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1 **Sensitivity to cadmium of the endangered freshwater pearl mussel *Margaritifera***  
2 ***margaritifera* from the Dronne river (France): experimental exposure**

3

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14

15 **Abstract**

16 *Margaritifera margaritifera* is a critically endangered species in Europe. Among the causes  
17 explaining its decline, metal pollution had never been deeply studied. Thus, an ecotoxicological  
18 investigation was developed on this species which comes from the Dronne river (South-West of  
19 France). Cadmium (Cd) exposure of mussels at 2 and 5 µg/L for 7 days was conducted to test their  
20 vulnerability to this metal, but also the potential endocrine disruption power of Cd. Morphometric  
21 analyses, gonad histological observations, metal bioaccumulation, metallothionein (MTs) production,  
22 measures of malondialdehyde (MDA) and finally quantitative relative expression analysis of genes  
23 involved in various metabolic functions were performed.

24 The main results showed Cd accumulation increasing in a dose dependent manner, especially  
25 in the gills. The same trend was observed for gene expression relative to oxidative stress. Histological  
26 analysis of the gonads highlighted a predominance of hermaphrodite individuals, but after 7d of  
27 exposure to Cd, the percentage of female was largely increased compared to controls, from 17 to  
28 33%. These results demonstrate the endocrine disruption effect of Cd on freshwater pearl mussels.

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30

31 The pearl mussel *Margaritifera margaritifera* is sensitive to cadmium since the metallothioneins are  
32 poorly induced, gene expression reveals oxidative stress and gonads tend to be feminized.

33

34 **Key words**

35 *Margaritifera margaritifera*, cadmium, metallothionein, gene expression, endocrine disruption,  
36 gonad histology

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**1. INTRODUCTION**

Indigenous freshwater mollusks are threatened by extinction in most parts of the world (Vaughn and Taylor, 1999; Strayer et al., 2004), and because little is known about their physiology and behavior, conservation efforts are difficult (Rotchell and Ostrander, 2003; Geist, 2010). Among them, the freshwater pearl mussel *Margaritifera margaritifera* was in the past the most common mussel type in Europe (Bauer, 1988). But populations have declined by more than 90% and only a small number of recruiting (functional) populations remain (Cosgrove and Hastie, 2001; Ziuganov, 2005; Hastie et al, 2008).

Several factors can be advanced to explain their disappearance during the XIX<sup>th</sup> and XX<sup>th</sup> centuries: pearl fishing, general deterioration of water quality, reduced host fish density, reduction of the habitats (Gumpinger et al., 2015), filling of sediments (Hauer, 2015), pollution by pesticides, phosphates and nitrogen responsible for eutrophication (Scheder et al., 2015), siltation and impoundments (Addy et al., 2012). Indeed, this species has particular ecological requirements (see appendix 1 in Varandas et al., 2013). The quality of the water must be excellent and the substrate healthy in particular for the survival of the juvenile mussels which are sensitive to the changes of their surrounding environment (Geist and Kuehn, 2005). For adults, the top of the valves (the oldest part of the mussel pearl-bearing) is the most damaged part and can prove to be fragile with the eventual chemical or physical attacks of water (Bensettiti & Gaudillat, 2004; Motte, 2005). Moreover, this mussel has a very particular life cycle since it has a very long lifespan (150 years on average in Europe) and requires the presence of a fish host. This life cycle is divided in four stages of development: glochidia (larval stage), parasitic stage, juvenile and adult stage. The sexes are normally separated, but females can become hermaphrodite when isolated and under stress (Bauer, 1987). First of all, the male releases sperm (June/July), which are trained by the current and then recovered by the female through its filtration system, after producing eggs. Fertilization produces a cell that evolves into a larva called glochidia. These larvae are incubated for four to six weeks in a part of the mussel mantle called marsupium. Then the glochidia are released into the current and, to continue their development, must be fixed in the week on the branchial system of a salmonid with a preference for brown trout (*Salmo trutta f. fario*) in central European populations (Wächtler et al., 2001). This favorable parasitic part of the cycle constitutes both a phase of larval development, but also a phase of dissemination of the species with the displacement of the host fish (Treasurer et al, 2006). Once fixed, glochidia parasitize their host for a period of several months. When they leave the gills of the host, the young mussels measure about half a millimeter and have become true bivalves. They then bury themselves in the sediment for a period of four to five years in order to continue

1 their growth and they progressively rise to position at the sediment-water interface. The fish host  
2 can also be affected by water quality and a lack in ecological continuity, which participate in the  
3 disappearance of the freshwater pearl mussel. Among pollutants introduced in aquatic systems by  
4 anthropogenic activities, metals could weaken populations of *M margaritifera* and jeopardize its  
5 survival.

6 Currently, this species is only little represented in the rivers of France. Approximately  
7 100,000 individuals are listed. The department of the Dordogne (South-West of France) shelters two  
8 of these populations, on the Bandiat and the Dronne rivers. The population of the Dronne is  
9 estimated at 15,000 individuals. Until now, the catchment area of the Dronne was relatively free of  
10 pollution. However, some observations made by agents of the AFB (Agence Française de la  
11 Biodiversité) or PNRPL (Parc Naturel Régional Périgord-Limousin) question a possible deterioration of  
12 the river quality.

13 In this catchment area, there are several potential sources of impacts (agriculture, release of  
14 suspended matter, artificial ponds which generate eutrophication,...). Among them, a source of  
15 specific pollution was listed in the commune of Saint Saud Lacoussière: an illegal waste dump which  
16 had received all kinds of waste for many years, including for instance batteries and drugs. This  
17 discharge is located on a slope on which streams the resurgence and rainwater. The site was  
18 destabilized by work and trace metals or chemicals can be brought out by scrubbing in the Dronne  
19 river downstream. However at this place the river shelters a population of approximately 1,000 to  
20 1,500 individuals of freshwater pearl mussels. An exceptional authorization (Agreement N°60/2008)  
21 delivered by the French ministry of the environment allowed us to collect several individuals in order  
22 to conduct an experiment and to evaluate if this discharge may have an impact on mussels. Recently,  
23 a study using some of these collected mussels demonstrated the presence of metals (As, Cd, Co, Cr,  
24 Ni and Zn) accumulated in tissues of *M. margaritifera* located downstream from this dump and  
25 impacting their gene expressions (Bertucci et al, 2017).

26 In fact, filter-feeders such as bivalves are known to accumulate metals and other pollutants  
27 in their tissues (Couillard et al, 1993, 1995; Baudrimont et al., 1999; Giguère et al, 2003; Marie et al.,  
28 2006a,b; Paul-Pont et al, 2010; Arini et al., 2014). After accumulation, metals can generate toxic  
29 effects, like in particular the production of oxidative stress which can poison the animal until death  
30 (Cossu et al, 2000; Gonzalez et al., 2006; Farcy et al, 2011; Gillis, 2012; Gillis et al, 2014). Among  
31 these metals, cadmium (Cd) is considered as a priority substance in Europe (European Water  
32 Framework Directive 2008/105/EC ) as it is a known toxic metal that accumulates and exerts its toxic  
33 action primarily in the kidneys (nephrotoxicity) and liver (hepatotoxic) in aquatic organisms, in  
34 conditions of chronic exposure. Cd is well known to provoke cellular or physiological dysfunctioning  
35 in varying degrees according to the species (Viarengo, 1989; Perceval et al. 2004). It is responsible for

1 a disturbance of the calcium metabolism in the freshwater pearl mussel (Frank and Gerstmann,  
2 2007), because of its ability to compete with  $\text{Ca}^{2+}$  for membrane carriers of this trace element, due to  
3 an atomic radius very close to the latter (0.97 and 0.99 Å). Cd is one of the main factor correlated  
4 with genes transcription levels, with effects on translation, apoptosis, immune response, response to  
5 stimulus, and transport pathways in *M. margaritifera* (Bertucci et al., 2017). Cd has also been  
6 described as an endocrine disruptor in fish (Pierron et al, 2008; Pierron et al, 2009), and as described  
7 in *Margaritifera margaritifera*, a gene of estrogen receptor was present (Bertucci et al., 2017) which  
8 can ask the question of this mode of action in the pearl mussel too. Among the possible lines of  
9 defense, the synthesis of small proteins such as the metallothioneins (MTs) can be induced under the  
10 influence of metals (Baudrimont et al., 2003; Amiard et al., 2006). These cytosolic proteins are able  
11 to sequester the metal ions and thus to neutralize their potential toxic impacts on other cellular  
12 targets. They are in the same way able to act directly against the generation of oxidative stress by  
13 protecting the cell from the presence of free radicals (Yang et al., 2009). This defense mechanism can  
14 be measured as well at the protein as at the gene level in tissues of aquatic organisms (Marie et al.,  
15 2006a,b; Paul-Pont et al., 2010). Among the effects of oxidative stress, the oxidative degradation of  
16 fatty acids (lipid peroxidation) at the level of the cellular membranes leads to the formation of an end  
17 product, malondialdehyde (MDA) which accumulates in the cell, and that we can easily quantify in  
18 tissues, as a lipidic marker of peroxidation (Legeay et al., 2005). Metals exert other toxic effects, like  
19 the disturbance of the mitochondrial metabolism, the apoptosis (programmed cellular death), or the  
20 attacks of the DNA. All these effects can be followed by quantitative real time RT-PCR of specific gene  
21 mRNA implied in all these functions (Gonzalez et al., 2006; Paul-Pont et al., 2010).

22 The objectives of this work were thus to study the potential impacts of cadmium on several  
23 individuals of the freshwater pearl mussel *Margaritifera margaritifera* collected around the dumping  
24 site during the reproductive period. Owing to the fact that Cd can be a potential endocrine disruptor,  
25 in parallel we exposed freshwater pearl mussels to estradiol as a positive control to compare its  
26 effect with Cd. The synthesis of detoxification proteins such as MTs was quantified, in parallel with  
27 MDA concentrations and relative gene expressions by quantitative PCR. Some histological  
28 preparations were also carried out in order to observe the gonads of the freshwater pearl mussels  
29 during Cd exposure in controlled conditions.

30

## 31 **2. MATERIALS AND METHODS**

32

### 33 2.1. Sampling of the freshwater pearl mussels in the Dronne river

34 Adult *Margaritifera margaritifera* sampling was carried out during July 2009, upstream and  
35 downstream from the illegal waste dump of Saint Saud Lacoussière (44°33.019; 44°51.008), following

1 a request for authorization of sampling which was given by the DIREN (French Ministry of the  
2 Environment, Agreement N°39/2016) in 2008.

### 4 2.2. Experimental study of Cd exposure during the reproductive period

5  
6 Thirty individuals of *M. margaritifera* were used to conduct the exposure of mussels to Cd under  
7 controlled conditions. Pearl mussels were  $89 \pm 7$  mm long (ranging from 70 to 99 mm) and their  
8 mean age was estimated at 39 years old according to Bertucci et al., 2017. Five experimental  
9 conditions were performed, with 6 individuals per experimental unit chosen in order to have the  
10 same average size in each of them: « Upstream control, UC », corresponding to individuals collected  
11 upstream from the wild waste dump; “Downstream control, DC”, corresponding to individuals  
12 collected downstream from the wild waste dump ; Cd exposure in a direct way with 2 or 5 µg/L with  
13 individuals coming from upstream of the wild waste dump; and estradiol exposure with 100 µg/L  
14 with individuals coming from upstream of the wild waste dump, in order to get a positive control of  
15 endocrine disruption, and notably of reproductive hormones (Flynn and Spellman, 2009). The  
16 contamination exposure lasted 7 days, after an acclimatization period of 14 days. Note that just  
17 before the beginning of the experiment, one individual died in the DC experimental unit,  
18 corresponding to only 3% of mortality for the entire experiment.

19 An experimental glass unit (volume = 40 L) consisted of 7 cm of ultrapure sand SILAQ (0,7-1,4 mm)  
20 which was rinsed beforehand and deposited on the bottom of the unit previously protected with a  
21 food standard plastic bag (Plastiluz). Each unit was filled using water of the Dronne river taken  
22 upstream from the dump and was placed at 19°C, near to the temperature measured in the river at  
23 this period, using a system of thermic regulation. The photoperiod was controlled with 16h/8h  
24 light/dark and oxygenation was ensured thanks to a system of ventilation by air pumps. Water was  
25 permanently filtered in order to avoid the intoxication of the freshwater pearl mussels by their own  
26 waste and the mussels were fed for the period of acclimatization using phytoplanktonic green algae  
27 *Scenedesmus subspicatus*. Cd was added in the experimental units every day using a stock solution  
28 of CdCl<sub>2</sub>. The concentration was measured in water samples collected every morning and adjusted  
29 daily by the compensated additions method, in order to maintain it constant for all the duration of  
30 the experiment. Temperature, pH and oxygen concentrations were daily measured in the  
31 experimental units and remained constant (T°C =  $18.76 \pm 0.13$ ; pH =  $7.2 \pm 0.05$ ; O<sub>2</sub> =  $9.11 \pm 0.03$   
32 mg/L).

### 34 2.3. Cd bioaccumulation in tissues of *Margaritifera margaritifera*

1 Cd bioaccumulation in 3 tissues of mussels (gills, visceral mass and rest of the body) was analyzed by  
2 atomic absorption spectrophotometry (AAS) with graphite furnace (M6 solar AA spectrometer,  
3 Thermoptec). After dissection, weighing and drying at 50°C during 48h00, samples were analyzed.  
4 Tissues were first digested for 3h at 100°C after addition of nitric acid 63% (3mL for 100 mg of dry  
5 tissue) in polypropylene tubes. Samples were diluted 6 fold with pure water (15 mL) and stored at  
6 4°C until analysis. For each mineralization procedure, 6 certified samples (50 mg of Tort-2 - Lobster  
7 Hepatopancreas Certified Reference Material for Trace Metals - and 50 mg of Dolt-3 - Dogfish Liver  
8 Certified Reference Material for Trace Metals -, NRC-CNRC) and 3 blanks were analyzed in order to  
9 test the validity of the method. The values obtained were systematically within the expected range.  
10 Results are expressed in µg of metal per gram of dry tissue. The detection limit is 0.1 µg/L for Cd.

11

#### 12 2.4. Histological analyses

13 In order to determine precisely the sex of individuals, histological analyses were carried out on all the  
14 individuals used for the experiment (6 individuals per condition). Samples of gonads not exceeding 5  
15 mm thickness were delicately cut using a scalpel, in a standardized manner. The fixing of tissues was  
16 carried out immediately after sampling by immersion of samples in liquid of Bouin (picric acid 0.9% +  
17 formol 7.4% + acetic acid 5%) for one minimal period of 48 H at 4°C. After several rinsings, the  
18 samples were dehydrated by successive alcohol baths of increasing degree. Complete dehydration  
19 was obtained by immersion of the samples during one day in a pure butanol bath renewed every 2 h.  
20 The samples were then placed in three successive paraffin baths (56°C) at a rate of 15 min per bath.  
21 At the end of the last paraffin treatment, samples were left on the straw mattress at ambient  
22 temperature. After solidification, blocks of paraffin were cut out in ribbon of 10 µM thickness using a  
23 microtome (Reichert). Before coloring, the paraffin was eliminated by immersion of the blades in two  
24 baths of xylene for 5 min. The cuts were then rehydrated. Gonads were colored according to the  
25 Hematoxylin/Eosin method described by Gabe (1968). Determination of the stage of evolution:  
26 Stage 0: sexual rest; Stage 1: proliferation of the gonies; Stage F: female sexual maturity; Stage MF:  
27 male and female sexual maturity.

28

#### 29 2.5. Metallothioneins (MTs) quantification

30 Aliquots of tissues of *M. margaritifera* stored beforehand at -80°C under nitrogen atmosphere were  
31 analyzed by the method of saturation by inorganic mercury, coupled with Hg determination by cold  
32 vapor AAS (Baudrimont et al., 2003). After defrosting and drying on absorbing paper, the tissues  
33 aliquots were weighed then immersed in 500 µL to 1 mL of Tris 25mM (pH 7,2) buffer. Under  
34 nitrogen atmosphere, they were homogenized using an ultra-Turrax mechanical crusher (IKA T10).  
35 The homogenates were centrifuged to 20,000 g at 4°C during 1h, the supernatants were then

1 saturated with mercury (50 mg/L Hg (II) - TCA 10%). The mercury not fixed to MTs was recovered by  
2 addition of porcine hemoglobin in excess. After centrifugation of 20 min to 20,000 g, the supernatant  
3 containing only MTs fixing mercury was recovered, weighed, and 100  $\mu$ L were analyzed by cold vapor  
4 AAS under O<sub>2</sub> flow (LECO AMA 254, Altec, Prague, Czech Republic) in order to determine the  
5 quantities of mercury contained in the samples. The detection limit of the device is 1 ng mercury. By  
6 calculation, the number of nmoles of sites having fixed Hg per gram of fresh weight of sample was  
7 deduced as an estimation of the MT concentration.

## 8 9 2.6. Malonedialdehyde (MDA) determination

10 In experiments, 200  $\mu$ L of supernatant was mixed with 650  $\mu$ L of solution of NMPI (N-methyl-2-  
11 phenylindole, according to the LPO-586 method), the whole was homogenized using a vortex, then  
12 150  $\mu$ L of chloridric acid (HCl) 12N was added. The mixture was incubated 1h at 45°C then  
13 centrifuged at 15,000 g at 4°C during 10 min. The supernatant was recovered and its absorbance  
14 (corresponding to the presence of carbocyanine formed by the presence of a molecule of MDA and  
15 two molecules of NMPI) was measured by spectrophotometry at 586 nm, thus giving the quantity of  
16 MDA present in  $\mu$ mol per liter of supernatant. To be able to compare various samples, the  
17 concentration of MDA was reported to the quantity of proteins ( $\mu$ mol per gram of protein; Legeay et  
18 al., 2005).

## 19 20 2.7. Gene analyses

### 21 22 *2.7.1. Total RNAs extraction*

23 Total RNAs were extracted from 20 to 40 mg of gills (preserved beforehand in RNA-later and glycerol  
24 at -80°C) by using the agilent kit "Absolutely RNA Miniprep Kit" following the manufacturer's  
25 instructions.

### 26 27 *2.7.2. Reverse transcription of RNA into cDNA*

28 Reverse transcription was carried out with the "Stratascript first strand synthesis system" kit  
29 (stratagene) using total RNA (3 to 5  $\mu$ g). 1  $\mu$ L of oligo-dT (1  $\mu$ M), 1  $\mu$ L of hexanucleotides (1  $\mu$ M), 0.8  
30  $\mu$ L of dNTP (10 mM) and 2  $\mu$ L of 10X activity buffer was added to the RNA. This mix spent 5 minutes  
31 at 65°C in the thermocycler in order to linearize RNAs. Then, 1  $\mu$ L of reverse transcriptase (1 U) and  
32 0.5  $\mu$ L of RNase-block (0.5 U) are added then heated at 42°C during 1h with the thermocycler to  
33 allow reverse-transcription.

34



### 1                   2.7.3. *Real time quantitative PCR (qPCR)*

2   The qPCR was carried out using the thermocycler LightCycler (Roche) and LightCyclerRun software  
3   5.32. In this device, the follow-up in real time of the quantity of products of amplification is made  
4   possible by the addition of an intercalating agent: SybrGreen in the reactional medium. The reaction  
5   takes place in capillaries of glass of 20  $\mu$ L prepared with: (I) 17  $\mu$ L of a mix of reagent consisted 12.8  
6    $\mu$ L water, 3.2  $\mu$ L of  $MgCl_2$  (25 mM) and 1  $\mu$ L of reactional mixture (Taq with 5 U/mL, dNTP, 10X buffer  
7   and SybrGreen); (II) 2  $\mu$ L of a mix primers with 3  $\mu$ M specific from each gene (Table 2) and (III) 1  $\mu$ L of  
8   cDNA resulting from the RT. The protocol used is as follows: activation of the enzyme (95°C, 10 min),  
9   then 50 cycles of PCR (denaturation 95°C, 5s; fixing of the primers 60°C, 5s and elongation 72°C, 20s).  
10   Reaction specificity was determined for each reaction from the dissociation curve of the PCR  
11   product. This dissociation curve was obtained by following the SyberGreen fluorescence level during  
12   gradual heating of the PCR products from 60 to 95°C. Relative quantification of each gene expression  
13   level was normalized according to the  $\beta$ -actin gene using the  $2^{-\Delta CT}$  method as described by Livak and  
14   Schmittgen (2001).

### 15                   2.9. Statistical treatment of data

16   The data processing was carried out using Statistica software (Statistica 12). After checking of the  
17   homogeneity of the variances, the factors “conditions” and “tissues” were tested by a two-way factor  
18   ANOVA or a one-way ANOVA for histological analyses. In the event of significance for one or the  
19   other of the factors taken into account, the mean comparison test (LSD Fisher) was carried out. In the  
20   event of nonconformity of the homogeneity of the variances, the non-parametric test of Kruskal  
21   Wallis was used in order to detect the significant differences between the sites or the tissues or  
22   conditions and this, for each parameter. The different letters mentioned on the graphs correspond to  
23   significant differences with the threshold  $\alpha = 0.05$ .

## 24                   25                   26                   27   **3. RESULTS AND DISCUSSION**

### 28                   29   3.1. Cadmium bioaccumulation

30   When exposed during seven days to Cd, freshwater mussels showed significant accumulations  
31   in all the studied organs. In gills, Cd concentrations reached values of 12.4 and 17.2  $\mu$ g/g (dw) under  
32   the conditions 2 and 5  $\mu$ g/L respectively, compared to 1.1  $\mu$ g/g for the upstream control individuals  
33   (Figure 1A). These accumulations corresponded to a direct route which involved exposure to the  
34   metal transferred from the dissolved phase of the water through the gill epithelia during filtration for  
35   respiratory and nutritional purposes. Cd bioaccumulation was also effective in the visceral mass and

1 the rest of the tissues, but less pronounced, attesting a blood transfer of Cd from the gills to the  
2 internal organs. Such concentrations were dependent on the exposure dose with highest values  
3 measured for the condition of 5 µg/L compared to 2 µg/L, especially in the gills and the rest of the  
4 body. In contrast, concentrations measured in the conditions "Downstream Control" and "Estradiol"  
5 showed no accumulation of this metal. The freshwater pearl mussel *M. margaritifera* therefore  
6 accumulated relatively large quantities of Cd, since in seven days, it multiplied its concentration in  
7 the gills by a factor of about 15. According to Frank and Gerstmann (2007) the freshwater pearl  
8 mussel indeed accumulated trace metals including Cd easily, with concentrations in Germany of  
9 about 50 µg/g (dw) in some contaminated sites in the visceral mass, compared to measures  
10 conducted in Finland, showing concentrations of this metal around 7 µg/g (dw) in these tissues. By  
11 comparison, here we measured values in control individuals acclimated for 14 days in the laboratory  
12 at around 2.5 µg/g (dw), which appears to be relatively low and consistent with measurements made  
13 *in situ* in the Dronne river upstream from the dump (Bertucci et al, 2017).

14

### 15 3.2. Metallothionein response

16 In freshwater pearl mussels *M. margaritifera*, concentrations of MTs measured in organs  
17 exposed to Cd 2 or 5 µg/L, showed a weak response to this metal in comparison to the condition  
18 "Upstream Control", which seemed very surprising given the bioaccumulation results of this metal  
19 for the species (Figure 1B). This means that unlike most species, the freshwater pearl mussel seemed  
20 to react very little in terms of detoxification by these proteins in the presence of Cd after a 7 days  
21 exposure period. This result could suggest a particular sensitivity of the freshwater pearl mussel to  
22 this metal, given the very strong bioaccumulation levels measured in this species in the field (Frank  
23 and Gerstmann, 2007).

24 However, a strong induction of these proteins was measured in the visceral mass of *M.*  
25 *margaritifera* downstream from the dump on the Dronne river. The induction of these proteins was  
26 at a factor of 3 compared to individuals from upstream, which was very high. Given the absence of  
27 Cd accumulation in these individuals downstream relative to those upstream, we were not able to  
28 explain this very strong induction which was probably related to the presence of other metals as  
29 observed by Bertucci et al (2017) *in situ*, or organic contaminants.

30

### 31 3.3. Malondialdehyde (MDA) quantification

32 MDA is a marker of oxidative degradation of membrane lipids (peroxidation) following the  
33 generation of oxidative stress. The accumulation of this molecule demonstrates a severe breach of  
34 physiological functioning at the cell level, which can lead to death. In our experiment under  
35 laboratory conditions, we did not observe any accumulation of this marker, whatever the conditions

1 studied (data not shown). This result is positive in the sense that it shows no severe toxic attack of Cd  
2 after 7 days of laboratory exposure on membrane lipids.

3

#### 4 3.4. Gene expression

5 Five major genes were analyzed (Table 1). Whatever the organ, the expression of the main  
6 gene involved in mitochondrial metabolism (*cox1*) was higher in individuals from downstream  
7 compared to upstream individuals (Table 2). For the *mt* gene, the highest overexpression was noted  
8 in the kidney with a very significant induction factor (1085), followed by the gills where this gene was  
9 found 36 times more expressed than in individuals from upstream, and the visceral mass (15 fold  
10 higher). The *mt* gene expressions are consistent with MT synthesis we observed before, notably in  
11 the visceral mass. Some differences are on the contrary observed in the gills where MT proteins are  
12 not significantly produced compared to upstream despite a significant gene induction. These results  
13 can be explained by the delays between gene expression and MT production, which was already  
14 observed in mollusks (Paul-Pont et al, 2010). In gills, a higher expression of the *sodMn* gene (factor 6)  
15 was also demonstrated. The results in these two groups of individuals suggested different levels of  
16 basic expression. This suggests that animals from downstream were probably adapted to  
17 contamination, but the nature of which remains unclear. This contamination impacted the  
18 mitochondria and detoxification systems. The fact that the gills and kidney were the two most  
19 affected organs suggests contamination by both the direct and trophic routes. Indeed, gills constitute  
20 the first biological barrier crossed by contaminants present in the water. The kidneys in turn are the  
21 organs involved in the elimination and thus the detoxification of these compounds. The fact that  
22 *sodMn* was overexpressed in the gills of downstream individuals also strengthens this hypothesis.  
23 This overexpression reflects a greater generation of ROS in the mitochondria of this organ.

24

25 The expression levels observed in conditions of Cd contamination were compared with those  
26 obtained from control individuals from upstream. A dose-dependent response was determined in the  
27 three organs studied. For example, in the gills Cd caused overexpression of *cox1* and *mt* genes, but  
28 also a repression of cytoplasmic *sod*. The same trends were found in the kidney. However, once  
29 again, the factors of inductions raised for this organ showed the highest values; 22 compared to 18  
30 for the *mt* gene and 181 compared to 19.5 for the *cox1* gene in the kidney and gills respectively after  
31 exposure to 5 µg/L. In the visceral mass, the same dose dependent overexpression of mitochondrial  
32 and detoxification genes was found. However, an induction of *sodMn* for a factor of 6 was also  
33 visible. These results were consistent with previous exposure to Cd studies in model organisms such  
34 as the zebrafish *Danio rerio* (Gonzalez et al, 2006; Orieux et al, 2011), the amphibian *Xenopus laevis*  
35 (Mouchet et al, 2006) or bivalves *Corbicula fluminea*, *Crassostrea gigas* or even *Cerastoderma edule*

1 (Baudrimont et al, 2003; Legeay et al, 2005; Marie et al, 2006a, b; Paul Pont et al, 2010). Thus, the  
2 production of defense proteins such as metallothioneins allows the sequestering of large quantities  
3 of metal ions at intracellular level in order to reduce the toxicity of these elements with respect to  
4 other cellular targets. These proteins are inducible by many other factors, such as cytotoxic agents,  
5 reproductive factors, the presence of parasites, bacterial infections, other xenobiotics etc ... (Kägi,  
6 1991; Baudrimont et al, 1997; 2006), which leads to then being considered more as representative of  
7 a comprehensive response to stress (Amiard et al., 2006). Cd, once in the cell, is able to inhibit the  
8 transfer of electrons along the mitochondrial respiratory chain, but also to induce the production of  
9 reactive oxygen species (Wang et al 2004; Gonzalez et al, 2006). The oxidative stress thus generated  
10 is at the origin of DNA breaks and induction of apoptotic mechanisms leading to cell death (Bagchi et  
11 al 2000; Chan and Cheng 2003; Mouchet et al, 2006). The differential expression observed in *M.*  
12 *margaritifera* can account for these phenomena.

13 Unlike the Cd, the response to estradiol was fundamentally different depending on the  
14 organ. In the gills, a single increase of the 12S gene (factor 4), indicating a greater number of  
15 mitochondria and therefore an increasing energy demand, was visible. Instead, repression of this  
16 gene and of the *sod* gene was observed in the kidneys, associated with an overexpression of *coxI*.  
17 This suggests an inhibitory effect of estradiol on this organ. The increase of *coxI* surely compensates  
18 for this decrease in activity and thus maintains a sufficient level of ATP in the cells. These results are  
19 consistent with studies of estradiol and its metabolites which showed that these compounds can  
20 inhibit mitochondrial respiration (Hagen et al, 2004). In the kidney, estradiol caused in hamsters,  
21 through deletion and mutation mechanisms, a decline in the number of copies of mitochondrial DNA  
22 leading to a decrease in energy capacity of these organelles and an increase in the sensitivity of the  
23 kidney (Bhat, 2002). Thus, this type of damage would be involved in the onset and progression of  
24 cancers in this organ. In the liver, only *coxI* and *sodMn* genes were found induced after exposure to  
25 estradiol, suggesting a greater demand for energy and the generation of oxidative stress in  
26 mitochondria. In this context, the production of ROS has recently been observed in the metabolism  
27 of estradiol, especially in the liver (Spencer et al, 2012). This production can generate ROS damage to  
28 DNA.

29

### 30 3.5. Histological analyses

31

32 The histological analysis of gonads was performed on individuals from this experiment of exposure to  
33 estradiol and Cd to evaluate the potential endocrine disruptive effect of Cd, compared to the positive  
34 control condition represented by "Estradiol" condition (Table 3).

1           The results obtained allowed us to demonstrate for control individuals, whether from  
2 upstream or downstream, a predominance of hermaphrodite individuals (0% of female individuals).  
3 It is largely accepted that freshwater pearl mussels have separate sexes, however, in adverse  
4 conditions (poor water quality, for example, or low population density), the female individuals can  
5 develop hermaphroditism to maintain their reproduction (Bauer, 1987). Concerning the mussels of  
6 the Dronne river, it seems that we are in this situation, which is positive in the sense that these  
7 individuals are able to reproduce (mature gonads oocytes and sperm - Figure 2) contrary to what we  
8 would have expected following the direct observation of the gonads (data not shown) showing a  
9 predominant presence of female individuals. However, these results suggest a reaction or adaptation  
10 of populations to their current location in the river, which can be judged in a rather unfavorable  
11 manner. We can also add from this point of view that the estradiol and Cd (2 and 5µg/L) conditions  
12 of exposure are less adverse conditions than downstream condition, which is also confirmed by gene  
13 expressions (Table 2). Nevertheless, we must consider carefully these first results since only 11  
14 individuals were analyzed (6 in the condition "Upstream Control" and only 5 in the condition  
15 "Downstream Control").

16           For the results obtained from exposure to estradiol, the female sex hormone stimulating  
17 oocyte development, we saw that after only 7 days' exposure, the percentage of strictly female  
18 individuals was largely increased compared to control conditions (33% against 0%). We have  
19 therefore a marked effect of this hormone on the oocyte development of *M. margaritifera*, as has  
20 already been demonstrated by Flynn and Spellman (2009) in the freshwater bivalve *Elliptio*  
21 *complanata*. Following exposure to Cd, we observed the same phenomenon, with a range effect of  
22 this metal, since the percentage of female individuals increased from 17 to 33% for 2 and 5 µg/L  
23 respectively. From this analysis we can observe that the Cd mimics the effect of estradiol on the  
24 development of female sexual characteristics of the freshwater pearl mussel, which attests to an  
25 endocrine disruptor effect in this bivalve. This is important given the effects already observed  
26 previously in terms of cell and molecular impact on the tissues of the freshwater pearl mussel, and  
27 facing significantly higher accumulations of Cd downstream relative to upstream from the waste  
28 dump of St Saud Lacoussière on the Dronne river in April 2009. Measured Cd accumulation still  
29 remained low compared to levels found in Germany or Finland, but may become a problem,  
30 especially concerning endocrine disruption, because of other metals accumulated in parallel in the  
31 field, or other potential contaminants, including organics that might amplify this phenomenon as  
32 previously observed in freshwater mussels exposed to municipal effluents (Blaise et al, 2003; Gagné  
33 et al, 2004).

34

#### 35 **4. CONCLUSION**

1            This study features a novel approach in the potential toxicological impacts of trace metals,  
2 and especially Cd, on the freshwater pearl mussel *Margaritifera margaritifera* from the Dronne river  
3 in France. We measured metallothionein concentrations and malondialdehyde production for the  
4 first time in this species and we quantified the relative expression of 5 genes implied in mitochondrial  
5 metabolism, oxidative stress and detoxification. The results showed, after Cd exposure of the  
6 freshwater pearl mussels in the laboratory, significant ability of mussels to accumulate this metal  
7 after only 7 days of exposure, but at the same time, the relative slow response of MTs (only at the  
8 gene level but not at the protein level) suggesting a certain sensitivity of these mussels to metals. We  
9 observed also that the majority of the mussels from the Dronne river seemed to be hermaphrodite,  
10 probably in relation with the decrease of the water quality of the river or with the low density of the  
11 population. But after exposure to Cd or estradiol, we observed an increase of the amount of female  
12 individuals, confirming for this species the endocrine disruptor role of Cd. These results thus bring  
13 new knowledge on the sensitivity of this species to metal contamination, which can help to improve  
14 reintroduction strategies on the European scale, taking into account the presence of this type of  
15 contamination in the field.

16

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21 analyses.

22

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- 15

- 1 Table 1: Nucleotidic sequences of the primer-pairs used for the qPCR of *Margaritifera margaritifera*.  
 2 a: sens primer, b: anti-sens primer

Function	Gene	Nucleotidic primer sequence (5'-3')
Reference gene	<i>β-actin</i>	TATCCACGCTCCGTGAGG <sup>a</sup> GTTGGGATTCAGGCTGTGTT <sup>b</sup>
Oxydative stress	<i>sod</i>	ATGTGCATGAATTTGGAGATAAACT <sup>a</sup> TTGGCCCTGTGAGGGT <sup>b</sup>
	<i>sodMn</i>	TGGGAGGTGCTCAGCC <sup>a</sup> GTAGTGTTTATACGCAGTCTGCC <sup>b</sup>
Mitochondrial metabolism	<i>12S</i>	CTTGCTCAGAGAAGACCCAACA <sup>a</sup> GGAGTGACGGGCGATT <sup>b</sup>
Detoxification	<i>mt</i>	AGATTTTGAAGCACCGAAGATGT <sup>a</sup> CTGTGCAGCCCTACCA <sup>b</sup>

3

- 4 Table 2: Differential expression factors relative to control individuals (> 1 induced, < 1 repressed) in  
 5 the organs of *Margaritifera margaritifera* (n = 6) after exposure (i) to the water of the Dronne river  
 6 downstream an illegal waste dump, (ii) to Estradiol at 100 µg/L and (iii) to cadmium at 2 and 5 µg/L  
 7 during 7 days.

	Downstream	Estradiol	Cd 2 µg/L	Cd 5 µg/L
<b>Gills</b>				
<i>sod</i>	0.51	0.15	0.34	0.12
<i>sodmt</i>	6.00	1.34	0.62	0.55
<i>12S</i>	1.76	3.85	0.56	7.63
<i>mt</i>	36.32	0.99	3.65	18.12
<i>coxI</i>	3.31	1.36	4.41	19.51
<b>Digestive gland</b>				
<i>sod</i>	0.47	0.72	0.71	0.64
<i>sodmt</i>	1.94	4.33	6.05	1.77
<i>12S</i>	16.88	2.26	0.96	0.64
<i>mt</i>	15.19	1.60	4.77	9.38
<i>coxI</i>	10.51	6.52	3.41	7.44
<b>Kidney</b>				
<i>sod</i>	0.51	0.04	0.81	0.48
<i>sodmt</i>	1.08	0.80	0.33	0.73
<i>12S</i>	13.67	0.17	0.82	1.37
<i>mt</i>	1085.04	0.57	6.81	22.48
<i>coxI</i>	3.31	2.61	35.25	181.20

8

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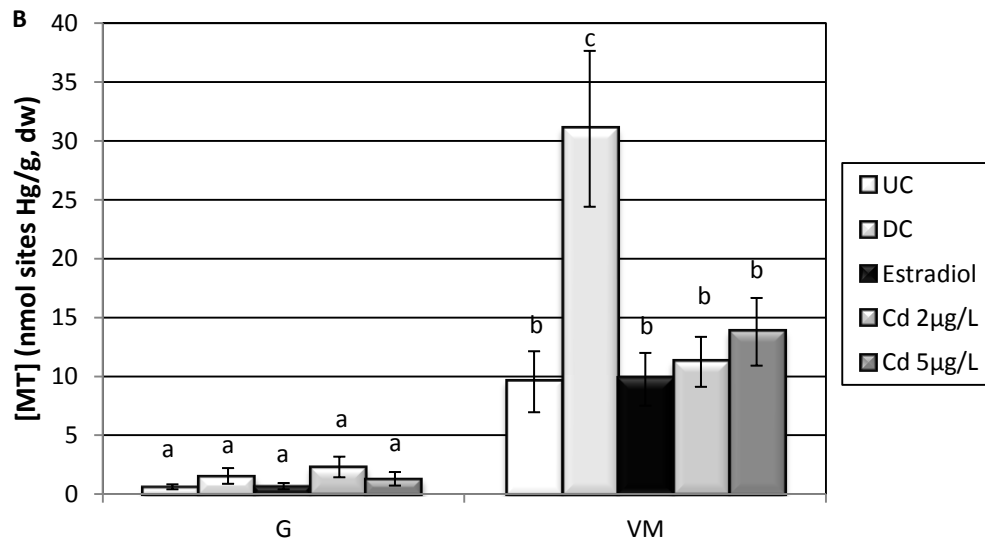
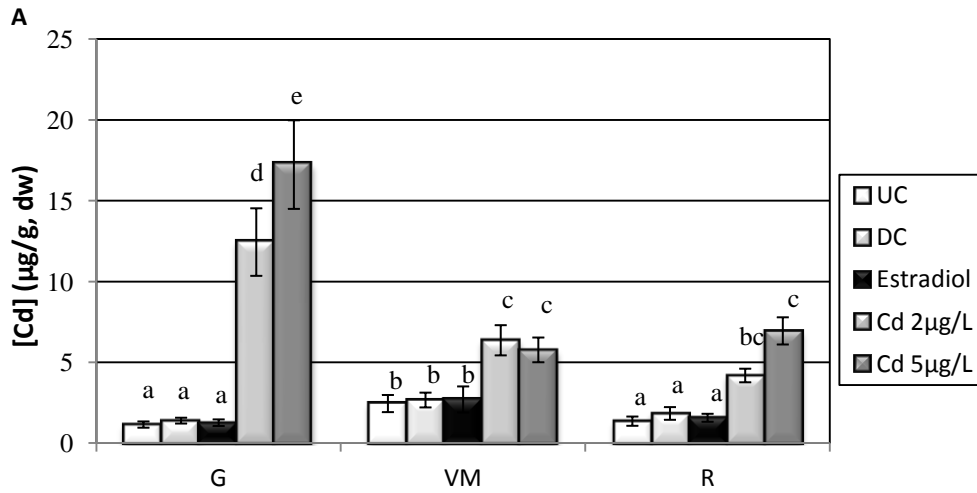
13

- 1 Table 3: Sex determination of individuals of *Margaritifera margaritifera* according to histological
- 2 analyses. The percentage of females is significantly dependent of the condition (ANOVA,  $p = 0.0277$ ).

<b>Condition</b>	<b>Hermaphrodites</b>	<b>Females</b>	<b>Undetermined</b>	<b>Percentage of females</b>	<b><i>n</i></b>
<b>Upstream</b>	6	0	0	0	6
<b>Downstream</b>	4	0	1	0	5
<b>Estradiol</b>	4	2	0	33	6
<b>Cd 2 µg/L</b>	5	1	0	17	6
<b>Cd 5 µg/L</b>	4	2	0	33	6

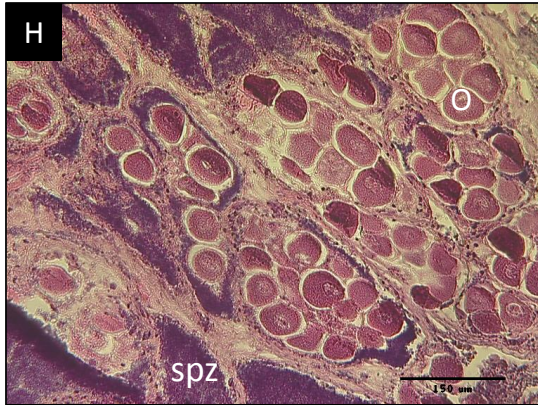
3

1 Figure 1: Cadmium bioaccumulation (A) and metallothionein concentrations (B) in tissues (G: gills,  
 2 VM: visceral mass and R: rest of the tissues) of *Margaritifera margaritifera* for control individuals  
 3 upstream (UC) and downstream (DC) the wild waste dump in the Dronne river, the exposure  
 4 conditions with estradiol (100 µg/L) and Cd (2 and 5 µg/L) (mean ± SE, n = 6). The letters on the graph  
 5 indicate significant differences between conditions.

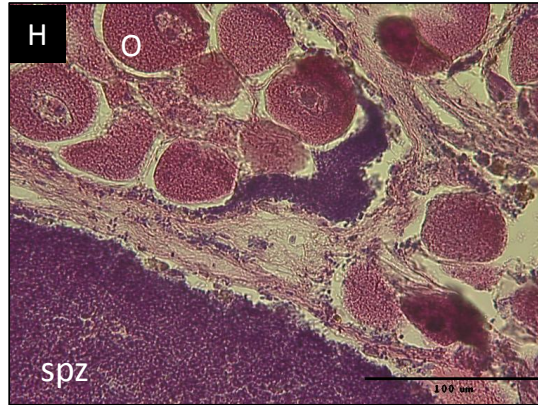


- 1 Figure 2: Histological observations of the gonads of freshwater pearl mussels *Margaritifera margaritifera* in function of the different exposure conditions : Upstream control, Estradiol, Cd 5µg/L.
- 2
- 3 H : hermaphrodite individual, F : female, O : oocytes, SPZ : spermatozooids.

Upstream Control



Upstream Control



Estradiol



Estradiol



Cd 5 µg/L



Cd 5 µg/L



- 4
- 5
- 6
- 7