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RESEARCH ARTICLE

Responses of cytochrome P450, GST, and MXR in the mollusk *Corbicula fluminea* to the exposure to hospital wastewater effluents

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Abstract Pharmaceutical products are a major group of chemical compounds that are continuously released into the environment. The primary pathway of pharmaceuticals to the aquatic environment is the discharge of wastewater effluents. The Psychiatric hospital of Montpon (Dordogne, France) operates with its wastewater treatment plant. We first evaluated the presence and concentrations of 27 pharmaceuticals compounds in these effluents. All of the 27 compounds were detected in these wastewater effluents at concentrations ranging between 37,500 ng L^{-1} (paracetamol) and 150 ng L^{-1} (citalopram). The aim of the study was then to evaluate the exposure effects of the effluents on cytochrome P450, GST, and MXR responses in *Corbicula fluminea* gills and digestive glands. Experiments on clams exposed during 1, 3, 7 14, and 21 days revealed a strong and continuous overexpression of *mdr1* (multidrug resistant 1) gene expression in gills and transitory variations in pi-gst expression and GST activity. EROD activity increased also transitory after 1 day in the digestive gland of exposed clams. These results indicated that in the effluent, some molecules have undergone metabolism of phase 1 and/or phase 2.

Keywords Hospital wastewater effluents · Psychotropics · Freshwater molluscs · *Corbicula fluminea* · Cytochrome P450 · GST · MXR

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Introduction

Freshwater contains many xenobiotics derived from human activities such as pesticides, hydrocarbons, and trace metals. Pharmaceutical products (PPs) are a class of xenobiotics emerging as freshwater pollutants. Human consumption and veterinary activity represent the main source of PPs found in the environment (Jørgensen and Halling-Sørensen 2000). After therapeutic or diagnostic use, PPs and/or their metabolites are excreted into aquatic environments primarily from sewage treatments plants (STP) which cannot always completely eliminate them with a removal rate ranging from 0 to 97 % (Cardoso et al. 2014). They may therefore reach surface water at concentrations range in the nanogram per liter to microgram per liter (Vasieva 2011). These drugs can cause toxic effect even in low nanomolar concentrations; (Cardoso et al. 2014). It is fundamental to evaluate the ecotoxicological risk of these mixtures. Biomarkers involved in detoxification reactions are often used to evaluate the impact of organic pollutants on different aquatic animals. Among these, three biomarkers are particularly used to report an activation of

the detoxification systems. These enzymes participating in the metabolic transformation of xenobiotics are classified into three phases, namely phases I, II, and II pathways. Cytochrome P450s (CYP) are one of the major phase I-type classes of biotransformation enzymes and are generally involved in the functionalization of exogenous and endogenous compounds thereby creating a more polar and water-soluble compound (Nelson et al. 1996; Rewitz et al. 2006). The cytochrome P450 4 (CYP4) family is one of the oldest P450 families, having evolved about 1.25 billion years ago, just after the formation of the steroid biosynthetic genes (Simpson 1997). The CYP4 family is divided into 72 subfamilies and the majority is found in invertebrates. In invertebrates, CYP4 has been identified in several phyla such as Arthropoda, Mollusca, Annelida, and Echinodermata (Snyder 1998). Several studies have demonstrated that they are involved in xenobiotics metabolism. Indeed, *cyp4* expression is modified by different types of organic xenobiotics (pesticides, phenolic compounds, drugs). In *Chironoma tentans*, the *cyp4* gene is induced by atrazin, biocide tributylin (TBTO) and reduced by nonylphenol (alkylphénols) and bisphenol A (Londoño et al. 2004; Martínez-Paz et al. 2012). In Annelida, xenobiotics such as B[a]P and phenobarbital induce the expression of *cyp4* genes (Chen et al. 2012; Rewitz et al. 2004). In Mollusca, *cyp4* cDNA have been cloned in seven marine species *Perna viridis*, *Mytilus galloprovincialis*, *Venerupis philippinarum*, *Cyphoma gibbosum*, and recently in *Ruditapes philippinarium*, *Chlamys farreri*, and *Crassotrea angulata* (Miao et al. 2011; Pan et al. 2011) but little is known about their functions. Induction of cytochrome P450 by xenobiotics exposure can also be studied at enzyme activity level. EROD is commonly used to evaluate CYP1A in vertebrates (Jung et al. 2011; Sureda et al. 2011). But to date, there is no evidence of *cyp1A 1* gene expression in bivalves. Actually, only predicted sequences are available for c*yp1a1* and *a2* or *cyp1-like* cDNA in bivalves *Crassotrea angulata*, *Mytilus californianus*, and *Crassotrea gigas* (Zhang et al. 2012a; Zanette et al. 2010). Therefore, several studies investigate phase I xenobiotic metabolism in invertebrates (polychaetes, bivalves) exposed to organic chemicals in environment with EROD activity (Binelli et al. 2006; Falfushynska et al. 2012; Ramos-Gómez et al. 2011).

Glutathione *S*-transferase (GST) are phase II detoxification enzymes and catalyze the attachment of glutathione (GSH) to a variety of electrophilic species for the elimination of potentially toxic xenobiotics. GST are localized in cytosolic or microsomal fraction of cells and have been grouped in several classes. Among these classes, cytosolic pi-GST are known to be involved not only in drug metabolism but also in antioxidant system by inactivating lipoperoxidation product, lipid hydroperoxide, and their derivates. The pi-GST can also directly inactivate ROS with their SH group (Vasieva 2011). pi-GST has been identified in several bivalves. In *Corbicula fluminea*, *pi-GST* is expressed in digestive glands, excretory systems, and gills (Doyen et al. 2005). In bivalves, *pi-GST* gene expression or GST global activity has been used in several studies to evaluate the impact of environmental stresses (Bigot et al. 2010; Gonzalez-Rey and Bebianno 2011; Rola et al. 2012). An additional crucial step in detoxification is the excretion of xenobiotics and/or their metabolites by the ATP-binding cassette (ABC) transporters named multixenobiotic resistance (MXR) system. They are involved in phase III detoxification pathways and are often used as biomarkers to evaluate exposure effects with several classes of xenobiotics in bivalves.

Bivalve molluscs are relevant species to study the freshwater quality. Indeed they are sedentary and filter large amounts of water to meet their respiratory and nutritional needs. The clam *Corbicula fluminea* is a freshwater bivalve originating from Asia. This species is invasive in many rivers and lakes from North America and Europe and is often used as a biological model in ecotoxicology in freshwater pollution assays (Brandão et al. 2014; Chen et al. 2014). The aim of this study is to assess the molecular responses of *Corbicula fluminea* clams exposed to wastewater from a psychiatric hospital. In this effluent, many drugs and especially neuroleptics are likely to modify *cyp4*, *pi-GST*, and multidrug resistant 1 (*mdr1*) expression and/or CYP and GST activity. In this study, *Corbicula fluminea cyp4* complete cDNA and *mdr1* partial cDNA sequence were characterized. We also determined tissue-specific expression of *mdr1* and *cyp4* genes. Afterwards, *cyp4*, *pi-GST*, and *mdr1* mRNA expression and cytochrome P450 and GST enzyme activity in response to wastewater containing many pharmaceuticals were measured by real-time PCR, EROD, and measure of GST activity.

Materials and methods

Wastewater sampling

The study site is a regionally psychiatric hospital with 250 used beds and 120 places in ambulatory services (Dordogne, France). In 2010, approximately 25 kg of psychotropic drugs were consumed in the hospital. The wastewater of the hospital is discharged to a wastewater treatment plant (WWTP), which treats up to 600 m^3/day. This wastewater treatment plant is composed of a primary decanter, a bacterial filter, and a secondary decanter. This WWTP does not operate continuously. When the volume in the lifting area reaches its maximum, waters fall over by pouring into the primary decanter. So, wastewater effluents are, more or less, always with the same composition.

The wastewater samples are the result of equal mixtures of six discharges of effluent. Samples are collected twice a week during the experiment.

Analysis of pharmaceuticals

The multi-residue analytical procedure was adapted from Togola and Budzinski (2008) and Devier et al. (2013). Briefly, each effluent sample (200 mL) was percolated on Oasis® MCX (60 mg, 3 mL) cartridges. Then cartridges were dried under vacuum and elution consisted in a three-step combination of solvents: 3 mL of ethyl acetate, 3 mL of ethyl acetate/acetone (50/50 *v*/*v*), and 3 mL of methanol/dichloromethane (50/50 *v*/*v*) with 5 % of ammonium hydroxide. After complete evaporation to dryness under a gentle stream of nitrogen, samples were reconstituted with 300 μL ultra-pure water/acetonitrile (90/10 *v*/*v*).

Instrumental analysis was performed using a Waters Ultra Performance Liquid Chromatography (UPLC) ACQUITY coupled to Waters Quattro Premier XE triple quadrupole (Waters, Saint Quentin en Yvelines, France), equipped with a electrospray ionization (ESI). The chromatographic separation was achieved with a Kinetex C18 column (100×2.1 mm; 1.7 μm) in 17 min. Identification of each compound was ensured through the monitoring of two transitions, except for Norfluoxetine where fragmentation pattern gives a unique fragmentation (Table 1, supplementary data). The ion ratio was used as a confirmation criterion.

Instrumental blank and procedural blank samples were performed for each batch experiment in order to prevent any contamination.

In addition, in each batch extraction experiment, Vittel mineral water was spiked with native compounds (200 ng L^{-1}) and extracted as a sample to calculate extraction recoveries. For the majority of the compounds analyzed, the values were greater than 50 %.

The instrumental method was validated through estimation of linearity, repeatability, and sensitivity. Method detection limits (MDLs) were extrapolated from signal to noise ratio (S/N=3) measured on enriched Vittel water samples extracted in triplicate. Thus, the MDLs of the compounds studied ranged from 0.1 to 1.1 ng L^{-1}.

Organisms and wastewater exposure experiment

Corbicula fluminea clams (20 to 30 mm in length) were collected from a local lake (la Roucarié, Carmaux, Midi-Pyrénées, France) and acclimated in the laboratory for 1 week in tanks with dechlorated and aerated tap water with 2 cm of sterile sand substrate at 17 °C under natural photoperiod. While there, clams were fed twice a week with JBL Korall Fluid. For the exposure, clams (115 per tank) were then randomized in three tanks per experiment (control and exposed) with 40 L of dechlorated and aerated water (controls) or effluent (exposed) and 2 cm of sterile sand substrate. The sand had been sterilized by baking at 300 °C for 12 h. For tissue analysis and cloning experiments, clams were collected directly after the acclimatization step. The tank water was changed by half every 2 days with dechlorated and aerated potable water from the hospital for the control tanks or with wastewater for exposed organisms. The concentration of psychotropics in tanks (with and without mollusk) was determined after 2 days of exposure.

RNA isolation and reverse transcription

As regards the exposure experiment, five clams of each condition were sampled at 0, 1, 3, 7, 14, and 21 days according to Bigot et al. (2009). For tissue expression analysis, ten clams were sampled. For cDNA cloning, three clams were sampled. Gills, digestive gland, adductor muscles, and mantle were then immediately dissected. The gills and digestive gland of exposed and control clams were stored at −80 °C in RNA latter. The gills, digestive gland, muscles, and mantle used for tissue expression analysis were processed for RNA extraction immediately after dissection.

Total RNA was extracted using TRIzol® reagent (Invitrogen, USA) according to the manufacturer's protocols. Contaminating genomic DNA was removed using DNA-free™ kit (Applied Biosystem, USA) following the manufacturer's instruction. RNA quantity and purity were evaluated using a nanodrop (NanoDrop 2000, Thermo, USA) and electrophoresis on 1.5 % agarose gel in TBE (pH 8.3 Tris-borate-EDTA). cDNA was synthesized from 1 μg of total RNA extracted from the digestive gland for cDNA cloning experiments and from 500 ng of total RNA for the real-time PCR experiment by M-MLV reverse transcriptase (Invitrogen, USA) at 37 °C for 50 min with random hexamers (Invitrogen, USA) following the protocol of the manufacturer.

cyp4 and *mdr1* cDNA cloning

An internal fragment of *cyp4* and *mdr1* cDNA was amplified using degenerated primers (CYP4for, CYP4rev, Mdr1Fow and Mdr1rev, see Table 1). CYP4 primers were designed based on CYP4 amino acid sequences alignment of *P. viridis* (B1A7T0), *Mytilus galloprovincialis* (O76505), *V. philippinarum* (B9VAW8), *Chlamys farreri* (B8YQ60), *Unio tumidus* (Q6QWQ4), *Mytella strigata* (Q2PPJ4), *Mactra veneriformis* (D3J8D8), and *Modiolus modiolus* (C6EQB8) and *Cyphoma gibbosum* (B3FYF8). *Mdr1* primers were designed based on MDR1 amino acid sequence alignment of *Mus musculus* (P06795), *Homo sapiens* (P08183), *Caenorhabditis elegans* (P34712), and *Mytilus galloprovincialis* (G4RJB1). The primers were obtained from Invitrogen (Fisher Bioblock, France). The PCR reaction was performed in a total volume of 25 μL using thermocycler (Multigene-gradient, Labnet) containing recombinant taq DNA polymerase (Invitrogen, USA), 2 mmol L^{-1} $MgCl_2$, 2 μmol L^{-1} of each primer, and 2 μL of cDNA. Gene

Table 1 Primers used for *cyp4* and *mdr1* mRNA isolation and expression analysis

Primer name	Primer sequence	Product length
Mdr1For	GGSTGYGGNAAAWSTACA	327 bp
Mdr1rev	GCTTGTTTCTGYCCNCC	
Mdr1ForDef	AGTGGTTGAGACGGCAGATT	177 bp
Mdr1revDef	CATATCCCTGAGGCAAGGAA	
CYP4For	TGTTYGARGGBCAYGAYAC	~400 bp
CYP4rev	TGWCCDATRCARTTTCYTG	
CYP4ForDef	TTGGTGGGAGTGACAGAATG	192 bp
CYP4RevDef	GAATAGCTGCCGGTGAAATC	
CYP43'RACEgsInner	ATTTTCTGCTGGTGCCAGG	
CYP43'RACEgsOuter	ACATCCGT TTGCTTACATACC	
CYP45'RACEgsInner	GGCCAAATACTGGCACTGAT	
CYP45'RACEgsOuter	GAATAGCTGCCGGTGAAATC	
piGSTFor	CAACACCGAATCTACGACCA	206 bp
piGSTRev	TACGTCCAGATCTCCGTCCT	
EF1alphaFor	CGTTGGTGTCAACAAGATGG	202 bp
EF1alphaRev	TACAGCCCAACCCTTGTACC	

amplification was carried out as follow: 95 °C for 5 min; 40 cycles in three steps: 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min; 72 °C for 10 min. The amplification products were analyzed on 1.5 % agarose gel. The target gene bands were purified using agarose gel DNA purification kits (Invitrogen, USA). The fragments were cloned into pCR®II vector using TA-cloning® Kit Dual promoter (pCR®II) (Invitrogen, USA). Vectors containing cloned insert were transformed into One shot®INVαF' chemically competent *Escherichia coli* and incubated overnight at 37 °C. Positive clones were screened by blue/white selection, verified by EcoRI digestion, and then sequenced with M13 reverse and forward(-20) primers (Get-TQ, Purpan, Toulouse, France).

Amplification of *cyp4* cDNA ends

Full-length *cyp4* cDNA was obtained using FirstChoice® RLM-RACE Kit (Invitrogen, USA) according to the manufacturer's instructions. Gene-specific primers (Table 1) were designed from the partial *cyp4* cDNA sequence. For 5′ RACE, PCR was performed as follow: 3 min at 95 °C followed by 7 cycles of 95 °C for 30 s, 65 °C for 1 min minus 1 °C per cycle, 72 °C for 1 min, followed by 33 cycles of 95 °C for 30 s, 58 °C for 1 min and 72 °C for 1 min with an increase of 20 s per cycle, then an additional extension time for 10 min. For 3′ RACE, PCR was performed as follow: 5 min at 95 °C followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min then an additional extension time for 10 min. Specific 1,200 pb 5′ PCR and 600 pb 3′ PCR products were then purified after gel migration with MinElute Gel Extraction Kit (Qiagen, Netherlands). The RACE products were cloned using the same TA-cloning® protocol described above and sequenced with M13 reverse and forward(-20) primers (Get-TQ Purpan, Toulouse, France).

Sequence analysis

Partial and complete sequences were analyzed using Mobyle pasteur site (http://mobyle.pasteur.fr/). The amino acid sequences were deduced using Transeq software. The homology searches of nucleotides and protein sequences were conducted with Blast program on NCBI and Uniprot (http://www.ncbi.nlm.nih.gov/, www.uniprot.org/).

Quantification of *cyp4*, *pi-gst*, *mdr1* gene expression after wastewater exposure

For *cyp4*, *mdr*1, and *pi-gst* primer design, we used Primer3 software. Primers were designed to obtain about 200 pb cDNA-specific product. The primers were first tested by real-time PCR to verify their efficiency. The efficiency of all the primers used was around 100 % (96 % for *pi-gst* primers, 104 % for EF1alpha primers and 103 % for *cyp4* primers, 98 % for *mdr1* primers). The expression of *cyp4*, *pi-gst*, and *mdr1* mRNAs in gills and digestive gland was quantified using a SYBR Green real-time PCR method. The real-time PCR was carried out in replicates on 96-well STEP-one+ (Applied biosystem) and analyzed with StepOne+v2.2.2 software. The PCR reactions were performed in a final volume of 12 μL containing 6 μL of 2× EvaGreen® qPCR Real Time Master Mix Plus (Biorad), 2 μL of cDNA template, 0.6 μL of 10 μmol L^{-1} of each primer (Table 1), and 2.8 μL of ultrapure water. Elongation factor 1-alpha (*Ef1alpha*) was chosen as an internal gene reference to normalize the expression level between samples. *Ef1alpha* primers are listed in Table 1 and are the same as in Martins et al. (2011). All data were relatively calculated and expressed for *Ef1alpha* to compensate any difference in initial RNA quantity and in Reverse transcriptase efficiency. The relative gene expression fold change was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Tissues expression analysis

We proceeded by following the same protocol described above.

Enzymatic activities

The gills, digestive gland, mantle, and muscles of the molluscs were dissected. Pools were made with the tissues of three organisms. Each pool of tissue was homogenized in 100 mM phosphate buffer; pH 7.4 containing 250 mM sucrose, 100 mM KCl, 1 mM EDTA, and protease inhibitors

Fig. 1 Nucleotide and deduced amino acid sequences of *Corbicula fluminea* Clam cyp4 (accession number: JQ678818)

according to commercial advice (SIGMA FAST, S8820) using TissueLyser II (Qiagen) with 25 frequencies/s for 3 min. Eight pools were prepared and aliquots of each homogenate were sampled for total protein, GST, and EROD analysis. In the GST fraction, dithiothreitol was added (100 mM). Aliquots were frozen at −80 °C until analysis. For GST, EROD, and protein analyses, samples were defrosted and centrifuged at 15,000×*g* for 30 min and at 4 °C. Pellets were discharged and supernatants were used in the enzymatic assays.

EROD activity

EROD activity was measured using the method described by Gagné and Blaise (1993) with ethoxyresorufin as substrate. The reaction was initiated by the addition of NADPH. The produced 7-hydroxyresorufin was recorded fluorometrically (λ_{ex}=520 nm; λ_{em}=590 nm) for 120 min. Each pool was analyzed in triplicate and EROD activity was expressed as picomole per minute per milliliter (volumetric acidity) or picomole per minute per milligram proteins (specific activity).

Glutathione *S*-transferase activity

GST activity was measured using the method described by Habig et al. (1974) with 1-chloro-2,4-dinitro-benzene (CDNB) as substrate of spectrophotometrical measurement at 340 nm and 37 °C.

Protein content was measured according to Bradford (1976) using bovine serum albumin (BSA) as standard.

Statistics

For both enzyme activity and RNA quantification, the equality of variances and normality were tested (using Bartlett test and

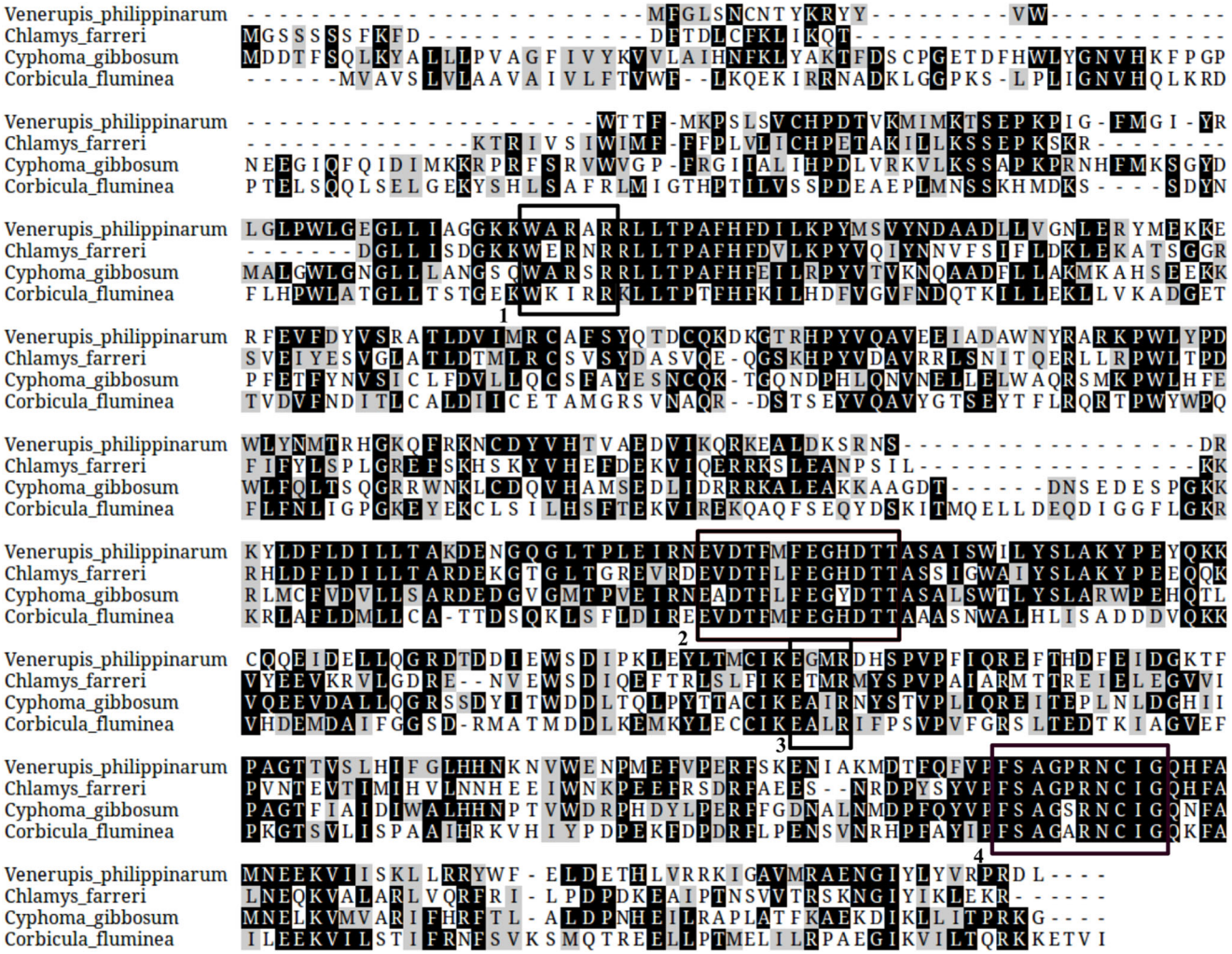

Fig. 2 Multiple alignments of deduced amino acid sequences of *Corbicula fluminea* clam with other known mollusca CYP4: *V. philippinarum* (B9VAW8), *Chlamys farreri* CYP4 (B8YQ60), *Cyphoma gibbosum* (B3FYF4). *First box*: WxxxR: (C-helix). *Second box*: characteristic of the family 4 cytochrome p450, not shared by other CYP genes. (GxxTT part of helix-I). *Third box*: ExxR (K-helix, heme stabilization) The four box represent the heme binding site: FxxGxxxCxG

Shapiro-Wilk normality test, respectively). Differences among transcript levels in different organs and enzyme activity among control and treated groups relative to the controls and time exposure were evaluated using one-way analysis of variance (ANOVA), followed by Tukey's test for multiple comparisons. Differences in RNA quantification among control and contaminated groups were evaluated using non-parametric Mann–Whitney test. The probability level for statistical significance was $P<0.05$. All statistical analysis was performed using R software version 2.14 (www.r-project.org/).

Results

Cloning and sequence analysis of *cyp4* cDNA

cyp4 cDNA was amplified using degenerated primers CYP4For and CYP4Rev (Table 1) which are based on highly conserved regions of mollusca CYP4 proteins. A complete cDNA sequence was then obtained using RACE PCR strategy. The full length of mRNA is 1,946 bp with an open reading frame of 516 amino acid (GenBank accession number: JQ678818) (Fig. 1). A blast search on uniprot revealed that this sequence was similar to those of Cytochrome p450 family 4 of other molluscs and other vertebrates or invertebrates with a higher homology with cytochrome p450 4 V2 subfamily of *Crassostrea gigas* (65 %, E value=0) and cytochrome p450 family variant 2 (CYP4V10 gene) of *Cyphoma gibbosum* (53 %, E value=0). An alpha helice was predicted at the N terminal end (probably the signal sequence of import in endoplasmic reticulum). Alignment with other mollusc CYP4 revealed many conserved motifs on *Corbicula fluminea* CYP4 (Fig. 2). A typical heme-binding motif takes place near the C-terminus FxxGxxxCxG (F^{450}SAGARNCIG^{459}) followed by a QK(F/Y)AxLE sequence specific to family 4 (Q^{460}KFAIL^{465} for *Corbicula fluminea* CYP4 protein). A typical WxxxR motif is also present near the N-terminus (W^{125}KIRR^{129}) together with the consensus sequence GxxT located in the I-helix and the 13 invariant amino acid residues E^{312}VDTFMFEGHDTT^{324} unique to family 4. Finally, the charge pair consensus motif ExxR (E^{377}ALR^{380}) is found within the K helix. The predicted 2D structure reveals the potential existence of 12 alpha-helix and 5 beta-sheet (data not shown). Phylogenetic analysis with 25 CYP4 complete amino acid sequences of molluscs, *Drosophila melanogaster*,

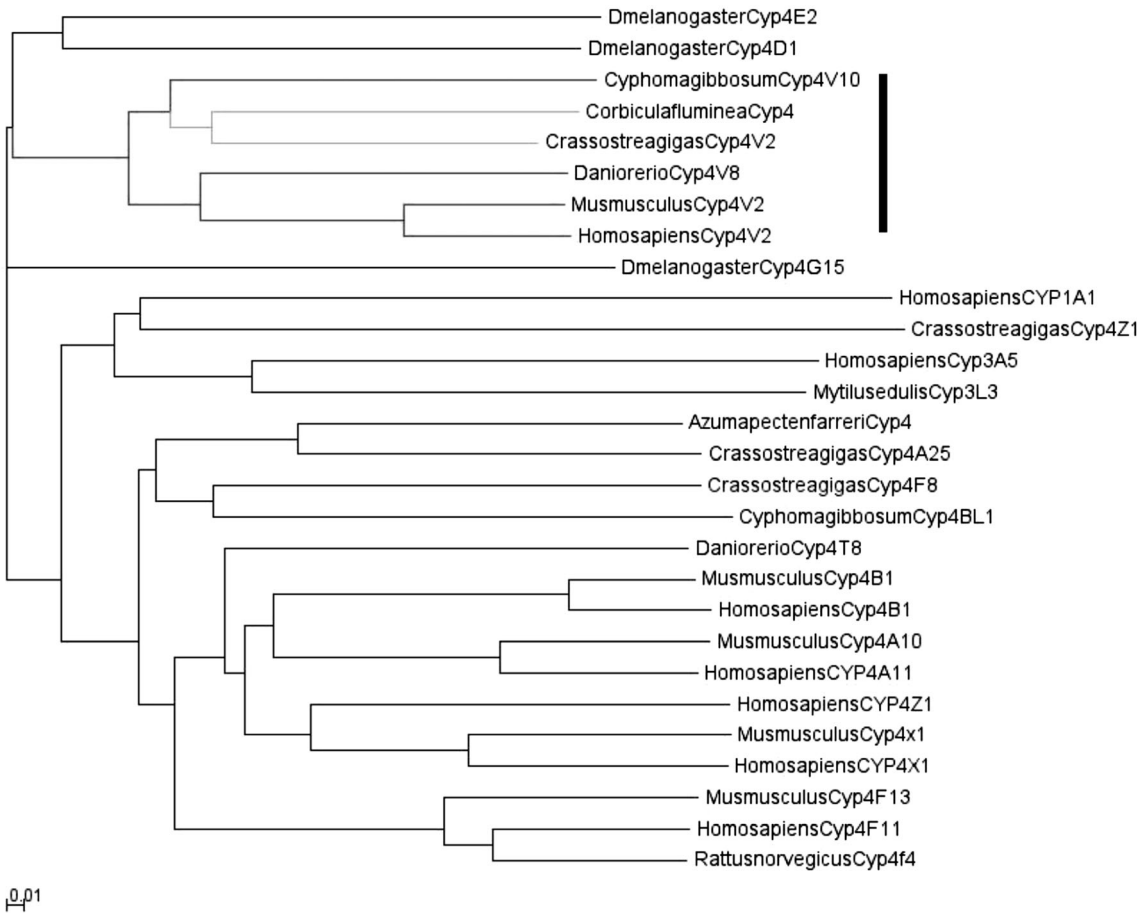

Fig. 3 Neighbor joining phylogenetic tree of *Corbicula fluminea* deduced amino acid sequence CYP4 and other CYP4 sequences

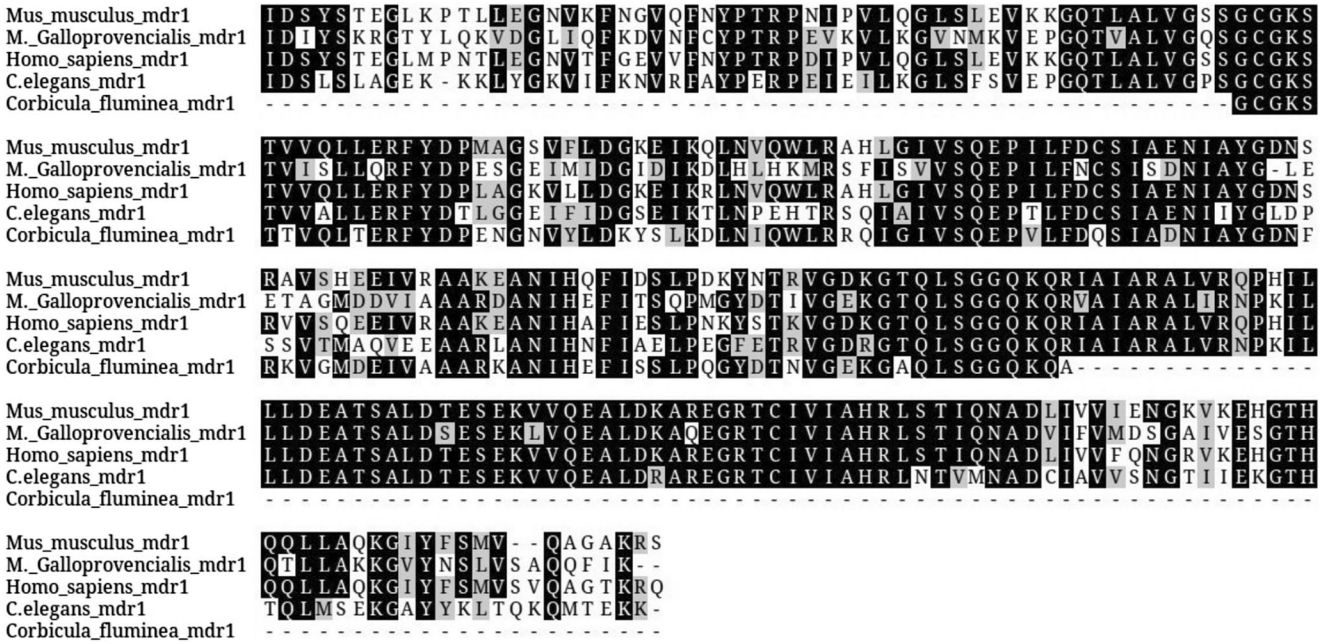

Fig. 4 Multiple alignments of MDR1 deduced amino acid sequences of *Corbicula fluminea* clam with other known MDR1: *Mus musculus* (P06795), *Mytilus galloprovencialis* (G4RJB1), *H. sapiens* (P08183), and *Caenorhabditis elegans* (P34712)

Mus musculus, *H. sapiens*, and *Danio rerio* revealed that *Corbicula fluminea* CYP4 shows more similarities with CYP4V subtype of *Cyphoma gibbosum* and *Crassostrea gigas* but also with the CYP4V subtypes of vertebrate phylum (Fig. 3).

Cloning and sequence analysis of partial *mdr1* cDNA

We cloned a 333-bp *mdr1* cDNA fragment encoding a predicted ORF of 111 amino-acid. A blast on uniprot databank revealed that this sequence was similar mdr1 of many

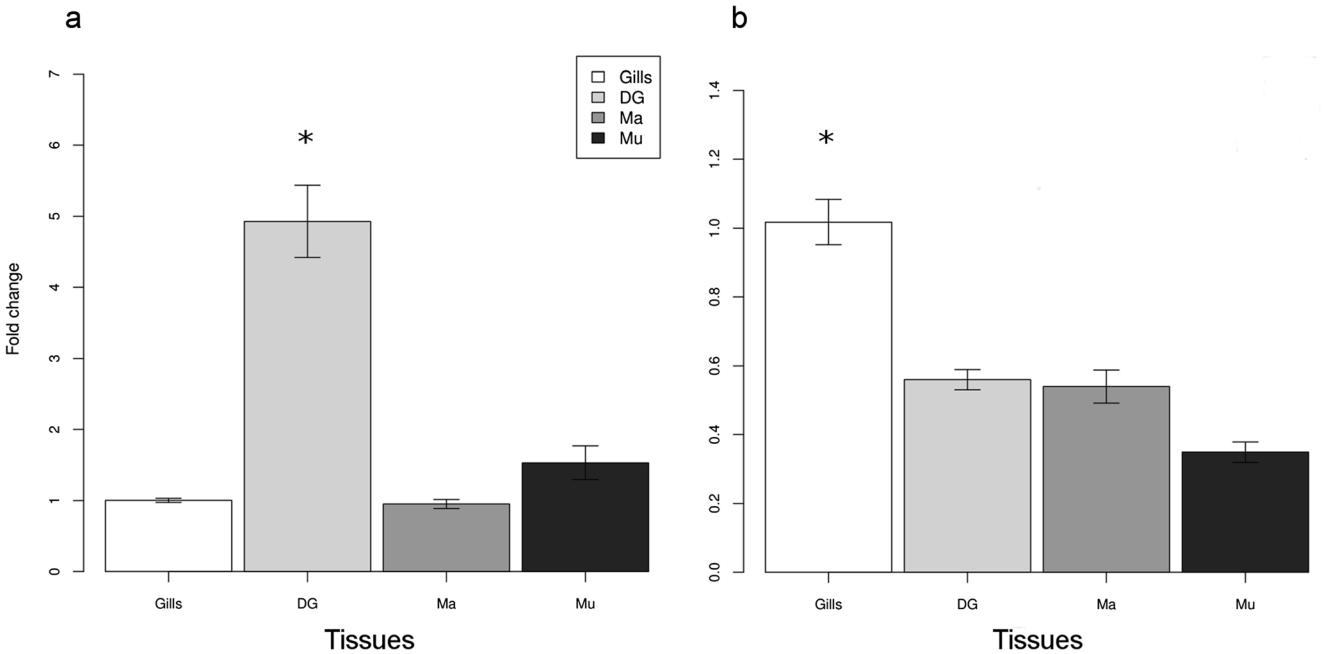

Fig. 5 Expression changes of *cyp4* (**a**) and *mdr1* (**b**) genes in digestive gland (*DG*), mantle (*Ma*), and adductor muscles (*Mu*) compared to gills. Levels of expression are measured by EvaGreen quantitative real-time PCR, *cyp4* and *mdr1* expression is measured relative to EF1alpha expression. Values are represented as means±SE of relative expression ratio between digestive gland, mantle, or adductor muscles and gills. Significant differences from all the other tissues are indicated with an *asterisk* (ANOVA followed by Tukey's test, p value <0.001)

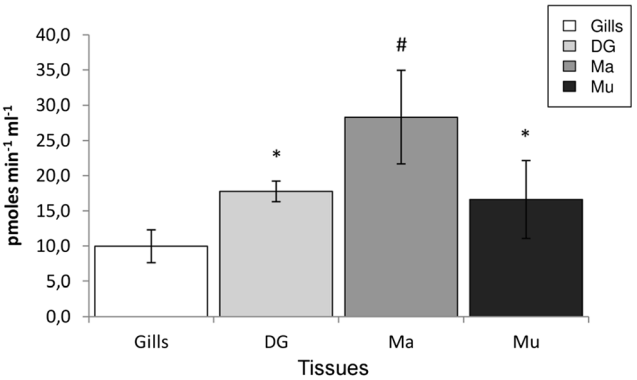

Fig. 6 Tissular distribution (gills, mantle, digestive gland, and muscles) of EROD activity in *Corbicula fluminea*. *Asterisk* and *hashtag*: EROD activity was significantly different in the tissue (ANOVA followed by Tukey's test, p value <0.05)

bivalves, *V. philippinarum*, *Crassostrea gigas*, *Mytilus galloprovencialis*, around 80 %. Figure 4 shows alignment of *Corbicula fluminea* MDR1 with other bivalve MDR1.

Tissue distribution of *cyp4* and *mdr1* mRNA

We evaluated the tissue distribution of *cyp4* and *mdr1* mRNA in clams. We tested the level of expression in gills, digestive gland, adductor muscles, and mantle. The results are shown in Fig. 5. As expected, the expression level of *cyp4* was significantly higher in digestive gland (fourfold, p value <0.001) than in other tissues. No significant difference was observed between the levels of expression in mantle, adductor muscles, and gills, despite a slightly higher expression in muscles. For *mdr1*, we observed the higher expression level in gills (twofold, p value <0.001). No significant difference was observed between expression level in mantle and adductor muscles.

Tissular EROD activity

The results of tissular EROD activity are presented in Fig. 6. Cytosolic EROD activity was assessed in mantle, gills, muscles, and digestive gland. Mantle exhibited the highest value followed, in the order, by the digestive gland, muscles, and gills. By using a ANOVA post hoc Tukey HSD test, significant differences were found between any pairs of tissues except between digestive gland and muscles ($p<0.05$).

Pharmaceutical concentrations in effluents after mollusk exposure

The psychotropic concentrations in the effluent used for the experiment (at T0, immediately after introduction to the tanks, and after 72 h (with or without *Corbicula fluminea*)) are presented in Table 2.

The results obtained after 24 and 72 h showed significant decreases in the concentrations of all compounds when compared to those at T0. However, we observed that after 72 h of exposure and before the change of water, psychotropics were still concentrated in effluent. Mollusks did not totally accumulate or did not degrade pharmaceuticals in 72 h and were always in touch with pharmaceuticals.

Quantification of *cyp4*, *pi-gst*, and *mdr1* mRNA expression in gill and digestive gland after wastewater exposure

We studied the expression pattern of *cyp4*, *pi-gst*, and *mdr1* mRNA in clams tissue exposed to wastewater from a psychiatric hospital's sewage treatment plant. In gills and digestive gland, we observed no significant difference in *cyp4* expression between exposed and unexposed clams (Fig. 7). *Pi-gst* gene expression was significantly decreased after 14 days

Table 2 Psychotropic concentrations in the effluent used for the experiment—at T0, after 24 h, and 72 h with or without *Corbicula fluminea*

	Effluent composition (T0) ng L^{-1}	Effluent composition (T=24 h) without *C. fluminea* ng L^{-1}	Effluent composition (T=72 h) without *C. fluminea* ng L^{-1}	Effluent composition (T=24 h) with *C. fluminea* ng L^{-1}	Effluent composition (T=72 h) with *C. fluminea* ng L^{-1}
Citalopram	24.7	5.8	2.5	4.8	2.9
Carbamazepine	2743.5	2339.4	375.2	431.2	442.9
Cyamemazine	141.7	66.2	18.7	29.3	21.1
Hydroxyzine	4.8	<LD	0.6	0.6	<LD
Oxazepam	6,879.6	6,751.6	1,508.8	1,920.9	2,030.0
Norfluoxetine	<LD	7.4	1.3	5.1	2.1
Lorazepam	<LD	<LD	<LD	<LD	<LD
Fluoxetine	13.2	16.8	9.0	20.1	11.3
Sertraline	<LD	<LD	<LD	<LD	<LD
Diazepam	34.2	20.5	16.4	24.6	26.4

(−0.64, ±0.07, p value <0.05) of exposure in gills and showed no significant variation in digestive gland. *Mdr1* gene expression was significantly overexpressed in gills during the whole experiment (×4.86±1.2; × 13.13±3.7; × 7.76±1.75; × 4.96± 0.53; × 3±0.3; p value <0.01). In the first times of exposure, individual responses were variables but always significantly increased. At 14 and 21 days of exposure, these individual variations were lower and overexpression maintained in exposure context. *Mdr1* expression was also significantly upregulated in digestive gland at 3 days of exposure (×4.4, ±1.7 p value <0.05).

EROD and GST activity after wastewater exposure

We evaluated EROD activity in the digestive gland of exposed and unexposed clams. The results are presented in Fig. 8. After 24 h of exposure, EROD activity in *Corbicula fluminea* increased significantly in exposed clams when compared with

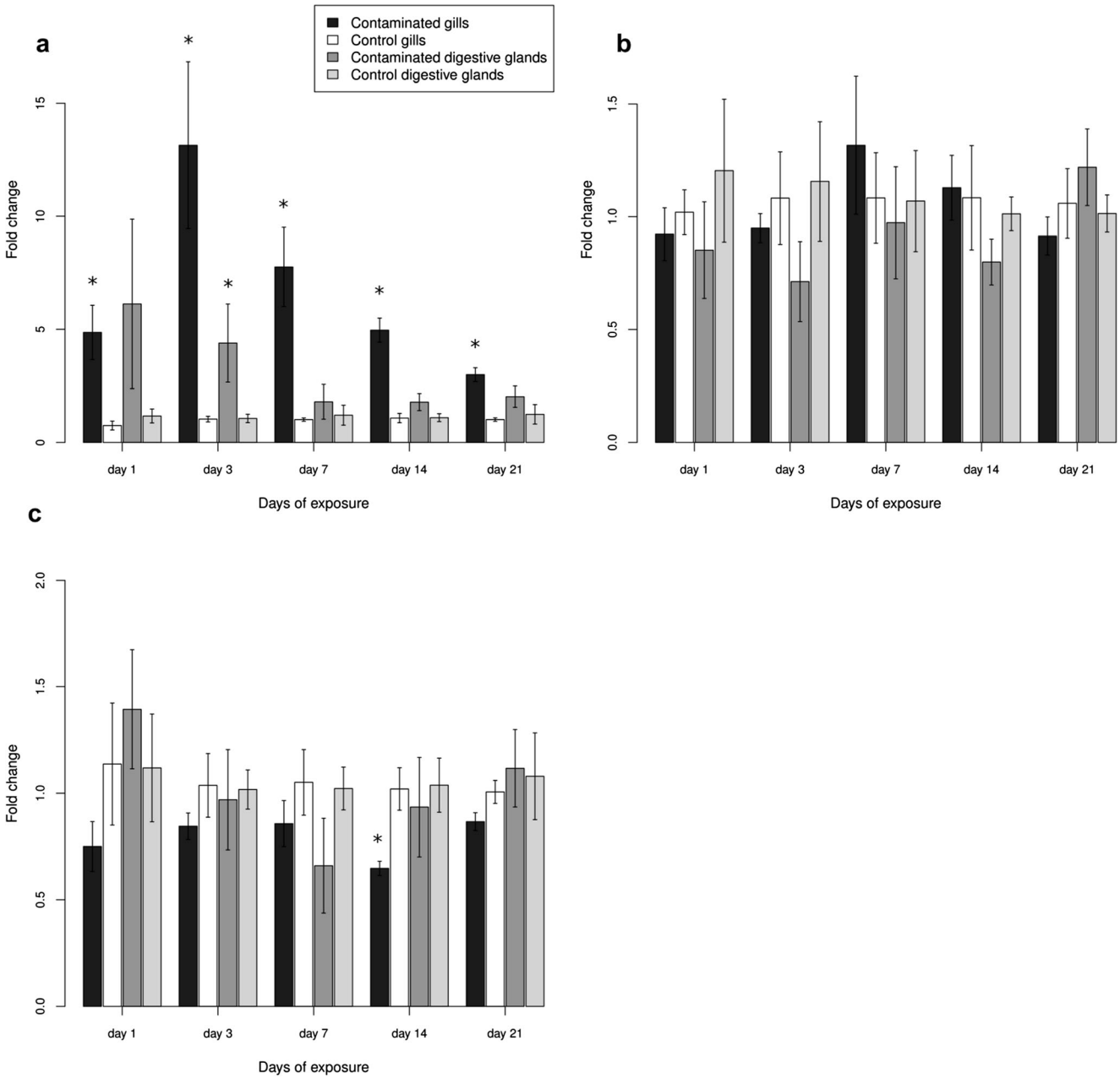

Fig. 7 Expression changes of *mdr1* (**a**) *pi-gst* (**b**), and *cyp4* (**c**) in digestive gland and gills of unexposed clams at 1, 3, 7, 14, and 21 days of experiment. Levels of expression are measured by EvaGreen quantitative real-time PCR and *mdr1*, *pi-gst*, and *cyp4* expression is measured relative to EF1alpha expression. Values are represented as means±SE of relative expression ratio between control sample at 3, 7, 14, and 21 and control sample at 1 day. Significant differences from control in the same time and tissue are indicated with an asterisk (Mann–Whitney non-parametric test, p value <0.05)

control. No significant difference was observed between the exposed clams and controls for the other duration of exposure. GST activity was followed in gills and digestive gland of exposed and control *Corbicula fluminea*. GSTs activity in gills of *Corbicula fluminea* was significantly enhanced when compared with control clams after 14 days of exposure. In the digestive gland, GSTs activity decreased significantly compared to the controls after 3 and 7 days of exposure and increased after 21 days of exposure.

Discussion

We identified full-length cDNA of a cytochrome p450 family 4 member in *Corbicula fluminea*. Sequence analysis reveals that *Corbicula fluminea* CYP4 protein exhibits a high degree of conservation of consensus motifs specific to other cytochrome p450 family 4 in particular the heme motif binding site near the C-terminus and the conserved 13 amino acid residue E^{312}VDTFMFEGHDTT^{324} of the I-helix. The typical E^{319} residue is known to bind covalently the heme group in certain member of CYP4 family through an ester linkage (Baer et al. 2005). This covalently link with the heme group is unique in family 4. Indeed the other cytochrome p450 bind their heme with non-covalent link. C*yp4* gene is expressed in all tissues analyzed but it expression level is significantly higher in digestive gland, which is consistent with results obtained in *Ruditapes philippinarum* (Pan et al. 2011). The digestive gland is known to be the major place for detoxification pathways in molluscs (Zanette et al. 2013). We can hypothesize that CYP4 is involved in these processes in *Corbicula fluminea* digestive gland. However, EROD activity is higher in the mantle than in the digestive gland. So, EROD is not only correlated to the activity of CYP4 in molluscs. Cytochromes p450 are involved in the metabolism of steroids in molluscs (Fernandes et al. 2011). However, sterols are distributed mainly in the mantle and digestive gland of the bivalve mollusc *Scapharca inaequivalvis*. In February, when the experiment was conducted, the sterol quantity was higher in the mantle than in the digestive gland of S. inaequivalvis (Piretti and Pagliuca 1989). EROD may reflect the activity of several cytochromes P450 involved in this metabolism.

After clam exposure to wastewater, no variation of *cyp4* gene expression was observed in gills or digestive gland. In *U. tumidus*, no change in *cyp4* gene expression was observed after exposure to diethylhexylphthalate (DEHP) known to be an inducer of *cyp4* in vertebrate (Chaty et al. 2004). In *Ruditapes philippinarum*, *cyp4* gene shows a significant increase only after 10 days of exposure with 0.2 μg L^{-1} of BaP and no variation with 0.01 g/L of BaP (Pan et al. 2011). In other bivalves as *Chalmys farreri*, exposure to BaP at the same doses induced a high decrease in *cyp4* gene expression at 3 and 10 days of exposure. Hence, the response of *cyp4* gene expression in bivalves appears to be very variable. Interestingly, when we analyzed the cytochrome p450 global activity, we showed that at 1 day of exposure this activity was significantly increased in the digestive gland of exposed clams. At all the other times, EROD activity showed no significant variation when compared to control. Indeed, cytochrome p450 response is very early and transitory. Usually EROD activity is modified during all exposure time with organic pollutants (Curtis et al. 2011; Martín-Díaz et al. 2009) but some authors also showed a transitory response of EROD activity in case of exposure with different xenobiotics (PaB, PCP, pharmaceutics compounds, caffeine) in several organisms (bivalves, fishes) (Binelli et al. 2006; Li et al. 2012; Monari et al. 2009; Thibaut and Porte 2008).

In gills, *pi-gst* is significantly downregulated after 14 days of exposure therefore no significant variation is observed in digestive gland. We have also observed

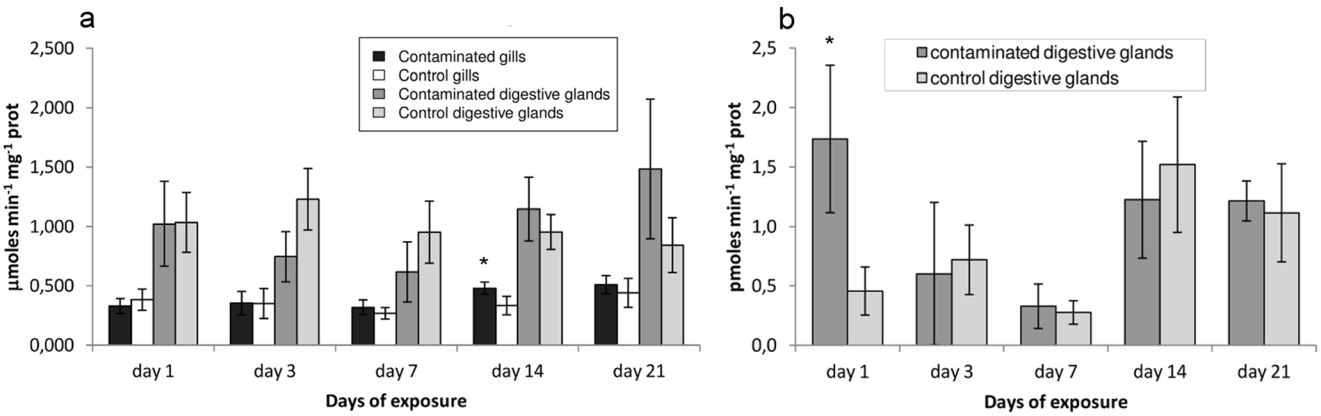

Fig. 8 Mean variation (±SD) of GST (**a**) and cytochrome p450 (**b**) activities in digestive gland and gills of *Corbicula fluminea* exposed to effluent for 1, 3, 7, 14, and 21 days. Significant difference between control and exposed clams is indicated with an *asterisk* (ANOVA followed by Tukey's test, p value <0.05)

changes in GST activity from 3 days of exposure. The GSTs activity decreases at 3 and 7 days of exposure and increases at 21 days in digestive glands and at 14 days in gills. So the wastewater exposure induced changes in enzyme activities in both gills and digestive glands. The effluent from the wastewater treatment plant can be an inhibitor of GST. Gagné et al. showed this type of effect in mussels exposed to wastewater effluent for 7 weeks (Gagné et al. 2007). The metabolic activity of cytochrome p450 may cause the production of metabolites inhibiting GST. It has been shown that the metabolites of exogenous estradiol inhibited human GST (Tian et al. 2012). Moreover, the metabolism of paracetamol or carbamazepine using cytochrome p450 produces quinone (Ju and Uetrecht 1999) that may inhibit GST (van Ommen et al. 1991).

cyp4 and *pi-gst* gene expression cannot be linked to enzyme activity. The difference between variation of gene expression and enzyme activity may be due to the gap in gene expression time and protein activity time and to the multiple points of regulation that may occur between translational level and enzyme activity level. In the case of *cyp4* gene expression, it will be necessary to test an anterior time as in Anwar-Mohamed and El-Kadi (2008).

Another possibility to explain these differences is that the protocols used to evaluate enzyme activity measure the global cytochrome p450 or GST families activity. This can explain this absence of correlation between the two types of analysis. The changes observed in GST or cytochrome p450 activity could be due to another class of GST such as GST-O, GST-S, or GST-M or to another family of cytochrome p450 such as cytochrome p450 family 3, 2, or 1-like that are also expressed in gills or digestive gland of other bivalves (Umasuthan et al. 2012; Zanette et al. 2013; Zhang et al. 2012b). For example, in *Ruditapes philippinarum*, Cyp3a65 and Cyp3u2 are upregulated in gills after 3 days of exposure to 1,000 μg L^{-1} of ibuprofen (Milan et al. 2013).

Mdr1 was expressed in all examined tissues but higher levels were found in gills. *Mdr1* gene shows also a strong overexpression in gills of exposed clams compared to control. MXR are members of the phase III system and are involved in excretion of xenobiotics or conjugated metabolites (Luedeking and Koehler 2004). In aquatic mollusks, gills are the first tissue in contact with xenobiotics. Several authors showed the presence of MXR in the gills of bivalve molluscs (Galgani et al. 1996; Keppler and Ringwood 2001; Minier et al. 2000). Achard et al. (2004) related the induction of MXR in gills of Corbicula fluminea exposed to cadmium. These results revealed that MXR plays an important role in detoxification pathway after wastewater exposure and that *mdr1* overexpression is an indicator of pollution exposure.

In conclusion, wastewater exposure modified expression and/or activity of phase I, II, and III proteins in both organs (gills and digestive gland). Except for *mdr1* that is permanently overexpressed in gills, others biomarkers are very transitory increased or decreased either at transcriptional or enzymatic activity levels.

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Conflict of interest The authors declare that they have no conflict of interest.

References

Achard M, Baudrimont M, Boudou A, Bourdineaud JP (2004) Induction of a multixenobiotic resistance protein (MXR) in the Asiatic clam *Corbicula fluminea* after heavy metals exposure. Aquat Toxicol 67: 347–357. doi:10.1016/j.aquatox.2004.01.014

Anwar-Mohamed A, El-Kadi A (2008) Down-regulation of the carcinogen-metabolizing enzyme cytochrome P450 1a1 by vanadium. Drug Metab Dispos 36:1819–1827. doi:10.1124/dmd.108.021154.nase-3

Baer BR, Schuman JT, Campbell AP et al (2005) Sites of covalent attachment of CYP4 enzymes to heme: evidence for microheterogeneity of P450 heme orientation. Biochemistry 44:13914–13920. doi:10.1021/bi051267j

Bigot A, Doyen P, Vasseur P, Rodius F (2009) Metallothionein coding sequence identification and seasonal mRNA expression of detoxification genes in the bivalve *Corbicula fluminea*. Ecotoxicol Environ 72:382–387. doi:10.1016/j.ecoenv.2008.04.014

Bigot A, Vasseur P, Rodius F (2010) SOD and CAT cDNA cloning, and expression pattern of detoxification genes in the freshwater bivalve *Unio tumidus* transplanted into the Moselle river. Ecotoxicology 19: 369–376. doi:10.1007/s10646-009-0419-x

Binelli A, Ricciardi F, Riva C, Provini A (2006) New evidences for old biomarkers: effects of several xenobiotics on EROD and AChE activities in Zebra mussel (Dreissena polymorpha). Chemosphere 62:510–519. doi:10.1016/j.chemosphere.2005.06.033

Brandão FP, Pereira JL, Gonçalves F, Nunes B (2014) The impact of paracetamol on selected biomarkers of the mollusc species *Corbicula fluminea*. Environ Toxicol 29:74–83. doi:10.1002/tox.20774

Cardoso O, Porcher J-M, Sanchez W (2014) Factory-discharged pharmaceuticals could be a relevant source of aquatic environment contamination: review of evidence and need for knowledge. Chemosphere 115:20–30. doi:10.1016/j.chemosphere.2014.02.004

Chaty S, Rodius F, Vasseur P (2004) A comparative study of the expression of CYP1A and CYP4 genes in aquatic invertebrate (freshwater mussel, *Unio tumidus*) and vertebrate (rainbow trout, *Oncorhynchus mykiss*). Aquat Toxicol 69:81–94. doi:10.1016/j.aquatox.2004.04.011

Chen X, Zhou Y, Yang D et al (2012) CYP4 mRNA expression in marine polychaete *Perinereis aibuhitensis* in response to petroleum hydrocarbon and deltamethrin. Mar Pollut Bull 64:1782–1788. doi:10.1016/j.marpolbul.2012.05.035

Chen H, Zha J, Liang X et al (2014) Effects of the human antiepileptic drug carbamazepine on the behavior, biomarkers, and heat shock proteins in the Asian clam *Corbicula fluminea*. Aquat Toxicol 155:1–8. doi:10.1016/j.aquatox.2014.06.001

Curtis L, Garzon C, Arkoosh M (2011) Reduced cytochrome P4501A activity and recovery from oxidative stress during subchronic benzo pyrene and benzo pyrene treatment of rainbow trout. Toxicol Appl 254:1–7. doi:10.1016/j.taap.2011.04.015.Reduced

Dévier M-H, Le Menach K, Viglino L et al (2013) Ultra-trace analysis of hormones, pharmaceutical substances, alkylphenols and phthalates in two French natural mineral waters. Sci Total Environ 443:621–632. doi:10.1016/j.scitotenv.2012.10.015

Doyen P, Vasseur P, Rodius F (2005) cDNA cloning and expression pattern of pi-class glutathione *S*-transferase in the freshwater bivalves *Unio tumidus* and *Corbicula fluminea*. Comp Biochem Physiol C Toxicol Pharmacol 140:300–308. doi:10.1016/j.cca.2005.02.013

Falfushynska HI, Gnatyshyna LL, Golubev AP, Stoliar OB (2012) Main partitioning criteria for the characterization of the health status in the freshwater mussel *Anodonta cygnea* from spontaneously polluted area in Western Ukraine. Environ Toxicol 27:485–494. doi:10.1002/tox.20663

Fernandes D, Loi B, Porte C (2011) Biosynthesis and metabolism of steroids in molluscs. J Steroid Biochem Mol Biol 127:189–195. doi:10.1016/j.jsbmb.2010.12.009

Gagné F, Blaise C (1993) Hepatic metallothionein level and mixed function oxidase activity in fingerling rainbow trout (*Oncorhynchus mykiss*) after acute exposure to pulp and paper mill effluents. Water Res 27:1669–1682

Gagné F, André C, Cejka P et al (2007) Toxicological effects of primary-treated urban wastewaters, before and after ozone treatment, on freshwater mussels (*Elliptio complanata*). Comp Biochem Physiol C Toxicol Pharmacol 145:542–552. doi:10.1016/j.cbpc.2007.01.019

Galgani F, Cornwall R, Toomey BH, Epel DD (1996) Interaction of environmental xenobiotics with a multixenobiotic defense mechanism in the bay mussel *Mytilus galloprovincialis* from the coast of California. Environ Toxicol Chem 15:325–331. doi:10.1897/1551-5028(1996)015<0325:IOEXWA>2.3.CO;2

Gonzalez-Rey M, Bebianno MJ (2011) Non-steroidal anti-inflammatory drug (NSAID) ibuprofen distresses antioxidant defense system in mussel *Mytilus galloprovincialis* gills. Aquat Toxicol 105:264–269. doi:10.1016/j.aquatox.2011.06.015

Habig W, Pabst M, Jakobi W (1974) Glutathione S-transferase: the first enzymatic step in mercapturic acid formation. J Biol Chem 249:7130–7139

Jørgensen S, Halling-Sørensen B (2000) Drugs in the environment. Chemosphere 40:691–699

Ju C, Uetrecht JP (1999) Detection of 2-hydroxyiminostilbene in the urine of patients taking carbamazepine and its oxidation to a reactive iminoquinone intermediate. J Pharmacol Exp Ther 288:51–56

Jung J-H, Kim M, Yim UH et al (2011) Biomarker responses in pelagic and benthic fish over 1 year following the Hebei Spirit oil spill (Taean, Korea). Mar Pollut Bull 62:1859–1866. doi:10.1016/j.marpolbul.2011.04.045

Keppler C, Ringwood A (2001) Expression of P-glycoprotein in the gills of oysters, *Crassostrea virginica*: seasonal and pollutant related effects. Aquat Toxicol 54:195–204

Li Z, Lu G, Yang X, Wang C (2012) Single and combined effects of selected pharmaceuticals at sublethal concentrations on multiple biomarkers in *Carassius auratus*. Ecotoxicology 21:353–361. doi:10.1007/s10646-011-0796-9

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–408. doi:10.1006/meth.2001.1262

Londoño DK, Siegfried BD, Lydy MJ (2004) Atrazine induction of a family 4 cytochrome P450 gene in *Chironomus tentans* (Diptera: Chironomidae). Chemosphere 56:701–706. doi:10.1016/j.chemosphere.2003.12.001

Luedeking A, Koehler A (2004) Regulation of expression of multixenobiotic resistance (MXR) genes by environmental factors in the blue mussel *Mytilus edulis*. Aquat Toxicol 69:1–10. doi:10.1016/j.aquatox.2004.03.003

Martín-Díaz ML, Gagné F, Blaise C (2009) The use of biochemical responses to assess ecotoxicological effects of Pharmaceutical and Personal Care Products (PPCPs) after injection in the mussel *Elliptio complanata*. Environ Toxicol Pharmacol 28:237–242. doi:10.1016/j.etap.2009.04.009

Martínez-Paz P, Morales M, Martínez-Guitarte JL, Morcillo G (2012) Characterization of a cytochrome P450 gene (CYP4G) and modulation under different exposures to xenobiotics (tributyltin, nonylphenol, bisphenol A) in *Chironomus riparius* aquatic larvae. Comp Biochem Physiol C Toxicol Pharmacol 155:333–343. doi:10.1016/j.cbpc.2011.10.001

Martins JC, Machado J, Martins A et al (2011) Dynamics of protein phosphatase gene expression in *Corbicula fluminea* exposed to microcystin-LR and to toxic *Microcystis aeruginosa* cells. Int J Mol Sci 12:9172–9188. doi:10.3390/ijms12129172

Miao J, Pan L, Liu N et al (2011) Molecular cloning of CYP4 and GSTpi homologues in the scallop *Chlamys farreri* and its expression in response to benzo[a]pyrene exposure. Mar Genomics 4:99–108. doi:10.1016/j.margen.2011.03.002

Milan M, Pauletto M, Patarnello T et al (2013) Gene transcription and biomarker responses in the clam *Ruditapes philippinarum* after exposure to ibuprofen. Aquat Toxicol 126:17–29. doi:10.1016/j.aquatox.2012.10.007

Minier C, Borghi V, Moore MN, Porte C (2000) Seasonal variation of MXR and stress proteins in the common mussel, *Mytilus galloprovincialis*. Aquat Toxicol 50:167–176. doi:10.1016/S0166-445X(99)00104-6

Monari M, Foschi J, Matozzo V et al (2009) Investigation of EROD, CYP1A immunopositive proteins and SOD in haemocytes of *Chamelea gallina* and their role in response to B[a]P. Comp Biochem Physiol C Toxicol Pharmacol 149:382–392. doi:10.1016/j.cbpc.2008.09.008

Nelson DR, Koymans L, Kamataki T et al (1996) P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. Pharmacogenetics 6:1–42

Pan L, Liu N, Xu C, Miao J (2011) Identification of a novel P450 gene belonging to the CYP4 family in the clam *Ruditapes philippinarum*, and analysis of basal- and benzo(a)pyrene-induced mRNA expression levels in selected tissues. Environ Toxicol Pharmacol 32:390–398. doi:10.1016/j.etap.2011.08.005

Piretti MV, Pagliuca G (1989) Systematic isolation and identification of membrane lipid oxidation products. Free Radic Biol Med 7:219–221

Ramos-Gómez J, Coz A, Viguri JR et al (2011) Biomarker responsiveness in different tissues of caged *Ruditapes philippinarum* and its use within an integrated sediment quality assessment. Environ Pollut 159:1914–1922. doi:10.1016/j.envpol.2011.03.030

Rewitz KF, Kjellerup C, Jørgensen A (2004) Identification of two *Nereis virens* (Annelida: Polychaeta) cytochromes P450 and induction by xenobiotics. Comp Biochem Physiol C Toxicol Pharmacol 138:89–96. doi:10.1016/j.cca.2004.05.007

Rewitz KF, Styrishave B, Løbner-Olsen A, Andersen O (2006) Marine invertebrate cytochrome P450: emerging insights from vertebrate and insects analogies. Comp Biochem Physiol C Toxicol Pharmacol 143:363–381. doi:10.1016/j.cbpc.2006.04.001

Rola RC, Monteiro MDC, Reis SRDS, Sandrini JZ (2012) Molecular and biochemical biomarkers responses in the mussel *Mytilus edulis* collected from Southern Brazil coast. Mar Pollut Bull 64:766–771. doi:10.1016/j.marpolbul.2012.01.004

Simpson AECM (1997) The cytochrome P450 4 (CYP4) family. Gen Pharmacol Vasc Syst 28:351–359. doi:10.1016/S0306-3623(96)00246-7

Snyder MJ (1998) Cytochrome P450 enzymes belonging to the CYP4 family from marine invertebrates. Biochem Biophys Res Commun 249:187–190. doi:10.1006/bbrc.1998.9104

Sureda A, Box A, Tejada S et al (2011) Biochemical responses of *Mytilus galloprovincialis* as biomarkers of acute environmental pollution caused by the Don Pedro oil spill (Eivissa Island, Spain). Aquat Toxicol 101:540–549. doi:10.1016/j.aquatox.2010.12.011

Thibaut R, Porte C (2008) Effects of fibrates, anti-inflammatory drugs and antidepressants in the fish hepatoma cell line PLHC-1: cytotoxicity and interactions with cytochrome P450 1A. Toxicol In Vitro 22:1128–1135. doi:10.1016/j.tiv.2008.02.020

Tian X, Song E, Pi R et al (2012) Polychlorinated biphenyls and their different level metabolites as inhibitors of glutathione S-transferase isoenzymes. Chem Biol Interact 198:1–8. doi:10.1016/j.cbi.2012.04.002

Togola A, Budzinski H (2008) Multi-residue analysis of pharmaceutical compounds in aqueous samples. J Chromatogr A 1177:150–158. doi:10.1016/j.chroma.2007.10.105

Umasuthan N, Revathy KS, Lee Y et al (2012) A novel molluscan sigma-like glutathione S-transferase from Manila clam, *Ruditapes philippinarum*: cloning, characterization and transcriptional profiling. Comp Biochem Physiol C Toxicol Pharmacol 155:539–550. doi:10.1016/j.cbpc.2012.01.001

Van Ommen B, Ploemen JH, Bogaards JJ et al (1991) Irreversible inhibition of rat glutathione S-transferase 1-1 by quinones and their glutathione conjugates. Structure-activity relationship and mechanism. Biochem J 276(Pt 3):661–666

Vasieva O (2011) The many faces of glutathione transferase pi. Curr Mol Med 11:129–139

Zanette J, Goldstone JV, Bainy ACD, Stegeman JJ (2010) Identification of CYP genes in *Mytilus* (mussel) and *Crassostrea* (oyster) species: first approach to the full complement of cytochrome P450 genes in bivalves. Mar Environ Res 69(Suppl):S1–S3. doi:10.1016/j.marenvres.2009.10.013

Zanette J, Jenny MJ, Goldstone JV et al (2013) Identification and expression of multiple CYP1-like and CYP3-like genes in the bivalve mollusk *Mytilus edulis*. Aquat Toxicol 128–129:101–112. doi:10.1016/j.aquatox.2012.11.017

Zhang G, Fang X, Guo X et al (2012a) The oyster genome reveals stress adaptation and complexity of shell formation. Nature 490:49–54. doi:10.1038/nature11413

Zhang L, Qiu L, Wu H et al (2012b) Expression profiles of seven glutathione S-transferase (GST) genes from *Venerupis philippinarum* exposed to heavy metals and benzo[a]pyrene. Comp Biochem Physiol C Toxicol Pharmacol 155:517–527. doi:10.1016/j.cbpc.2012.01.002