of sea urchin embryos [59]. In the zebra mussel *Dreissena polymorpha* the *abcc* and *abcb1* homologues presented low RNA levels in eggs but these levels increased 3- and 10-fold, respectively, during the first 2 h post fertilization [60,61]. In zebrafish, the expression levels of the *abcc1*, *abcc2* and *abcc5* genes are also varying during embryogenesis [37,39,53]. In zebrafish embryos, *abcb4* and *abcb5* genes are constitutively expressed during the first 48h, but their expression varied one to two orders of magnitude among developmental stages, and their expressions were very low compared to *b-actin* gene expression that was 3 to 4 orders of magnitude higher [28]. Thus, one cannot exclude the possibility that some ABC genes only play a role during embryogenesis and larval stages and none at adulthood.

In a previous experiment, we reported the 100-fold over expression in gills of *abcb3l1* gene (encoding a TAP-like transporter) after 7 days of exposure of zebrafish to 86 nM Cd [41]. In the present work, after 7 days of exposure to 89 nM Cd, we could not observe the over expression of *abcb3l1* gene in gills but a 3.7-fold up-regulation in brain. The main difference between the experiments is the way water is renewed in the tanks: one-third of the water volume was changed every 2 days in the 2006 experiment versus permanent renewal of water in the present experiment). This results in differences in ionic conductivity of water in tanks. Indeed, in flow-through tanks (allowing a permanent renewal of water which is the case in the present work), a constant water conductivity of 305 ± 10 µS/cm was recorded, whereas in tanks in which water renewal was discontinuous, like in the 2006 experiment, the water conductivity was much higher and equal to 400 ± 10 µS/cm as recorded at days 7. This highlights the necessity to carefully report all the culture conditions because we cannot rule out that even the faintest parameter can exert an influence. This also means that the life conditions are likely to modulate the gene response to toxicants. That is why we began new experiments in which we want to compare the response of zebrafish ABC genes to cadmium and zinc when fish are cultured under two life styles to the antipodes of each other: difficult life style in which fish are fed an hypocaloric diet without water renewal; luxurious life style in which fish are fed a hypercaloric diet with permanent water renewal. In addition, some ABC candidate genes responding to metal contamination are being expressed in a bacterial mutant hypersensitive to metals in order to check whether the corresponding transporters can protect bacteria against a metallic challenge.

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