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RESEARCH ARTICLE

Transcriptomic responses of the endangered freshwater mussel *Margaritifera margaritifera* to trace metal contamination in the Dronne River, France

Anthony Bertucci[1] · **Fabien Pierron**[1] · **Julien Thébault**[2] · **Christophe Klopp**[3] · **Julie Bellec**[2] · **Patrice Gonzalez**[1] · **Magalie Baudrimont**[1]

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Abstract The freshwater pearl mussel *Margaritifera margaritifera* is one of the most threatened freshwater bivalves worldwide. In this study, we aimed (i) to study the processes by which water quality might affect freshwater mussels in situ and (ii) to provide insights into the ecotoxicological significance of water pollution to natural populations in order to provide necessary information to enhance conservation strategies. *M. margaritifera* specimens were sampled in two close sites located upstream or downstream from an illegal dumping site. The renal transcriptome of these animals was assembled and gene transcription determined by RNA-seq. Correlations between transcription levels of each single transcript and the bioaccumulation of nine trace metals, age (estimated by sclerochronology), and condition index were determined in order to identify genes likely to respond to a specific factor. Amongst the studied metals, Cr, Zn, Cd, and Ni were the main factors correlated with transcription levels, with effects on translation, apoptosis, immune response, response to stimulus, and transport pathways. However, the main factor explaining changes in gene transcription appeared to be the age of individuals with a negative correlation with the transcription of retrotransposon-related genes. To investigate this effect further, mussels were classified into three age classes. In young, middle-aged and old animals, transcription levels were mainly explained by Cu, Zn and age, respectively. This suggests differences in the molecular responses of this species to metals during its lifetime that must be better assessed in future ecotoxicology studies.

Keywords Freshwater mussel · Transcriptomics · RNA sequencing · Metal pollution · Sclerochronology · *Margaritifera margaritifera* · In situ study

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✉ Anthony Bertucci
anthony.bertucci@u-bordeaux.fr

[1] Univ. Bordeaux, UMR EPOC CNRS 5805, 33615 Pessac, France

[2] Université de Brest, Institut Universitaire Européen de la Mer, Laboratoire des sciences de l'environnement marin (LEMAR UMR 6539 CNRS/UBO/IRD/Ifremer), 29280 Plouzané, France

[3] Plate-forme bio-informatique Genotoul, Mathématiques et Informatique Appliquées de Toulouse, INRA, 31326 Castanet-Tolosan, France

Introduction

Despite their role in particle processing, nutrient release, and sediment mixing (Vaughn and Hakenkamp 2001), our knowledge about the complex biology of freshwater bivalves (order *Unionoida*) is still scarce (Lopes-Lima et al. 2014). Freshwater mussels form a species-rich group of bivalves, with about 900 species present on all continents except Antarctica (Carella et al. 2016). They are particularly sensitive to habitat alterations and water quality plays a key role in their distribution (Gillis et al. 2017). They are known to be amongst the most endangered groups of animals currently (Machordom et al. 2003). Due to the mussel's life history as sessile filter-feeding organisms that live in the substrata, their evasion capabilities are limited, making them particularly susceptible to environmental changes. Most of the main current threats are presumed to be chronic, low-level, and pervasive stressors, such as industrial and agricultural point- and nonpoint-source water pollution, sedimentation,

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construction of dams, and introduction of exotic species (Strayer et al. 2004). The vast number of potential factors contributing to their decline makes it difficult to determine the key chemical contaminants or set of environmental conditions to target in a regulatory framework for protection and conservation (Cope et al. 2008). Recent studies on freshwater mussels have documented the impairment of their physiological and biochemical status due to reduced water quality (Lummer et al. 2016; Kerambrun et al. 2016; Gillis et al. 2017). Amongst all the freshwater mussels' families, the Unionidae, in particular, has emerged as a critical group for consideration in the field of ecotoxicology over the past 20 years because of their high sensitivity to chemical exposures and a variety of other environmental stressors, with respect to other groups of organisms (Carella et al. 2016).

Like many nonmarine molluscs facing a global decline (Lydeard et al. 2004), the freshwater pearl mussel *Margaritifera margaritifera* (family Unionidae) is one of the most threatened freshwater bivalves worldwide. This species can be found in cool running waters of the Holarctic region. The European population decline was estimated to be 90% by the 1990s, due to pearl harvesting, predation, and habitat degradation, coupled with a decrease of host fish populations necessary for larval growth and dispersion (Geist 2010). Most unionids have indeed a complex reproductive cycle including an ectoparasitic stage on fish; the glochidia are released into the water and attach to the gills or fins of a suitable host fish (*Salmo trutta fario* for *M. margartifera*) for several weeks until transformation into juvenile mussels. Then, juveniles leave the host and become infaunal benthic organisms that typically remain burrowed beneath the sediment surface through the first 2 to 4 years of life depending on the species. The freshwater pearl mussel is currently protected internationally by the Bern Convention (Annex III) and the European Commission Habitats Directive (Annexes II and V), being listed as "Endangered" globally and as "Critically Endangered" in Europe by the IUCN Red List of Threatened Species, which contains 200 other unionid species (Cuttelod et al. 2011). The current distribution and populations' size of the species are difficult to estimate owing to rediscoveries, decline, and extinction of some populations. The last estimate of the French population was of a maximum of 100,000 individuals. While most of the populations are small with a maximum of 300 individuals, a large population of about 16,000 is found in the Dronne River (Geist 2010).

As a preliminary to a reintroduction program of *M. margaritifera* in the Dronne River, France, the current study aimed at measuring the potential impact of an illegal dumping site on local mussels. In this aim, we conducted a large-scale and without a priori RNA-Seq-based approach. RNA-Seq technology offers the opportunity for ecotoxicologists to investigate the effects of contaminants on transcriptome-wide response in non-model but environmentally relevant species (Regier et al. 2013; Uren Webster et al. 2013) for which molecular information are limited such as *M. margaritifera* (González et al. 2015). Gene expression profiling using RNA-seq could play a key role in identifying new biomarkers of exposure and adverse effects and in discovering new toxicity pathways (Connon et al. 2012; Gonzalez and Pierron 2015). This approach was coupled with the determination of the contamination level of nine trace metals, as well as the age and condition index of each individual. We then performed correlation analyses between the gene transcriptional levels and each biological factor.

Methodology

Specimen collection and sampling The two close sampling sites were located upstream and downstream from an illegal dump site along the Dronne River in Saint Saud-Lacoussière, France. The two sites were close enough, respectively located 50–100 m upstream and 10 m downstream, to ensure that the dumping site was the only contamination source and avoid any other difference between the two sites. We do not know since when this site has been used for the disposal of household waste including cans, motor oil, furniture, batteries, appliances, and pharmaceuticals, for example. Eight individuals were collected randomly at each site in April 2009 and March 2010, leading to a total number of 32 individuals. The low abundance of the species and its protected status prevented us from carrying out a higher sampling effort. Soft (body) tissues and shells were weighted in order to determine the condition index (Quayle and Newkirk 1989) with the following formula: tissue (g, fresh weight)/shell (g, dry weight) × 100. The shell was also measured and collected for age determination. The digestive glands and kidneys were dissected for metal content analysis and RNA extraction, respectively. Digestive glands were immediately placed at 4 °C before storage at − 20 °C until analysis. Kidney samples were collected in RNA-later solution and placed at 4 °C overnight before storage at − 20 °C until RNA extraction. Physicochemical parameters (temperature, pH, conductivity, and oxygen concentration) were measured in March 2010. These measurements were not determined in April 2009 owing to technical issues.

Age determination Shells were dried and their length (anterior to posterior margin) and height (dorsal to ventral margin) measured to the nearest 0.1 mm using a Vernier caliper. For each specimen, the left valve was mounted on a PVC cube using epoxy glue and subsequently embedded in a two-part metal-epoxy resin along the axis of minimum growth (dorso-ventral axis) in order to strengthen the shell and avoid fracture during sawing. After complete polymerization, a 0.6-mm-thick cross section was cut from each valve perpendicular to

the growth lines along the dorso-ventral axis using a low-speed precision saw (Struers Secotom-10) equipped with a 0.6-mm-thick diamond cut-off wheel (Struers MOD20) cooled with water (feed rate, 125 μm s^{-1}; rotation speed, 300 rpm). Cross sections were then glued on glass slides (Crystalbond 509 mounting adhesive), manually ground for 3 min on a 1200-grit size abrasive disk, more finely ground using a 2500-grit size abrasive disk on an automated polishing machine (Struers TegraPol-35), and finally polished with a diamond suspension (Struers DiaPro Dur 3 μm). In order to better resolve annual growth lines and increments, cross sections were cleaned with ethanol before being etched in the Mutvei's solution for 25 min at 38.5 °C (see Schöne et al. 2005 for a detailed description of the method). They were then gently rinsed with ultra-pure water and air-dried. Several high-resolution pictures of each of the 32 cross sections were taken using a stereomicroscope (Zeiss Lumar.V12) equipped with a color digital camera (Zeiss AxioCam MRc5). Images were then automatically stitched together into a single high-resolution picture using Zeiss AxioVision software. The number and width of each annual increment were finally measured in the outer shell layer using ImageJ software (Schneider et al. 2012). As all specimens were eroded close to the umbo, it was impossible to count the first growth increments, leading to a small underestimation of shell ages (between one and three missing years).

Metal analyses After drying at 50 °C for 48 h, the digestive gland samples were digested by 3 ml of pure trace metal-grade nitric acid (HNO_3) for 3 h at 100 °C, and after cooling, 15 ml of ultrapure water (Milli-Q®) was added. As, Cd, Co, Cr, Cu, Mn, Ni, Pb, and Zn were analyzed by inductively coupled plasma-mass spectrometry (ICP-MS). Blanks and standards (TORT-2 and DOLT-4) were submitted to the same procedure in order to verify the accuracy of the method.

RNA extraction, library construction and sequencing Total RNA was isolated from kidney tissues using the SV Total RNA isolation system kit (Promega) according to the manufacturer's instructions. RNA-seq libraries were prepared and sequenced by Genotoul (France) with the Illumina HiSeq 3000 technology. The RNA-seq reads used in this study have been deposited in the NCBI Gene Expression Omnibus (Edgar et al. 2002) under GEO Accession GSE94542.

Bioinformatics workflow The read quality of the RNA-seq libraries was evaluated using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Cleaned and filtered reads were de novo assembled using DRAP version 1.7 (Cabau et al. 2016) (de novo RNA-Seq Assembly Pipeline, https://www.sigenae.org/drap) using the Trinity assembler version 2.0.6 (Grabherr et al. 2011). Assembled contigs were filtered in order to keep only those with at least one fragment per kilobase of transcript per million reads (FPKM). The resulting contigs were aligned with NCBI blast (Camacho et al. 2009) (blastx program, -e 1e-5 parameter, version 2.2.26) on Refseq protein, Swissprot, and Ensembl protein reference files from *Crassostrea gigas*, *Lottia gigantea*, and *Lingula anatina* to retrieve the corresponding annotations. The contigs were also processed with rnammer (Lagesen et al. 2007) (standard parameters, version 1.2) to find ribosomal genes, with repeatmasker (Smith et al. n.d.) (-engine cross-match -gccalc -species *Danio rerio* parameters, version open-4-0-3) to list the contained repeats and with interproscan (Quevillon et al. 2005) (–goterms –pathways parameters, version 4.8) for gene ontology and structural annotation. Reads have been realigned back to contigs with bwa (Li and Durbin 2009) (standard parameters, mem algorithm, version 0.7.12). The resulting files were compressed, sorted, and indexed with samtools (Li et al. 2009) (view, sort, and index programs, standard parameters, version 1.1). The contig transcription counts have been generated with samtools (idxstats program, standard parameters, version 1.1) and merged with unix commands (cut, paste). The alignment files have then been filtered for duplicates with samtools (rmdup program, standard parameters, version 1.1) before variant calling. The resulting bam files have been processed with GATK (McKenna et al. 2010) (–glm BOTH parameter, version v3.0-0-g6bad1c6) following the best practices found on the GATK website.

Data accessibility Transcriptome sequences: This Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession GFHD00000000. The version described in this paper is the first version, GFHD01000000. Gene expression data: RNA-seq reads and raw count table are available at NCBI Gene Expression Omnibus (GEO) database, accession number GSE94542. Detailed annotation information of de novo assembly is listed in Online Resource 1. Moreover, all the results have been uploaded in a RNAbrowse instance (Mariette et al. 2014) and can be accessed from the web (Sigenae web address available upon publication http://ngspipelines2.toulouse.inra.fr:9007).

Real-time PCR Specific primers amplifying approximately 100 bp were designed for a set of 10 genes using the software primer3 (Rozen and Skaletsky 2000). Primer sequences used in this study are listed in Online Resource 2. Expected lengths of the amplicons were checked by agarose gel electrophoresis after regular PCR amplification using the GoTaq DNA Polymerase (Promega). Primer efficiencies were determined using standard curve analysis ($E = 10^{(-1/\text{slope})} - 1$) with a dilution series of pooled cDNA from all conditions and ranged from 90 to 99%. Transcript level quantification was performed using the GoTaq qPCR mastermix from Promega and a

Startagene Mx3000P system. PCR conditions were as follows: 1× GoTaq qPCR mastermix (Promega), 200 nM primers, and 10 ng of cDNA in a total volume of 25 μl. PCR parameters were 95 °C for 2 min, followed by 40–45 cycles of 15 s at 95 °C, 60 s at 60 °C, and a dissociation curve step (60–95 °C) to confirm the absence of nonspecific products. The dissociation curves showed a single amplification product and no primer dimers. Contigs LMarga_ACT2, LMarga_LOC105342395, and LMarga_LOC101857690.2.2, respectively, encoding β-actin, a histone acetyltransferase, and myosin-2 heavy chain, were selected as control genes based on their stability in the RNA-seq data set. The relative quantification for each gene was normalized by the geometric mean of control genes and relative to its upstream expression. Fold expression values were calculated as $(E^{\mathrm{dCT}})_{\mathrm{target}}/(E^{\mathrm{dCT}})_{\mathrm{control}}$ where E is the amplification efficiency for each pair of primers and $\mathrm{dCT} = \mathrm{CT}_{\mathrm{downstream}} - \mathrm{CT}_{\mathrm{upstream}}$.

Statistical analyses Concerning metal concentrations, condition index, and age, comparisons amongst the four mussel groups were performed by analysis of variance (ANOVA), after checking assumptions of normality and homoscedasticity. If significant effects were detected, the Tukey HSD test was used to determine whether means between pairs of samples were significantly different from one another. Comparisons of the two sites and years were performed by means of Mann-Whitney test. Computations were performed with the R package "Stats."

Differential gene transcription between sites was inferred based on the contig expression counts by using the DESeq2 R package (Love et al. 2014). p values for differential gene expression were corrected for multiple testing using the Benjamini and Hochberg method, and a false discovery rate (FDR) threshold of 0.05 was used. Data quality assessment was conducted according to the DESeq2 package documentation.

The factorial analysis for multiple testing (FAMT) approach described in Friguet et al. (2009) was adopted to increase statistical power between a variation in the transcription level of an individual gene and the contamination level of a given contaminant as well as some morphometric measurements (age and condition index (C.I.)). Since length is highly correlated with age, it was not added to the model. These variables were gathered in a matrix made of independent observations. In order to favor strong correlations, the R package FAMT (Causeur et al. 2011) was used with a FDR value of 0.01. This approach proved its usefulness in previous ecotoxicology studies (Baillon et al. 2015). The FDR was increased to 0.05 when comparing age classes due to the smaller number of individuals. These three classes were created by using the R command "kmeans."

GO annotations were obtained with an E value and a homology threshold set to 10^{-6} and 50%, respectively. GO-enrichment analyses were carried out with the topGO R package (Alexa and Rahnenfuhrer 2016). In order to reduce redundancy in GO terms and ease the interpretation, we used the REVIGO web server with a value of $C = 0.5$ (Supek et al. 2011).

Results and discussion

Biometric measurement and pollution analysis Biometric measurements, condition indices, and trace metal contamination levels measured in digestive glands, as well as their statistical differences, are presented in Table 1. In bivalves, the digestive gland functions both as a site for metal uptake and as an important reservoir for metal storage; thus, this organ is a main target of metal bioaccumulation (e.g., Sheir et al. 2013). Measurements showed significant differences between the two sites and the two times of sampling. The pollution level was higher downstream from the dump site for As, Cd, Co, Cr, Ni, and Zn. Our data also shows that this pollution was higher in the year 2009 compared to 2010 (As, Cr, Pb, and Zn). The largest differences between sites were found for Cd and Cr with downstream sites being on average 2.63 and 1.94 times more contaminated, respectively, while Cr and Pb show the largest annual changes with 1.90 and 3.38 times higher concentration in individuals sampled in 2009 compared to those from 2010 on average. Higher bioaccumulation levels were associated with poor health status of mussels as evidenced by weaker values of condition indices (C.I.). It seems that differences in trace metal contamination could result from the older age of the individuals from the "downstream 2009" group that are significantly more contaminated in As, Cd, Co, and Cr than the "upstream 2009" group or the "upstream 2010" group. Animals from the downstream 2010 group presented intermediate values, and no significant difference was observed in 2010 between the upstream and downstream sites. Cu and Mn are the only trace metals that showed no difference amongst all our samples. However, age does not preclude an effect of the dumping site, especially in year 2009, since individuals that do not show differences in terms of age, such as downstream 2009 and downstream 2010, show significant differences in their metal bioaccumulation levels (As, Pb, and Zn). As suggested by measurements performed in March 2010 (Table 1), the dumping site has no effect on physicochemical parameters.

Effects of the polluted site on gene expression in kidney In order to investigate the potential effects of this metallic pollution, we used a classical RNAseq approach using the R package DESeq2 to identify the genes that were differentially regulated in the kidneys of individuals from the upstream and the downstream sites (Love et al. 2014). In bivalves, environmental pollutants accumulate in various tissues and very often in the kidney (Seiler and Morse 1988)

Table 1 Means ± SE of biometric measures and metal concentrations (μg per g of dry weight) in digestive glands between the different sites and years

		Site		Year	
		Upstream	Downstream	2009	2010
Biometry	Length (mm)	87.03 ± 2.12	95.88 ± 1.81 (**)	95.24 ± 2.31	87.66 ± 1.76 (*)
	Age (years)	25.94 ± 4.03	41.56 ± 3.85 (**)	39.81 ± 4.46	27.69 ± 3.77
	C.I.	57.29 ± 4.06	42.69 ± 2.30 (**)	45.9 ± 3.09	54.08 ± 4.10
Metals (μg/g dry weight)	As	6.48 ± 0.38	8.49 ± 0.56 (**)	8.53 ± 0.50	6.44 ± 0.45 (**)
	Cd	1.36 ± 0.11	3.58 ± 0.68 (***)	3.03 ± 0.69	1.90 ± 0.34
	Co	0.98 ± 0.09	1.70 ± 0.12 (***)	1.44 ± 0.13	1.23 ± 0.15
	Cr	2.58 ± 0.46	5.01 ± 0.83 (***)	4.98 ± 0.83	2.62 ± 0.46 (*)
	Cu	12.22 ± 1.08	10.66 ± 1.43	11.53 ± 0.97	11.35 ± 1.54
	Mn	947.34 ± 190.01	1545.88 ± 222.88 (*)	1450.00 ± 210.33	1043.21 ± 218.14
	Ni	0.58 ± 0.05	0.9 ± 0.1 (**)	0.79 ± 0.06	0.69 ± 0.11
	Pb	1.46 ± 0.42	1.82 ± 0.43	2.54 ± 0.51	0.75 ± 0.06 (***)
	Zn	75.26 ± 1.72	81.78 ± 2.56 (*)	84.09 ± 2.06	72.95 ± 1.65 (***)
		Pairwise comparison			
		Upstream 2009	Downstream 2009	Upstream 2010	Downstream 2010
Physicochemical parameters	Temp. (°C)	n.d.	n.d.	6.10	6.00
	pH	n.d.	n.d.	7.36	7.26
	Cond. (μS)	n.d.	n.d.	85	84
	O_2 (mg/L)	n.d.	n.d.	11.72	12.05
Biometry	Length (mm)	89.44 ± 3.23 (a)	101.04 ± 1.62 (b)	84.61 ± 2.51 (a)	90.71 ± 1.96 (a)
	Age (y)	27.63 ± 5.78 (a)	52.00 ± 3.07 (b)	24.25 ± 5.63 (a)	31.13 ± 4.79 (a, b)
	C.I.	53.08 ± 4.64 (a, b)	38.71 ± 2.01 (a)	61.49 ± 6.39 (b)	46.66 ± 3.65 (a, b)
Metals (μg/g dry weight)	As	6.88 ± 0.43 (a)	10.17 ± 0.38 (b)	6.07 ± 0.59 (a)	6.81 ± 0.65 (a)
	Cd	1.37 ± 0.13 (a)	4.69 ± 1.11 (b)	1.34 ± 0.19 (a)	2.47 ± 0.59 (a, b)
	Co	0.99 ± 0.11 (a)	1.9 ± 0.07 (b)	0.97 ± 0.16 (a)	1.49 ± 0.22 (a, b)
	Cr	2.85 ± 0.63 (a)	7.11 ± 1.11 (b)	2.32 ± 0.65 (a)	2.92 ± 0.65 (a, b)
	Cu	12.93 ± 1.68	10.14 ± 0.71	11.51 ± 1.34	11.19 ± 2.78
	Mn	1020.66 ± 283.54	1879.35 ± 227.72	874.02 ± 253.74	1212.4 ± 347.8
	Ni	0.65 ± 0.07 (a, b)	0.94 ± 0.05 (a)	0.52 ± 0.08 (b)	0.85 ± 0.19 (a, b)
	Pb	2.19 ± 0.76 (a, b)	2.88 ± 0.67 (b)	0.74 ± 0.08 (a)	0.75 ± 0.09 (a)
	Zn	79.46 ± 2.44 (a, b)	88.73 ± 2.41 (a)	71.07 ± 1.25 (b)	74.84 ± 2.93 (b)

Sites and years were compared with Mann-Whitney test while the four groups were compared with univariate ANOVA tests. Pairwise comparisons were assessed with a Tukey HSD test and indicated with a and b letters, p value < 0.05. Physicochemical parameters were measured in 2010 only

Temp. temperature, *Cond*. conductivity, *n.d.* not determined

*p value < 0.05; **p value < 0.01; ***p value < 0.001

that serves, together with the pericardial glands, as an excretory organ. First, the 32 cDNA Illumina libraries were sequenced on a Hiseq3000 platform (genotoul, Toulouse, France). Three billion sequences averaging 150 bases in length were generated and assembled into a total of 51,392 contigs, with a N50 of 3,141 bp. A total of 22,288 contigs (43.3%) exhibited high homology with known sequences (blastx evalue 1.E-5) amongst which 11,375 (51.0%) were assigned to a gene ontology term (details are given in Online Resource 1). These relatively low percentages highlight the sparsity of sequences and functional information existing for bivalves. To validate the sequencing data, the transcription of seven genes that showed variations in their expression levels was measured by the RT-qPCR method. RNAseq and RT-qPCR gave consistent results with an R^2 value of 0.84 (Online Resource 3).

From the 51,392 contigs, only 68 transcripts (25 with known homologies) were differentially expressed (DE; full list supplied as Online Resource 4) between upstream and downstream organisms in year 2009. This result is consistent when we compare the two sites regardless of the year (41 DE genes amongst which 23 had Blast homologies). Examples of

Table 2 Example of annotated transcripts with differential expression between upstream and downstream sites. Columns from left to right: name of the contig, its description based on blastx comparisons (1e-5), fold change values downstream obtained by DESeq2 considering all samples or samples from 2009 only, and the biological function of the putative protein

Contig	Description	FC downstream		Function
		All	2009	
LMarga_CFH.3.3	Complement factor H	2.21		Immune response
LMarga_SVEP1.3.3	Sushi, vWA, EGF and pentraxin domain-containing protein 1	2.54		
LMarga_LOC103480842	Tripartite motif-containing protein 65-like		0.24	
LMarga_LOC105321584.2.2	Cysteine sulfinic acid decarboxylase-like	0.59		Response to oxidative stress
LMarga_LOC106070504.5.5	Glutathione peroxidase-like isoform	2.37		
LMarga_ppp1r15b	Protein phosphatase 1 regulatory subunit 15B		1.45	
LMarga_LOC106063684	Peptidyl-prolyl *cis-trans* isomerase-like		0.24	Protein folding
LMarga_plg.3.3	Plasminogen precursor		0.17	Proteolysis
LMarga_LOC105321824.1.3	Neprilysin-like		3.59	
LMarga_LOC106055697.1.2	Balbiani ring protein 3-like		4.05	
LMarga_LOC106873821.1.2	Zinc metalloproteinase nas-14-like		5.20	
LMarga_DIAP2.1.3	Death-associated inhibitor of apoptosis 2		0.53	Inhibition of apoptosis
LMarga_LOC105331304.3.3	Baculoviral IAP repeat-containing protein 7-like	0.48		
LMarga_LOC105323879.5.5	Baculoviral IAP repeat-containing protein 7-like	0.51		
LMarga_NHRF1	Na(+)/H(+) exchange regulatory cofactor NHE-RF1		0.41	Phosphate reabsorption
LMarga_LOC105318951	Putative all-*trans*-retinol 13,14-reductase	2.34		Adipose tissue physiology
LMarga_LOC105319316.1.2	Neo-calmodulin-like		3.62	Calcium binding
LMarga_LOC105330779.2.2	EMILIN-2-like	2.30		Cell adhesion
LMarga_contig_05524	Putative ferric-chelate reductase 1		0.14	Oxidoreduction
LMarga_LOC105324826	Tyrosinase-like protein 1		0.30	
LMarga_LOC102259112	Zinc finger protein 420-like	0.71		Transcription factor

transcripts with homologous protein and their fold change are presented in Table 2. These results suggest that in mussels located downstream from the polluted site, immune system and oxidative stress defenses were impacted. In fish, the transcription of genes encoding proteins involved in immunity is often negatively correlated to metal concentrations (Williams et al. 2006; Reynders et al. 2006; Pierron et al. 2011). Studies in bivalves are less abundant and reported little or no effect in *Cerastoderma edule* (Paul-Pont et al. 2010) and an increase in hemocyte density in *Mytilus edulis* (Coles et al. 1995) and *Crassostrea gigas* (Auffret and Oubella 1994). In our study, SVEP1, which showed a 2.54-fold increased expression in downstream animals in comparison to upstream animals, is a multi-domain protein of the extracellular matrix (ECM) (von Willebrand factor A—VWA; pentraxin; EGF-like; and "sushi" domains) (Sato-Nishiuchi et al. 2012). The association of these domains in the same molecule has been associated with immune functions such as the complement system, acute phase response of infection, and tissue injury and/or cell adhesion (Gilgès et al. 2000; Bouchut et al. 2006). While their function is unknown in bivalve molluscs, VWA-containing proteins are present in the hemolymph of terrestrial molluscs as part of the coagulant immune system, playing a key role in the processes of susceptibility/resistance in host-parasite interactions (Bouchut et al. 2006). The expression of a SVEP1-like protein was shown to be reduced in Pacific oyster *Crassostrea gigas* exposed to sanitary sewage discharges (Flores-Nunes et al. 2015). The overexpression of SVEP1 in our mussels in response to higher metal concentrations illustrates the complexity of the immune response amongst bivalve species. This alteration of the immune response is also suggested by the change in expression of two key regulators of the response to pathogens: a complement factor H homolog (Ferreira et al. 2010) and a tripartite motif-containing (TRIM) protein (Ozato et al. 2008).

Trace metals and organic xenobiotics are typical classes of environmental pollutants with prooxidant effects (Regoli and Giuliani 2014). The presence of an oxidative stress response was suggested by the differential expression of a glutathione peroxidase (GPx) and a cysteine sulfinic acid decarboxylase-like (CSAD) protein. It was previously shown in eels exposed to environmental pollutant that when catalase, the enzyme usually involved in hydrogen peroxide (H_2O_2) removal, is inhibited, glutathione peroxidases (GPx) is transcriptionally enhanced (2.37-fold in the present study) and uses reduced glutathione (GSH) as an electron donor to catalyze the reduction of H_2O_2 to H_2O (Regoli et al. 2003). GPx can also reduce lipid hydroperoxides to alcohols, with the concomitant

oxidation of GSH. CSAD is involved in hypotaurine (HTAU) synthesis from L-cysteine. The oxidation of HTAU by various biologically relevant oxidizing agents such as hypochlorite, hydroxyl radical, singlet oxygen, or peroxynitrite could represent a way to prevent oxidative cellular damages (Conrado et al. 2014; Nishimura et al. 2015). The repressed transcription of CSAD may thus deprive the cell of a part of its antioxidant defense. The last transcript involved in the response to stress encodes a homolog of the protein phosphatase 1 regulatory subunit 15B (Ppp1r15b) that targets the translation initiation factor 2 alpha (eIF2α) (Harding et al. 2009). Phosphorylation by stress-sensing kinases makes eIF less able to initiate translation, and so the cell builds fewer proteins and conserves more of its resources during times of stress like starvation or exposure to toxins (Chambers et al. 2015). Ppp1r15b removes the phosphate group once the stressful conditions are over.

This increased expression of stress-related genes in bivalves downstream from the dump site might eventually lead to increased protein denaturation and apoptosis. The need of chaperoning to stabilize unfolding proteins and the removal of denatured ones is a common response to environmental stress (Tomanek 2012) and is an indicator of severe cellular stress (Kültz 2005). For instance, contig LMARGA_LOC106063684 encodes a putative peptidyl-prolyl *cis-trans* isomerase-like protein (PPIase) that accelerates protein folding and that shows a 4-fold downregulation downstream. This could result in more denatured proteins that need to be removed from the cell, hence the up to 5-fold upregulation of several transcripts encoding proteins with peptidase activity such as a zinc metalloproteinase nas-14-like protein (LMARGA_LOC106873821.1.2).

Finally, the downregulation of transcripts encoding proteins such as BIRC-like and Death associated inhibitor of apoptosis suggests an effect of the polluted site on apoptotic processes (Leulier et al. 2006; de Almagro and Vucic 2012; Saleem et al. 2013). In addition to these cellular events, adipose tissue and renal physiology may also be altered as suggested by the response of a retinol saturase enzyme (RetSat, all-trans-retinol reductase) that has a > 2-fold increase in expression and by the > 2-fold repression of a Na^+/H^+ exchange regulatory factor (NHE-RF1). RetSat is probably involved in vitamin A (or retinol) metabolism and adipose tissue physiology through adipocyte differentiation (Moise et al. 2004, 2010). The lack of knowledge about this role precludes any further interpretation regarding a potential alteration of retinoid metabolism or adipose tissue physiology. However, an impairment in retinol metabolism and its impacts on lipid and energy metabolisms were identified as a potential mechanism of metal toxicity in fish (Pierron et al. 2011; Defo et al. 2012). Downstream mussels may experience deleterious effects on renal physiology as NHE transporters are involved in renal phosphate reabsorption.

The small number of differentially expressed genes (DEG) between sites, either when we analyze the year 2009 data or the entire data set, highlights the difficulty to apply a high-throughput RNAseq method to in situ studies in environmental toxicology where multiple biotic and abiotic stressors, including many unmeasured ones (such as organic pollutants), can interact and result in a high level of variance amongst individuals. This is especially true for organisms in water bodies receiving complex effluents, nonpoint source pollutants, or runoff from areas containing naturally high background chemical concentrations. This is evidenced by the distribution of the transcriptomic data. The gene expression pattern of our 32 samples was examined by heatmaps of sample-to-sample distances (example given in Online Resource 5), multidimensional scaling (MDS) method or principal component analysis (Fig. 1). These analyses showed a lack of separation between groups and high interindividual variability. The complex expression pattern that we observe likely results from a complex combination of mutually interacting and/or masking effects. Hence, we could expect a modest number of differentially expressed genes. Moreover, although no significant difference in metal bioaccumulation levels was detected between the two sites in 2010 (Table 1), we identified 736 DEG (220 with known homologs, Online Resource 4). This higher number might be explained by the fact that in Fig. 1a, 2010 samples showed a homogeneous transcription profile (dense clustering) facilitating the discovery of significant changes in gene expression. We choose not to further explore these genes as we could not relate them to a change in environmental variables. However, this result suggests that metals might not be the only factors affecting gene expression in our animals.

The present study indicates that when differences in contamination levels exist (in 2009), we observe fewer regulated genes, meaning that single or interacting factors might mask the global molecular response by creating inter-individual variability. We cannot exclude the possibility that this species might be tolerant to metal contamination. However, even if examples of local adaptation exist, many studies evidenced the susceptibility of *M. margaritifera* and related unionid mussels to metals (Young 2005).

Effects of individual factors on gene expression in kidney

In order to counter this issue, we then used a factor analysis for multiple testing (FAMT; Friguet et al. 2009) approach to explore the transcriptomic effect of individual pollutants, age, and C.I. to identify genes that are likely to be related to one single factor. A total of 13,953 transcripts from our transcriptome were significantly correlated (FDR ≥ 0.01) with at least one of the variables we measured. Out of these, 11,264 were related to one specific factor only (complete list provided as Online Resource 6). The

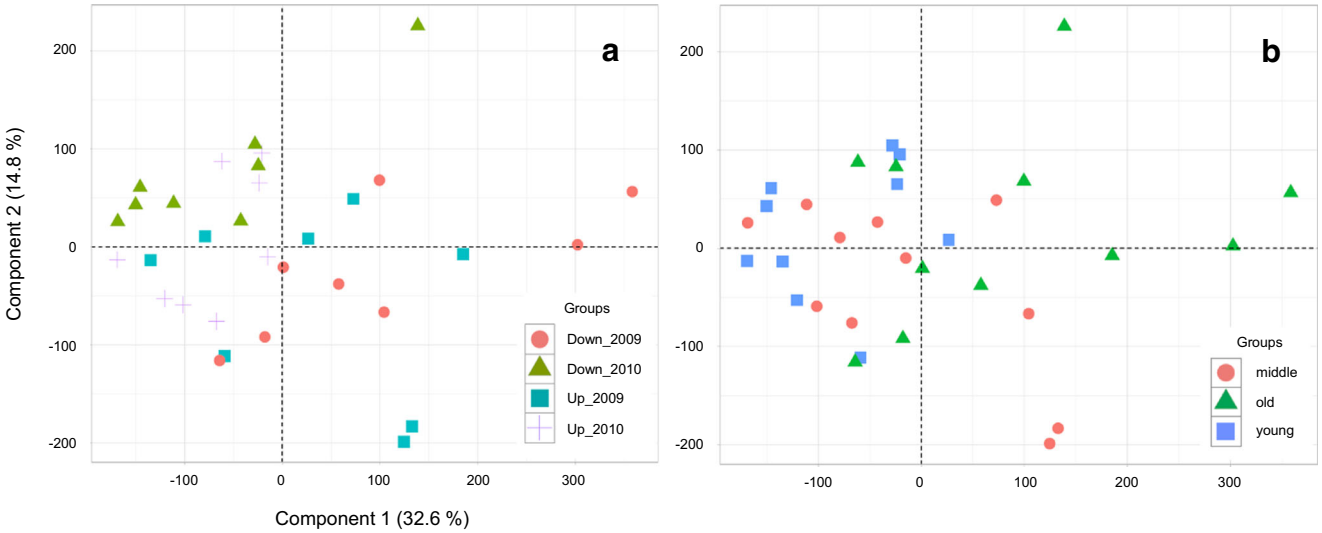

Fig. 1 Principal component analysis (PCA) plot of *M. margaritifera* RNAseq count data. The two first principal components are plotted with the proportion of variance explained by each component shown on the axis. Symbols correspond either to (a) sampling group or (b) age class. Down = downstream, Up = upstream

numbers of transcripts correlated with each specific factor are indicated in Fig. 2. Fifty-two percent of the transcripts (5,856) identified by the FAMT method significantly correlate with age. Metal concentrations and condition index correlate with the expression level of 5,047 (44.8%, ranging from 1,712 genes associated with Cr to 14 associated with Co) and 361 (3.2%) transcripts, respectively. In addition to metals, the age of the animals thus appears as a primary factor to consider.

Analysis of gene ontology enrichment (Table 3) showed that age-related genes are linked to DNA metabolism and transcription (GO:0006278, GO:0006352, and GO:0015074). Fifty-four transcripts are found in these GO categories (Online Resource 7) out of which 11 are homologous of reverse transcriptases encoded by retrotransposons related to the LINE-1 family (Ivanov et al. 1991). As dynamic components of genomes, retrotransposons are a source of genetic/epigenetic variation and novelty and are increasingly seen as implicated in fundamental genomic functions, in both normal and pathological contexts (Sciamanna et al. 2016). All these transcripts were negatively correlated with the age of animals. In several cancer cell lines, the reduction of LINE-1-derived RT was found to reduce proliferation, promote differentiation, and reprogram the global transcription profiles of coding and non-coding sequences. In spite of the abundance and the putative role of transposable elements in shaping genome variation in bivalves (Zhang et al. 2012), their response to environmental stressors remains poorly understood (Casacuberta and González 2013; Miousse et al. 2015). The expression of retrotransposable elements usually increases with age; however, it could be reduced by caloric restriction (De Cecco et al. 2013), and previous studies, though in marine mussels, showed that feeding rates are reduced with age (Bellas et al. 2014).

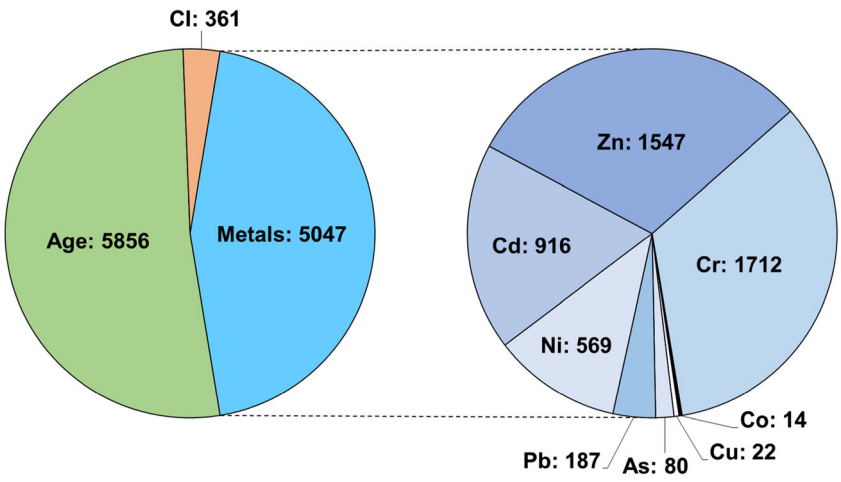

Fig. 2 Number of genes correlated with a single factor according to FAMT analyses (FDR ≤ 0.01)

Table 3 Gene ontology (GO) terms that were enriched (corrected p value ≤ 0.05) in genes correlated with a specific factor in FAMT analyses. Categories are P, biological process; F, molecular function; and C, cellular compartment

Factor	GO-ID	Term	Category	FDR	Number of sequences
Age	GO:0006278	RNA-dependent DNA replication	P	1.94E−11	37
	GO:0003964	RNA-directed DNA polymerase activity	F	1.94E−11	37
	GO:0003723	RNA binding	F	1.28E−9	68
	GO:0006352	DNA-templated transcription, initiation	P	3.08E−3	9
	GO:0015074	DNA integration	P	1.49E−2	12
CI	GO:0008083	Growth factor activity	F	3.78E−2	4
	GO:0030246	Carbohydrate binding	F	3.78E−2	9
All metals	GO:0008270	Zinc ion binding	F	3.72E−5	175
	GO:0003735	Structural constituent of ribosome	F	1.37E−3	38
	GO:0005840	Ribosome	C	1.62E−3	38
	GO:0006412	Translation	P	2.54E−3	51
	GO:0042981	Regulation of apoptotic process	P	6.08E−3	24
	GO:0005102	Receptor binding	F	1.44E−2	25
Cr	GO:0008270	Zinc ion binding	F	2.47E−11	87
	GO:0042981	Regulation of apoptotic process	P	7.63E−8	19
	GO:0005164	Tumor necrosis factor receptor binding	F	2.82E−4	11
	GO:0006955	Immune response	P	6.61E−4	11
	GO:0004197	Cysteine-type endopeptidase activity	F	3.70E−3	11
	GO:0005185	Neurohypophyseal hormone activity	F	7.38E−3	3
	GO:0005622	Intracellular	C	1.20E−2	103
Zn	GO:0003735	Structural constituent of ribosome	F	3.07E−13	36
	GO:0006412	Translation	P	2.29E−11	42
	GO:0015935	Small ribosomal subunit	C	2.13E−2	5

Moreover, it was documented that adult freshwater mussels have the ability to detect toxicants in the water and consequently close their valves to avoid exposure (Liu et al. 2016). This process could limit food intake too. Amongst genes whose expression was positively correlated with age, we could identify three transcription factors related to human TAF6 and TAF9 and two homologs of the TATA box-binding protein-like protein 1. The recruitment of transcription initiators and regulators is a common response of organisms to stress (de Nadal et al. 2011).

Metal-related genes show an enrichment in two biological processes that are "translation" (GO:0006412) and "regulation of apoptosis" (GO:0042981). A detailed analysis of each metal showed that the enrichment in translation is linked to Zn-related genes (n = 1,547), while regulation of apoptosis was linked to Cr-related genes (n = 1,712). This last group was also enriched in genes involved in "immune response" (GO:0006955). The enrichment in the biological process translation was due to 34 ribosomal proteins and 3 elongation factors (EF1β, EF1δ, and EF1γ) that were positively correlated with Zn (Online Resource 7). Regarding enriched biological processes involving Cr-related genes, these correspond to the expression of four caspase-like proteins, one TNF-like protein, and four TNF ligand superfamily members. Amongst genes related to the condition index, the molecular function carbohydrate binding (GO:0030246) can be related to genes encoding lectin-like proteins. Lectins are proteins of non-immune origin that can recognize and bind specific carbohydrate structures. They can play a role as pattern recognition receptors, recognizing the pathogens and initiating the stress response. Their role in the innate immune response of plants and animals is well established (De Schutter and Van Damme 2015). These transcripts were positively correlated to the condition index, suggesting an increased immune competence in mussels with better health conditions.

We found no enrichment in other metal-related categories, likely due to a smaller number of genes or the lack of annotation. Yet, amongst Ni-related genes (n = 569), "transport" (GO:0006810) was the most represented biological process with 10 transcripts, out of which eight were downregulated. Cd-related genes (n = 916) were mostly related to "response to stimulus" (GO:0050896) and "protein modification process" (GO:0036211) with 31 and 20 representatives, respectively. Most of those were upregulated when Cd concentrations increased. Finally, "protein metabolism" (GO:0019538) was the most represented process in Pb-related genes (n = 187; list of genes is given in Online Resource 8).

This second approach greatly increased the number of genes showing a differential expression in the kidney. It allowed us to unmask the effect of pollutants (the accurate characterization of multiple metal mixtures and the effects of biological factors being crucial regarding the protection of aquatic systems) and revealed the important role of age in gene expression. This latter parameter should hence be better assessed prior to sampling in future studies. Age, amongst other biological parameters, was already shown to alter or mask the response to pollution in aquatic organisms (e.g., in bivalves—Canesi and Viarengo 1997; Bellas et al. 2014—or in fish—Stubblefield et al. 1999). In Table 1, mussels sampled in 2009 were significantly older downstream whereas no difference was detected in 2010. Age could then have masked the effect of metals and this could explain the reduced number of DEG in 2009 compared to 2010. Other environmental parameters linked to the dumping site and that we were not able to measure in this study could also explain these results. As stated previously, no difference in any factor was detected in 2010, yet a relatively high number of DEGs are found. This result strongly suggests the existence of additional factors. RNAseq provides a large amount of data of which the full comprehension would require to measure many environmental variables (organic pollutants, for instance) that could explain a part of the changes in transcription levels.

Differences between age classes In order to get more insights into the effect of age on the response of our mussels to metal exposure, we created three classes of age through *k*-mean clustering (R command "kmeans"). The "old" class (n = 11, mean = 53.3 years), inside which we find seven out of eight downstream 2009 individuals, was more contaminated than the middle aged (n = 11, mean = 34.3 years) and "young" (n = 10, mean = 11.7 years) groups, the young mussels being the least contaminated animals (Table 4). The largest difference was observed for Cr that shows c.a. 6-fold higher accumulation in the oldest mussels compared to the youngest. In addition to site and year, age could hence explain a large part of the differences amongst individuals in terms of contamination levels. Interestingly, the occurrence of young individuals along the Dronne River may indicate successful recruitment over the last decade. Indeed, according to Geist (2010), the vast majority of European populations are extremely overaged, with the youngest individuals usually being 30–50 years old.

It is interesting to note that after grouping the samples' expression profiles by age class in Fig. 1b, young/relatively weakly contaminated mussels exhibit a more consistent expression pattern (more densely clustered in the PCA plot) than middle-aged and old individuals. The increasing variability in expression profiles with age/contamination level may explain the difficulty in identifying differentially expressed genes.

Previous research in the laboratory has suggested that in all aquatic organisms tested, the early life stages of freshwater mussels were highly sensitive to some chemicals, including Cu, but not to others, such as some pesticides and solvents. Similar studies showed that adults were less sensitive to toxicants than glochidia and 2–4-year-old juveniles (Cope et al. 2008), but there is no information regarding potential changes in susceptibility to environmental stressors during adulthood (from 4 up to 100 years and more). Increased susceptibility of aging organisms to a particular kind of stress could be explained either by differences in lifestyle between young and old mussels—for instance, in many unionids, juveniles remain burrowed in sediment for their first 2 to 4 years, mainly relying on deposit feeding while the main exposure route in adults is the water and suspended solids overlying the sediments (Cope et al. 2008)—or by a general lowering of cell metabolism. The FAMT method was conducted on each age class separately in order to investigate how age could affect the molecular response of *M. margaritifera* to trace metals. Results revealed that young, middle-aged, and old mussels were not affected by or did not respond to the same factors (Table 5).

A total of 6,095 transcripts were differentially expressed in young mussels, amongst which 5,593 were uniquely linked to Cu. Cu is an essential micronutrient required by all living organisms, acting as a cofactor of many enzymes and a component in other proteins (Taylor and Anstiss 1999). The GO enrichment analysis revealed an over-representation of the GO molecular function "catalytic activity" (GO:0003824) in contigs linked to Cu (Table 6). Out of the 567 contigs harboring this function, the vast majority (518) were positively correlated to Cu levels in young mussels (details in Online Resource 9). This could likely be a the sign that Cu is necessary to ensure a high metabolic rate in fast-growing young mussels. This hypothesis is reinforced by the positive correlation between Cu concentration and C.I. (Online Resources 10 and 13) and the positive effect of Cu on the transcription of genes involved in "fatty acid metabolism" (GO:0006631) (Table 6 and Online Resource 9). Inversely, at elevated concentrations, Cu can be toxic to aquatic organisms by catalyzing the formation of hydroxyl radicals from hydrogen peroxide via the Haber–Weiss reaction. Amongst the different physiological and biochemical processes negatively correlated with Cu in aquatic organisms, we can cite glycolysis, Krebs cycle, ionic and osmotic regulation, acid–base balance, ammonia excretion, oxygen consumption, and growth. Most of these effects can be directly or indirectly associated with an insufficient aerobic production of energy to maintain cell metabolism and homeostasis (for references, see Lauer et al. 2012). Due to the high reactivity of ionic ("free") Cu, cells have developed complex and elegant mechanisms for shuttling Cu into, through, and out of cells as needed for its many critical reactions, without allowing

Table 4 Means ± SE of biometric measures and metal concentrations (μg per g of dry weight) in digestive glands of young, middle-aged, and old mussels. When differences were detected with a univariate ANOVA test, pairwise comparisons were assessed with a Tukey HSD test and indicated with letters a, b, and c, p value < 0.05

		Young	Middle aged	Old
Biometry	Length (mm)	80.70 ± 1.22 (a)	93.25 ± 1.60 (b)	99.42 ± 1.48 (c)
	Age (years)	11.70 ± 1.20 (a)	34.27 ± 1.55 (b)	53.27 ± 1.81 (c)
	C.I.	69.05 ± 3.40 (a)	43.79 ± 1.78 (b)	38.85 ± 1.62 (b)
Metals (μg/g dry weight)	As	5.14 ± 0.26 (a)	8.09 ± 0.57 (b)	9.01 ± 0.4 (b)
	Cd	1.00 ± 0.09 (a)	1.95 ± 0.23 (a)	4.32 ± 0.88 (b)
	Co	0.72 ± 0.07 (a)	1.4 ± 0.12 (b)	1.84 ± 0.11 (c)
	Cr	1.10 ± 0.15 (a)	3.73 ± 0.45 (b)	6.32 ± 0.94 (c)
	Cu	14.23 ± 1.12	8.97 ± 0.86	11.37 ± 2.00
	Mn	398.97 ± 86.15 (a)	1634.51 ± 231.99 (b)	1629.29 ± 243.16 (b)
	Ni	0.45 ± 0.03 (a)	0.80 ± 0.06 (b)	0.95 ± 0.13 (b)
	Pb	0.71 ± 0.09	2.38 ± 0.73	1.75 ± 0.32
	Zn	73.38 ± 2.10 (a)	77.71 ± 2.58 (a, b)	84.02 ± 2.78 (b)

free Cu to exert cytotoxic effects. Amongst these mechanisms, it has been reported that Cu could be pumped into vesicles for release or intracellular storage (Petris et al. 1996; Gupta and Lutsenko 2009; Polishchuk and Lutsenko 2013). However, the relationship between Cu and vesicle processing is not well understood. In accordance with this hypothesis, contigs linked to Cu in young mussels show an enrichment in transport processes (Table 6 and Online Resource 9). Out of a total of 77 contigs belonging to these GO categories, 27 genes that were upregulated in response to Cu were identified as regulators of vesicle trafficking such as Rab proteins (12 contigs). This suggests that the young mussels from our sampling sites are able to regulate Cu homeostasis to avoid cellular damages. Cu provides a good example of the confounding effect of age as its effect was not detected in the first FAMT test where only 22 transcripts were related to it. Strong correlations actually appear when mussels were separated based on their age. Previous studies evidenced high toxicity of Cu to unionid mussels, especially in young stages (see Young 2005). Since freshwater pearl mussels do not reach sexual maturity until an age of 10 to 20 years (Thomas et al. 2010), the comprehension of the susceptibility of this age class to Cu pollution is of crucial interest for conservation planning actions.

Table 5 Numbers of contigs correlated to a specific factor according to FAMT analyses conducted on each age class (FDR ≤ 0.05)

Factor	Age class		
	Young	Middle aged	Old
Age	1	–	4,825
CI	1	16	18
As	82	1,022	–
Cd	–	1,044	–
Co	4	1	2
Cr	1	57	476
Cu	5,593	2	1,098
Mn	–	30	–
Ni	–	944	87
Pb	15	437	–
Zn	238	2,755	–
Total	6,095	8,064	6,777

In middle-aged individuals, gene expression was influenced by many factors including Zn, Cd, As, Ni, and Pb (with 2,755, 1,044, 1,022, 944, and 437 amongst 8,064 transcripts, respectively). The redox stability of zinc (contrasting with other transition metals) coupled with its ability to form polyhedral co-ordination complexes with a variety of ligands, notably histidine and cysteine, renders zinc a very useful component of cellular proteins. Zn has functions that can be characterized in three general areas: catalytic, structural, and regulatory. Each function can be related in some way to gene expression (Cousins 1998). Zn may play a structural role, typified by the zinc-finger domains of DNA-binding proteins such as transcription factors. In terms of enzyme catalysis, all six major enzyme classes contain examples of zinc-containing proteins, such as RNA polymerases. The regulatory function of Zn is accomplished through the direct interaction of Zn-binding transcription factors to specific DNA sequences (metal response elements; MRE). This importance of Zn is confirmed by the GO analysis of transcripts correlated to Zn (Table 6) that revealed an enrichment in the terms "Gene expression" (GO:0010467) and "response to chemical / endogenous stimulus" (GO:0042221/GO:0009719) harbored by transcripts encoding ribosomal proteins (18 transcripts), transcriptions regulators (25 transcripts), RNA polymerase subunits (3 transcripts), and nuclear receptors (8 transcripts)

Table 6 Examples of enriched GO categories (corrected p value ≤ 0.05) in the different age classes. Categories are *P*, biological process; F, molecular function; and C, cellular compartment. Details are given in Online Resources 9, 11, and 12

Age class	Factor	Term_ID	Description	Category	FDR	Number of seqs
Young	Cu	GO:0003824	Catalytic activity	F	7.40e− 05	567
		GO:0071702	Organic substance transport	P	5.71e− 04	77
		GO:0033036	Macromolecule localization	P	5.71e− 04	
		GO:0015031	Protein transport	P	5.71e− 04	
		GO:0051641	Cellular localization	P	2.14e− 03	
		GO:0051649	Establishment of localization in cell	P	1.54e− 03	
		GO:0006631	Fatty acid metabolic process	P	4.09e− 02	8
Middle aged	Zn	GO:0010467	Gene expression	P	1.64e− 02	63
		GO:0042221	Response to chemical	P	2.72e− 03	11
Old	Age	GO:0010467	Gene expression	P	3.93e− 16	157
		GO:0006412	Translation	P	5.62e− 17	72
		GO:0005739	Mitochondrion	C	1.27e− 06	27

(Online Resource 11). The role of MRE-binding transcription factors in the response of marine bivalves to Zn has already been suggested (Meng et al. 2015). However, further studies are necessary to better conclude about their role in affording protective mechanisms under zinc exposure in these animals. No GO enrichment was detected for transcripts correlated with trace metals other than Zn.

Finally, gene expression in older freshwater mussels was mainly influenced by age, Cu, and Cr (4,825; 1,098 and 476 transcripts amongst 6,506, respectively). The impact of age confirms the larger dispersion of transcriptomic profiles observed in Fig. 1b for this age class. Many aspects of the "primary metabolism" (GO:0044238; 343 transcripts) were correlated with this factor, like "gene expression" (GO:0010467; 157 transcripts) and "translation" (GO:0006412; 72 transcripts) (Table 6—detailed in Online Resource 12). Aging is one of the most important biological processes in animals. Studying age-related transcriptomic changes can provide valuable information for the understanding of this fundamental process. In humans for instance, and similarly to our study, functional annotation of aging genes points to a large collection of biological processes (Yang et al. 2015). However, one frequent GO category related to aging genes is the cellular component "mitochondrion", also enriched in our results (27 sequences). Mitochondria dysfunction in aging has been observed in multiple model organisms and is amongst the most recognized aging theories (Sahin and DePinho 2010). Previous studies on long-lived bivalves revealed a higher susceptibility to environmental stressors in older age classes (e.g., in the Antarctic bivalve *Laternula elliptica*; Husmann et al. 2014) that showed reduced metabolic rates, lower survival, limited ability to burrow into the sediment, and lower oxidative defense capacities. In this species, older animals exhibited less pronounced differences in transcriptional expression, indicating that age is a significant factor impairing the transcriptomic defense response to stress. Similarly, in older *M. margaritifera*, the absence of defense responses may lead to an alteration in the age structure of the population under environmental stress. However, with a maximum life span of 190 years (maybe more according to some studies on Northern European populations; e.g., Dunca et al. 2011), this species is amongst the masters of longevity (Philipp and Abele 2010), which suggests efficient molecular adaptations for oxidative damage mitigation, such as reduced nucleic acid oxidation and higher protein resistance to unfolding (Gruber et al. 2015), and could make the freshwater pearl mussel an interesting model for studies in other fields of research such as aging.

Thus, future ecotoxicological studies in unionoids, and most probably in other long-lived bivalves, should target individuals belonging to a restricted range of size (a good proxy of age) in order to limit age-related inter-individual variability able to mask or bias the effects of a contamination, and draw out correct interpretations and adopt appropriate conservation measures.

Conclusion

We have sequenced, assembled, and annotated a transcriptome for the kidney of the freshwater pearl mussel *Margaritifera margaritifera* in order to investigate the response of this endangered species to metals likely originating from an illegal dumping site along the Dronne River, France. A classical approach only revealed a small number of differentially expressed genes between the upstream and downstream sites.

The use of a factorial approach allowed us to correlate the expression of a much larger number or transcripts to specific factors. Thereby, the age of the individuals was identified as a major factor affecting gene expression in freshwater pearl mussels, likely being a masking factor that limited the discovery of responsive genes in the classical approach. This effect was particularly observed in old individuals where primary metabolism and mitochondrial physiology seemed to be affected. Few studies have investigated the influence of age in bivalves. The induction of a transcriptomic response to specific pollutants in young (Cu) and mostly middle-aged mussels (Zn, As, Cd, and Ni) might indicate a greater resilience and a better adaptive response of animals to exposure compared to older individuals. To our knowledge, the current work is the first example of a transcriptome-wide study of age-related changes in gene transcription levels in response to metal exposure in a freshwater bivalve.

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Author contributions AB, FP, JT, CK, PG, and MB wrote the manuscript; MB designed the experiment; AB, FP, and CK analyzed the results. JT and JB performed sclerochronological analysis. All authors approved the final version of the manuscript.

Compliance with ethical standards The collection of *M. margaritifera* for this study was allowed by the prefectoral decree 60/2008 delivered by the Préfecture de la Dordogne (DIREN Aquitaine) on the 31st of October 2008. In France, no ethical permits are required to carry out research on bivalves. Therefore, all procedures were conducted according to the ethical guidelines of France to ensure ethical appropriateness.

Conflict of interest The authors declare that they have no competing interests.

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