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## Identification and organ-specific patterns of expression of two metallothioneins in the sentinel species *Gammarus fossarum*

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### ABSTRACT

Metal pollution is a major concern for aquatic environments. Widespread contamination by various trace metal ions has been described in freshwater streams as well as their subsequent bioaccumulation, potentially leading to toxicity and trophic transfer. Metallothioneins constitute an evolutionary conserved family of low molecular weight, cysteine-rich, metal-chelating proteins, whose known physiological functions are the maintenance of the homeostasis of essential metals, the detoxification of non-essential metals, and the protection against oxidative stress and free radicals.

In this study, we identified two metallothionein-coding transcripts, *mt1* and *mt2*, in the transcriptome of the amphipod *Gammarus fossarum*, a sentinel species widely used to assess the quality of watersheds. For the first time, we investigated the organ-specific patterns of expression of these two *mt* transcripts at the individual level in the gills and the caeca of this small crustacean. *In silico* analysis and experimental exposures to environmentally relevant concentrations of cadmium, zinc and silver showed that *G. fossarum mt1* induction is stronger after Cd exposure compared to the other tested metals. *G. fossarum mt1* was more significantly induced in the caeca than in the gills of exposed organisms for any metal exposure, while *G. fossarum mt2* was, at least at the individual level, more inducible in the gills than in the caeca of *G. fossarum* exposed to Cd and Zn.

Our results provide new genetic resources that will help to improve the understanding of metal homeostasis in this sentinel species.

### 1. Introduction

Metal pollution has become a major concern for aquatic environments. Widespread contamination by various metal ions has been described in freshwater streams as well as their subsequent bioaccumulation, potentially leading to high toxicity and trophic transfer (Alric et al., 2019; Idrus et al., 2018; Liu et al., 2021). In particular, some of these metals, such as cadmium, mercury, nickel and lead are considered priority substances to be monitored in Europe (EU, 2000). Others, such as zinc or copper, are considered as essential metals as they are cofactors of several proteins and enzymes, but they may exert toxic effects at high concentrations, notably for aquatic animals (Slobodian et al., 2021; Liu et al., 2021; Prato et al., 2021).

In order to assess the environmental exposure to chemicals and to improve spatial and time comparisons of bioavailable contamination levels, sentinel species, such as the amphipod *Gammarus fossarum*, are increasingly used in active biomonitoring campaigns (Chaumot et al., 2015; Alric et al., 2019; Babut et al., 2020). This freshwater crustacean species is of particular interest for its ability to accumulate efficiently metals (Alric et al., 2019), thus being an organism key to identify the sources and origins of contamination in freshwater ecosystems (Sarkis et al., 2020). Multiplexed targeted mass spectrometry has recently fostered the development of protein biomarkers for *in situ* application in *G. fossarum* (Gouveia et al., 2017; Gouveia et al., 2019; Faugere et al., 2020; Leprêtre et al., 2022a, 2022b). However, mass spectrometry methods targeting specific protein families, especially small (<10 kDa)

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polypeptides, are only available for some human proteins (Mehus et al., 2014; Gunn et al., 2021). Moreover, little is still known about the relations between external exposure to a given pollutant and its distribution and molecular effects across different tissues and organs, notably in small invertebrate species. As an example, recent works have characterized the toxicokinetics of Cd and Zn ions in various organs of *G. fossarum* using radioactive isotopes, thus providing a multi-compartment model of Cd bioaccumulation (Gestin et al., 2021). Indeed, there is an increasing interest in identifying specific molecular biomarkers that may provide insights on tissue-specific toxicity (Forcella et al., 2022; Pinto et al., 2022) or specific chemical exposures (Wu et al., 2019; Reinwald et al., 2022).

A number of molecular defence mechanisms, such as oxidative stress response, DNA repair, or metal trafficking, might contribute to the response of living organisms towards metal exposure and toxicity (Amiard et al., 2006; Janssens et al., 2009). Among them, the role of a family of metal-binding proteins, known as metallothioneins, is evolutionarily conserved across the tree of life (Luo et al., 2020). Metallothioneins constitute a heterogeneous superfamily of low molecular weight, cysteine-rich, metal-chelating proteins (Capdevila and Atrian, 2011). Their main physiological functions are the maintenance of the homeostasis of essential metals such as zinc and copper, the detoxification of toxic metals such as cadmium or mercury, and the protection against oxidative stress and free radicals (Capdevila and Atrian, 2011; Isani and Carpenè, 2014). Metal preference is one of the criteria used for MTs classification, leading to the identification of zinc, copper or even cadmium thioneins. Metal-specific gene-induction or metal ion recovery in native protein preparations are some of the strategies used to classify MTs, since identifying the sequence features determining metal specificity is still challenging in this diverse protein family (Capdevila and Atrian, 2011; Luo et al., 2020).

In invertebrates, MTs are often quantified either directly at the transcript level using RT-qPCR, or indirectly by spectrophotometric or size-exclusion chromatography quantification of a partially purified metalloprotein fraction extracted from tissue homogenates (Viarengo et al., 1997; Pereira Righi et al., 2022; Geffard et al., 2010; Cosio et al., 2021). A recent transcriptomic study in the giant river prawn *Macrobrachium rosenbergii* has shown different expression patterns of several genes involved in the response to Cd exposure in the gills and the hepatopancreas (Liu et al., 2021). In particular, one MT was found more induced in the gills than in the hepatopancreas after 3 h and 3 days of exposure at 80 µg/l of Cd<sup>2+</sup> (Liu et al., 2021). Another study in the mangrove crab *Ucides cordatus* provided the response of few different biomarkers (e.g. DNA strand breaks, micronucleates cell or metallothioneins) together with lead bioaccumulation in two different population of crabs, showing that environmentally exposed organisms have different bioaccumulation and detoxification capacities, a possible strategy for developing a tolerance to this exposure (Duarte et al., 2020). In another study on the parasitic crustacean *Lamproglana clariae* (Crustacea:Copepoda), concentrations of MT in both host (the teleost fish *Clarias gariepinus*) and parasite correlated with site pollution (Ndaba et al., 2022). In other invertebrates, such as the bivalve *Mytilus coruscus*, MT content decreases in the gills when the mussels are co-exposed to 0.31 mg/l of Cd and 50 µg/l of benzo(a)pyrene, leading to an increased Cd toxicity (Xu et al., 2021). In oysters, MT concentration and soluble metal concentration are correlated with temporal variations of metal exposures, in contrast to the digestive gland MT concentrations, suggesting the importance of an organ-specific approach to correctly assess the response to metal pollution (Geffard et al., 2002). In smaller invertebrates, such as the daphnids or gammarids, organ dissection is not a common procedure due to their small size, thus leading to a lack of detailed information about metal homeostasis, detoxification or adaptation mechanisms at the organ level for these species (Tang et al., 2015; Chain et al., 2019; Cosio et al., 2021).

In this study, we first identified two *mt* transcripts in the amphipod *Gammarus fossarum* by mining RNA-seq data previously obtained (Cogne

et al., 2019) and characterized them by sequence and structure prediction analyses. We also investigated the organ-specific expression patterns of these two *mt* transcripts. To do this, we selected the gills, a major tissue for metal uptake in aquatic invertebrates (Cooper et al., 2010; Gestin et al., 2021), and the caeca, a major detoxification organ in the amphipods (Gismondi et al., 2017). We quantified *mt* expression levels under environmentally relevant concentrations of cadmium, silver and zinc and showed, for the first time in this small crustacean, different metal- and organ-dependent responses.

## 2. Materials and methods

### 2.1. Transcriptome resources and sequence homology search

Our previously published *G. fossarum* *de novo* transcriptome assembly (Cogne et al., 2019) served as search database to perform the sequence homology search. A searchable database from the assembly was created using the command-line BLAST function makeblastdb. *Tblastn* was used to identify homolog sequences to the amphipod *Hyalella atzeica* MTs (HAZT008668, a putative MT-like B; HAZT008669 a putative MT-like C). A search for the Open Reading Frames was performed using the NCBI tool ORFfinder.

### 2.2. Prediction of 3D structure from MT protein sequences

The 3D structures and the structure-based functional annotation of the two putative metallothionein ORFs identified in *G. fossarum* were predicted using the I-TASSER server (Yang et al., 2015). For each target, I-TASSER simulations generate tens of thousands of conformations (called decoys). To select the final models, I-TASSER uses the SPICKER program to cluster all the decoys based on the pair-wise structure similarity, and report up to five models that correspond to the five states of lowest free energy and therefore have the highest confidence. The confidence of each model is quantitatively measured by C-score. C-score is typically in the range of [-5, 2], where a C-score of a higher value signifies a model with a higher confidence. Ligand binding sites were predicted using the COFACTOR and COACH software (Zhang et al., 2017; Yang et al., 2013).

### 2.3. Sampling and maintenance of organisms and laboratory exposure to metal ions

Gammarids were collected from the River Le Pollon in France (45°57'25.8"N 5°15'43.6"E) and acclimatized to laboratory conditions as previously described (Leprêtre et al., 2023). Male gammarids were sorted two days before the beginning of the exposure and acclimatized in 50% Evian water and 50% drilled groundwater. The day before the beginning of the experiment, organisms were placed in 100% Evian water at 12 ± 1 °C. Organisms were retrieved from a previous experiment described in detail in Leprêtre et al. (2023). Briefly, gammarids were exposed to equivalent ion concentrations of 0.6 or 5.4 µg/l of Cd (as CdCl<sub>2</sub>), to 6.6 or 60 µg/l of Zn (as ZnSO<sub>4</sub>) or to 0.066 or 0.6 µg/l of Ag (as AgNO<sub>3</sub>) dissolved in Evian water. Both experimental concentrations of these metals are environmentally relevant. The highest concentrations did not induce mortality, while they affected the feeding behaviour of gammarids, an ecotoxicological relevant end-point (Leprêtre et al., 2023). For each condition, twenty calibrated (size ranging from 9.5 to 12.5 mm) male gammarids were then placed in 500 ml plastic beakers (10 gammarids per beakers, 2 beakers per condition). Control organisms were kept in Evian water. Organisms were fed *ad libitum* with conditioned alder leaves (*Alnus glutinosa*). Conditioned leaves are in fact kept in natural water for 7 days at 12 °C ± 1 °C before being provided to gammarids as food source. Renewal of exposure media was performed daily. After 8 days of exposure, five organisms per group were dissected and the gills and the caeca were individually collected for each organism as described in Gestin et al. (2021). Organs were flash-frozen in liquid

**Table 1**  
Identification of two metallothioneins in the amphipod *Gammarus fossarum*.

IDs	Coding DNA Sequence	Open Reading Frame	Primers qPCR
GFBF_DN110944_c4_g1_i2 putative MT-1	ATGCCTAACGACTGCTGCAAAGAGGACTGCAAATGCACCGC CGAGGAATGCGGGAAGGACTGCGGCTGCACGACTGCGACT GCCAGAAAGTGGGAGAATTGCAAGGGCAGCTGCGACTGCTCC TCTGTTAGCGCTGCGCTACCAACTGCGACACGCCATGCGAGC TGCTGCCCTACGGAGTAG	>MPNDCKEDCKCTAECEGKDCG CTDCDCQKCNCKGSCDCSSVDAC ATNCDTPCSCCPE	Forward: AACGACTGCTGCAAAGAGGA Reverse: CAGCTGCCCTTGAATTCTC
GFBF_DN110487_c1_g1_i4 putative MT-2	ATGCCTAACGACTGCTGTAAGAGTCTGCAAGTGCGCCGC CGCCAAGTGCAGGAAGGAATGTGCTGCACAGACTGCGACT GCCAGAAAGTGGGACAATGTGATGGCGCCTGCCCCCTGCTCC ACCAGCCAAGACTGCGCTACCAAGTGCAAAGATCCCTGCC CTGCTGCC	>MPKTCKESCKCAAACGKCEKAC TDCCDCQKCDKDGACPCSTSDCA TKCKDPCPCPATDTPKTPDTKAP	Forward: AAGTGCACAAATGTGATGG Reverse: CACTTGGTAGCGCAGTCTTG

nitrogen and kept at  $-80^{\circ}\text{C}$  until RNA extraction.

#### 2.4. RNA extraction, cDNA synthesis and quantitative PCR

RNA was extracted from each organ sample using the RNeasy fibrous tissues kit (Qiagen, Hilden, Germany) and quality and quantity were assessed by spectrophotometry using NanodropOne (ThermoFisher, Waltham, MA, USA). RNAs (about 100 ng per sample) were retro-transcribed using the high capacity cDNA reverse transcription kit (Applied Biosystems, Waltham, MA, USA). Specific primers were designed using the NCBI web tool Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) (Table 1). Diluted cDNAs (1:5) were amplified using the OneGreen SYBR Green mix (Ozyme, Montigny-le-Bretonneux, France) in a Bio-Rad CFX96 qPCR platform (Hercules, CA, USA) using the following conditions:  $95^{\circ}\text{C}$  for 3 min followed by 40 cycles at  $95^{\circ}\text{C}$  for 10 s,  $61^{\circ}\text{C}$  for 30 s and  $72^{\circ}\text{C}$  for 30 s. Melting curves were also established with a  $0.5^{\circ}\text{C}$  of resolution to evaluate the PCR specificity. MTs were quantified using the relative quantification method ( $\Delta\text{Ct}$ ) using the elongation factor (EF) transcript to normalize gene expression across samples (Gouveia et al., 2018). In the analysis, we exclude only two gill samples that failed technically (one from the  $0.6\ \mu\text{g}/\text{l}$  Cd group due to insufficient RNA yield; one from the  $5.4\ \mu\text{g}/\text{l}$  Cd group due to failed amplification).

### 3. Results and discussion

#### 3.1. Identification of two metallothioneins in *Gammarus fossarum* transcriptome

We mined the transcriptome of *G. fossarum* by BLASTt using *H. atzeca* MTs sequences (HAZT008668, a putative MT-like B; HAZT008669 a putative MT-like C) as queries. We identified two contigs with partial homology, each one containing several potential open reading frames (ORFs). We screened the various ORFs identified using InterProScan from InterPro database (Blum et al., 2021) and we identified one ORF in each contig predicted as a crustacean metallothionein (Table 1). In particular, the contig GFBF\_DN110944\_c4\_g1\_i2 codes for an ORF of 60 amino acids residues that we named as *mt1*. The contig GFBF\_DN110487\_c1\_g1\_i4 codes for an ORF of 71 amino acids that we named *mt2* (Table 1). These putative *mt* ORFs both contain 18 cysteine residues, similarly to the majority of described MTs in other crustacean, such as decapods or the amphipod *Hyalella atzeca* (Mahmood et al., 2009; Bain et al., 2016; Poynton et al., 2018). Amino acid sequence alignment of the two identified amino acid sequences to other crustacean MTs showed that the positions of the 18 cysteine residues are highly conserved, while the remaining residues (except two proline residues in position 2 and 57 in *G. fossarum* ORFs) did not show strong conservation (Fig. 1). Interestingly, *G. fossarum* MT2 shows a longer C-terminus sequence compared to most of the identified MTs, similarly to the MT of the clade F recently identified in *D. melanogaster* (Luo et al., 2020). These characteristics lead the authors to suggest that this MT

might be able to bind Zn ions in the *Diptera* (Luo et al., 2020).

To better characterize these two putative MTs in *G. fossarum*, we performed a 3D structure prediction using I-TASSER. The best models for MT1 (C-score =  $-1.59$ ) and MT2 (C-score =  $-2.49$ ) predicted the presence of a  $\alpha$ -helix of six amino acids (including one of the 18 Cys residues, Cys 46) at the C-terminus in each protein (most of the residues having a confidence score higher than 7 with 9 indicating the highest confidence) (Fig. 2). I-TASSER also provided a functional prediction based on the 3D models by integrating the COFACTOR and COACH softwares (Zhang et al., 2017; Yang et al., 2013). In both *G. fossarum* MT-1 and MT-2, a cadmium-binding site involving the same four cysteine residues (Cys 40, Cys 46, Cys 50, and Cys 57) was predicted (Fig. 2A-B). Moreover, a zinc-binding site was predicted only for MT-2, involving four different cysteine residues located in proximity of the N-terminal (Cys 5, Cys 10, Cys 17, Cys 21) (Fig. 2C).

#### 3.2. Metallothioneins induction in the caeca and gills of *G. fossarum*

While amphipods and other small aquatic invertebrates accumulate and are affected by environmental metal exposure (Alric et al., 2019; Dong et al., 2020; Filipović Marijić et al., 2016), to our knowledge, there is no available information on the expression profiles of amphipod *mt* genes at the organ level in this taxon. Thus, the lack of knowledge about the molecular physiology of these genes and corresponding coded-proteins in organisms used as sentinel species limits our capacity of use metallothionein transcript or protein level as informative molecular biomarker (Le et al., 2016). For this reason, the expression of these two new putative *G. fossarum mt* genes were measured in the caeca and the gills of calibrated male amphipods in absence of metal exposure. First, we observed that relative expression of *mt1* and *mt2* showed similar basal levels in either caeca or gills in non-exposed organism ( $p$ -value = 0.22 and 0.55, respectively, Wilcoxon test) (Supplementary Fig. 1; Table 2). Tissue-specific expression of *mt* genes has been studied in other invertebrates, such as decapods and bivalves, thanks to an easier access to the organs in these organisms (Mahmood et al., 2009; Liu et al., 2021). Interestingly, in the decapod *Macrobrachium rosenbergii*, basal levels of one MT were higher in the hepatopancreas compared with other organs and no expression was observed in the gills (Mahmood et al., 2009). Similarly, in the white shrimp *Litopenaus vannamei*, a high variability in basal expression in different tissues was observed, with highest level in the heart and hepatopancreas and lowest levels in the hemocytes and the gills (Felix-Portillo et al., 2014). Similar patterns of expression were observed in the bivalve *Mytilus galloprovincialis* exposed to dissolved Cd ion or Cd dots (Rocha et al., 2018). In particular, both the *mt* genes studied, *mt10* and *mt20*, showed similar basal level in the gills and in the digestive gland. Notably, the *mt20* gene was more induced in the gills than in the digestive gland of exposed organisms to both Cd forms (Rocha et al., 2018), indicating organ-specific pattern of *mt* expression. It is worthy of noting that other characteristics, such as age or developmental stage may influence *mt* gene expression or MT concentrations. In the shore crab *Carcinus maenas*, the inducibility of MT



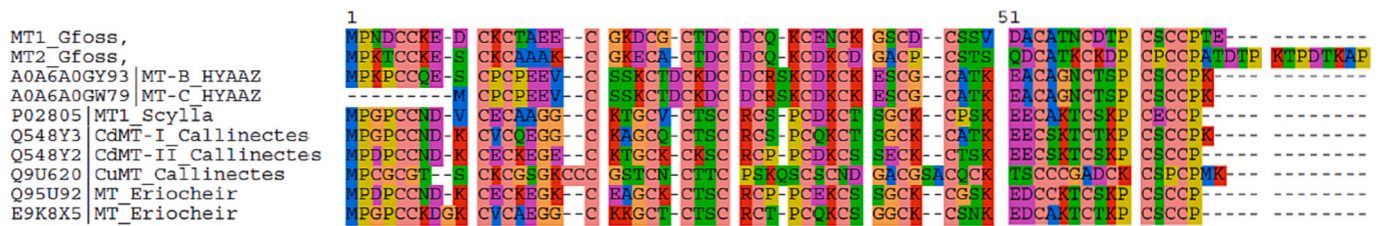


Fig. 1. Sequence alignment of the two putative *Gammarus fossarum* metallothioneins (MT-1\_Gfoss and MT-2\_Gfoss) with other crustacean metallothioneins (the amphipod *Hyalella atzeica*; the decapods *Scylla serrata*, *Callinectes sapidus* and *Eriocheir sinensis*).

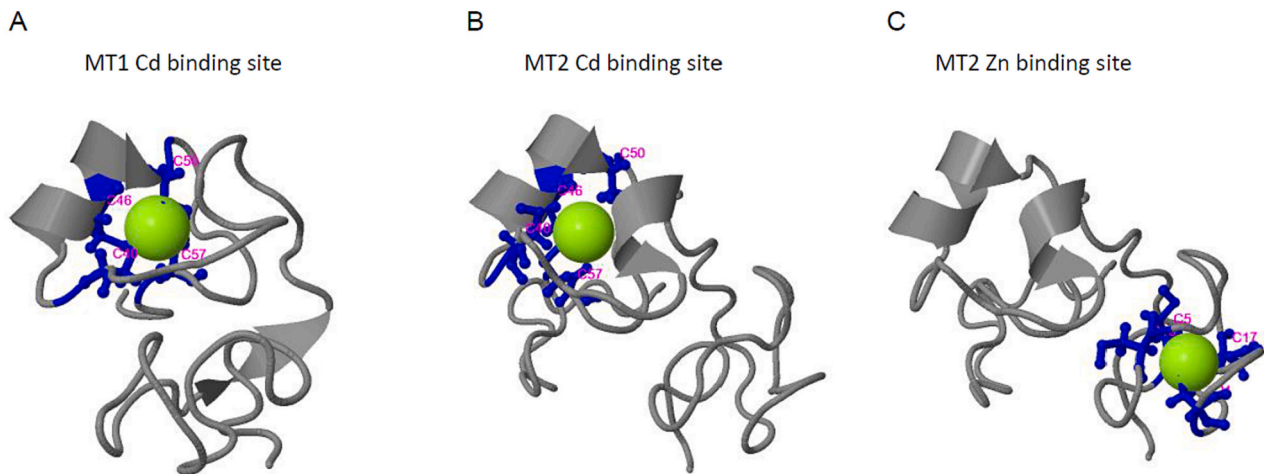


Fig. 2. *Gammarus fossarum* MT-1 and MT-2 predicted 3D structures and ligand predictions. A) MT-1 structure with the predicted Cd binding site. B) MT-2 structure with the predicted Cd binding site. C) MT-2 structure with the predicted Zn binding site.

upon Cd exposure was reported to be dependent on age, despite the baseline metallothionein concentrations did not change with age (Bjerregaard et al., 2021). In the snail *Cantareus aspersus*, it was reported that the capacity of a Cd-thioneins overexpression increased with the age of embryos (Baurand et al., 2016). Thus, there is evidence that basal levels of *mt* expression in different organs are likely to be species-specific and susceptible to changes due to other parameters such as age or developmental stage. The definition of reference level across age, life stages and organs is thus very relevant for an efficient development of molecular biomarkers particularly in sentinel species.

*G. fossarum mt1* was generally more significantly induced in the caeca than in the gills of exposed organisms for any metal exposure tested here, albeit high individual variability was observed (Table 2, Fig. 3). While in this study we did not evaluate metal concentrations in gills and caeca, previous works using radioisotopes of Cd and Zn provided accurate toxicokinetic models of these two metals in *G. fossarum* (Gestin et al., 2021; Gestin et al., 2022; Gestin et al., 2023). In particular, the distribution pattern of Cd is characterized by a higher

bioaccumulation in the gills followed by the caeca and the intestines that showed similar bioaccumulation (Gestin et al., 2022). Interestingly, the gills did not depurate Cd over an observation period of 3 weeks, while it was eliminated by the other organs (Gestin et al., 2022). The distribution pattern of Zn was different and was characterized by a higher bioaccumulation in the intestines, followed by the caeca, and then the gills (Gestin et al., 2022). In the caeca of amphipods exposed to Cd, *mt1* was significantly increased at 5.4 µg/l condition, an environmental relevant condition, while an increasing trend was observed at a concentration as low as 0.6 µg/l compared to the control (adjusted *p*-value = 0.014 and 0.06 respectively, Kruskal-Wallis test followed by a post-hoc Dunn's multiple comparison test). Results showed an increased median expression of *mt1* with a log2 fold change (logFC) of 7.9 and 9.4 in the caeca at 0.6 and 5.4 µg/l compared to the controls, respectively (Table 2, Fig. 3B). The increased expression observed in the gills did not reach statistical significance since a high level of individual variability was observed, whereas the observed *mt1* increase corresponded to a logFC of 6.6 and 8.3 at 0.6 and 5.4 µg/l compared to the controls, respectively

Table 2  
Relative expression of MT-1 and MT-2 in caeca and gill of *G. fossarum* exposed at different level of metal ions.

Group	MT-1			MT-2								
	Caeca			Gills			Caeca			Gills		
	1st Q	Median	3rd Q	1st Q	Median	3rd Q	1st Q	Median	3rd Q	1st Q	Median	3rd Q
Control	0.0016	<b>0.0072</b>	0.0275	0.0020	<b>0.0048</b>	0.0907	0.0791	<b>0.0815</b>	0.1017	0.0007	<b>0.0024</b>	0.0242
Cd 0.6 µg/l	0.6556	<b>1.7230</b>	5.5543	0.0434	<b>0.4572</b>	0.8738	0.0811	<b>0.0836</b>	0.1270	0.0007	<b>0.0017</b>	0.0032
Cd 5.4 µg/l	1.3620	<b>4.9490</b>	7.7670	0.8999	<b>1.4712</b>	3.9605	0.0802	<b>0.1401</b>	0.2849	0.0016	<b>0.1352</b>	0.3197
Zn 6.6 µg/l	0.0475	<b>0.0708</b>	0.0738	0.0037	<b>0.0072</b>	0.0116	0.0037	<b>0.0072</b>	0.0116	0.0008	<b>0.0215</b>	0.0360
Zn 60 µg/l	0.0237	<b>0.0237</b>	0.6828	0.0010	<b>0.0041</b>	0.0051	0.0010	<b>0.0041</b>	0.0051	0.0005	<b>0.3915</b>	0.6101
Ag 0.066 µg/l	0.0195	<b>0.0327</b>	0.0345	0.0006	<b>0.0010</b>	0.0025	0.0372	<b>0.0733</b>	0.0769	0.0011	<b>0.0016</b>	0.3684
Ag 0.6 µg/l	0.0071	<b>0.0191</b>	0.0276	0.0008	<b>0.0012</b>	0.0024	0.0867	<b>0.0985</b>	0.0990	0.0019	<b>0.0020</b>	0.1150

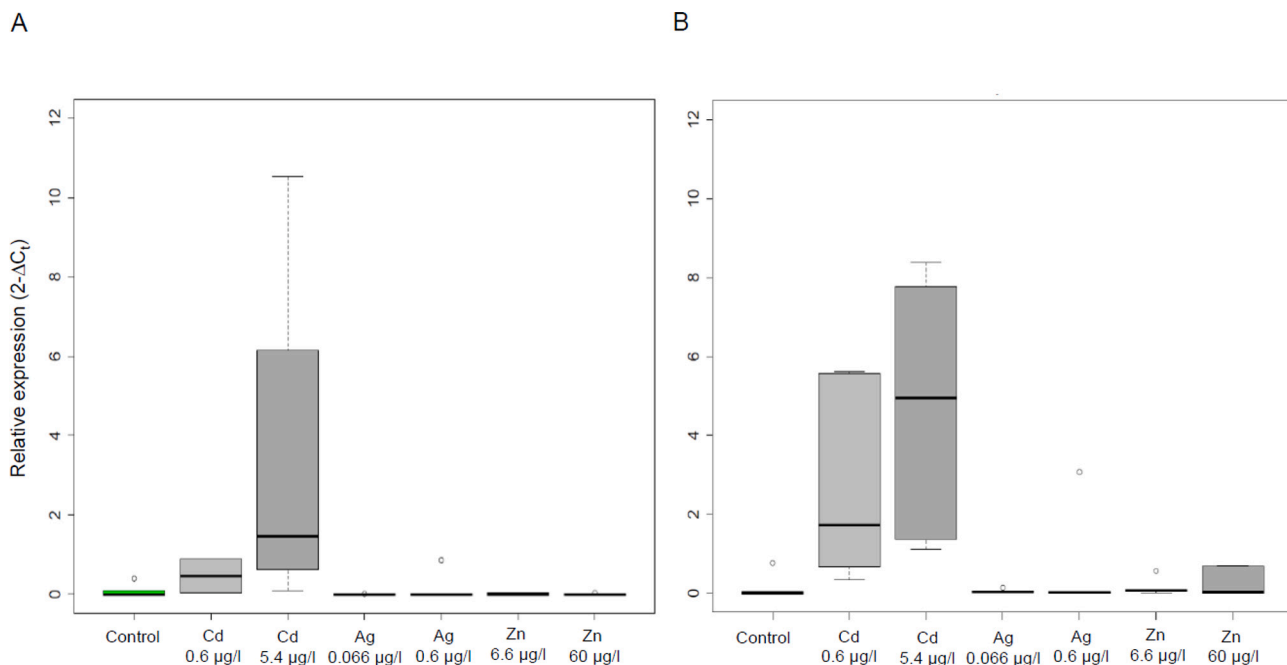


Fig. 3. MT-1 relative expression in *Gammarus fossarum* males exposed to Cd, Zn or Ag. (A) Results in the gills. (B) Results in the caeca.

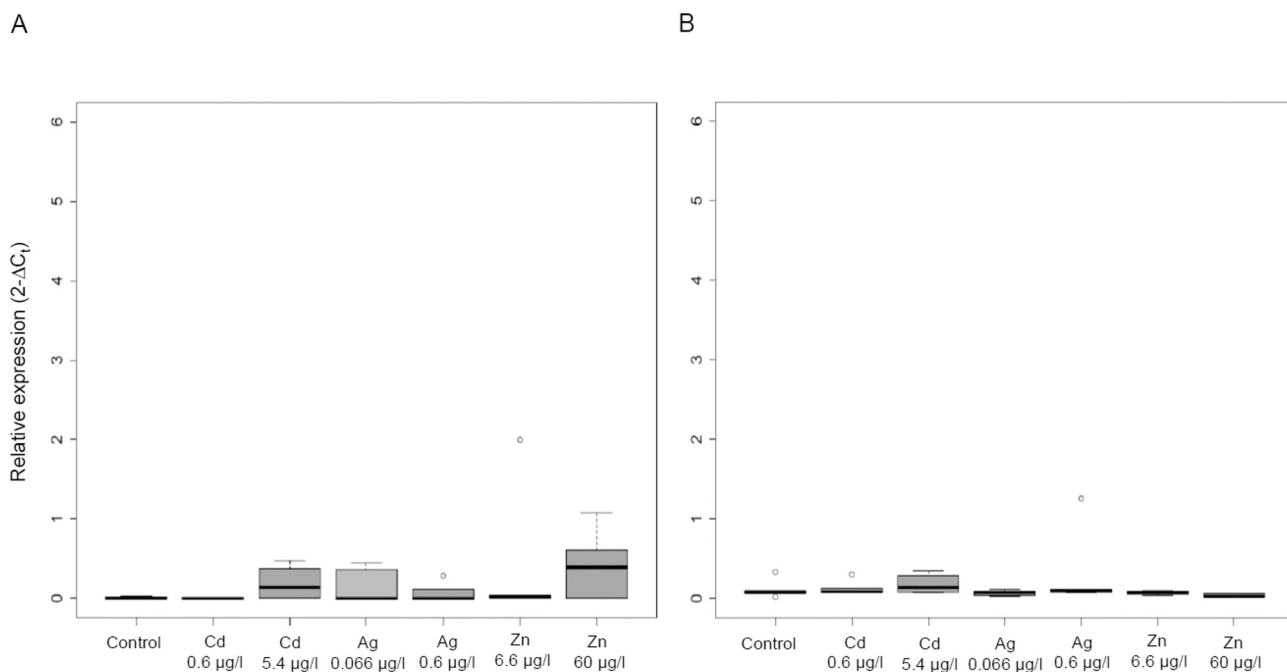


Fig. 4. MT-2 relative expression in *Gammarus fossarum* males exposed to Cd, Zn or Ag. (A) Results in the gills. (B) Results in the caeca.

(Fig. 3A). Thus, our data suggest a more dynamic inducibility of *mt1* expression in the caeca, although we cannot definitively conclude in this study whether trophic transfer of the metal may be involved in this pattern. Zinc and Ag exposures induced slightly but non-significantly *mt1* expression in the caeca (logFC ranged from 1.4 to 3.3), while no induction at all was observed in the gills in the same conditions (logFC ranged from -2.8 to 0.58). The high inter-individual variability observed in this study may be partially explained by the limit of our sampling strategy based on five biological replicates par group (however, a typical design to assess molecular biomarkers) that did not allow

us to clearly identify biological outliers. Thus, our results suggest that it is essential to better assess the individual variability in *mt* gene expression in non-exposed and metal exposed gammarids both in laboratory and in field conditions. This approach will help to establish a reference baseline and potential confounding factors affecting *mt* gene expression, as performed with other molecular biomarkers in this and other sentinel species (Leprêtre et al., 2022a, 2022b, Storhaug et al. 2019, Cuevas and Zorita, 2018). In this context, it merits noting that the quantification of MT enriched fractions as biomarkers of metal exposure has shown similar variability in other invertebrates, such as bivalves,

snails and crabs (Le et al., 2016; Baurand et al., 2016; Mouneyrac et al., 2001).

*G. fossarum mt2* did not show statistically significant differences in any of the tested conditions compared to the controls (Fig. 4). However, exposure at the highest Cd concentration induced a slight increase (logFC = 5.8) in *mt2* expression in the gills. Moreover, Zn exposure induced a higher *mt2* expression at both 6 and 60 µg/l conditions in the gills (Table 2), with a logFC induction of 3.2 and 7.3 respectively, but no induction was observed in the caeca. These results suggest that *mt2* is, at least at the individual level, more inducible in the gills than in the caeca of *G. fossarum* exposed to Cd and Zn.

These results indicate that *G. fossarum mt1* induction is stronger after Cd exposure compared to the other tested metals, while *mt2* expression shows high individual variability and it is not strongly influenced by the metal ions used in this study and at the described experimental conditions. Indeed, functional annotation based on structure prediction of the *mt2* CDS showed it might bind both cadmium and zinc (Fig. 2). Our results support the interest of *in vivo* experimental validation in parallel to *in silico* studies, in particular for proteins that exhibit high amino acid sequence divergence, such as those belonging to the MT superfamily. Experimental approaches focusing on the gene expression at the organ level help to characterize the specific ability of different metals to induce different *mt* isoforms and to elucidate their different patterns of expression. Indeed, it would be of great interest to develop specialized mass spectrometry methods to address the direct and specific quantification of MT proteins in sentinel species, since these methods are currently available only for human MTs (Mehus et al., 2014; Gunn et al., 2021). These methods would help the biological interpretation of *mt* gene expression and the different dynamics between transcript induction and protein expression.

#### 4. Conclusions

This study identified two metallothionein coding transcripts in the amphipod *G. fossarum*. Our results suggest that *G. fossarum mt1* codes for a Cd-thionein, providing a potential biomarker to investigate the mechanisms of metal detoxification, for example in population of gammarids naturally exposed to Cd (Vigneron et al., 2015; Lalouette et al., 2023). *G. fossarum mt2* was not significantly induced by any metal exposure, although higher variability was observed in the gills under both Zn concentrations and at the highest dose of Cd. Moreover, our results suggest that metallothionein expression might be regulated by tissue-specific mechanisms that can differ on the base of the metal exposure. The individual variability observed in laboratory conditions opens the question of the individual sensitivity to metal, in particular Cd, exposure. This aspect needs to be considered both for the interest of using MTs as biomarkers of exposure and for investigating the molecular mechanisms of phenotypic plasticity observed in a number of gammarids' population exhibiting tolerance to cadmic environments (Lalouette et al., 2023). Finally, our results suggest an important link between the uneven distribution of metals in different organs and the tissue-specific induction of the different members of *mt* genes (Gestin et al., 2021; Gestin et al., 2022). Thus, organ-specific expression patterns of metal-induced genes or proteins will allow a better understanding of metal detoxification mechanisms in sentinel species.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cbpb.2023.110907>.

#### Declaration of Competing Interest

The authors declare that they have no known conflict of interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data availability

Data will be made available on request.

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