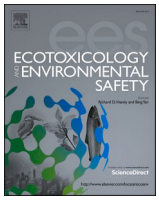
Ecotoxicology and Environmental Safety 209 (2021) 111815

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv



Research paper

Inorganic mercury effects on biomarker gene expressions of a freshwater amphipod at two temperatures

Madson Silveira de Melo [a,c], Krishna Das [b], Eric Gismondi [c,*]

[a] Laboratório de Reprodução e Desenvolvimento Animal, Departamento de Biologia Celular, Embriologia e Genética, Universidade Federal de Santa Catarina, Florianópolis, Santa Catarina, Brazil
[b] Laboratory of Oceanology, Freshwater and Oceanic Sciences Unit of ReSearch (FOCUS), University of Liège B6c, 11 Allée du 6 Août, 4000 Liège, Belgium
[c] Laboratory of Animal Ecology and Ecotoxicology (LEAE), Freshwater and Oceanic Sciences Unit of ReSearch (FOCUS), University of Liège B6c, 11 allée du 6 Août, 4000, Liège, Belgium

ARTICLE INFO

Edited by: Dr Yong Liang

Keywords:
Mercury toxicity
Gammarus Sp.
Transcriptomic
Multi-stress exposure

ABSTRACT

Mercury (Hg) is a global contaminant resulting of both natural processes and human activities. In aquatic environments, studies conducted on vertebrates highlighted changes of gene expression or activity of antitoxic and oxidative enzymes. However, although Hg is a highly toxic compound in aquatic environments, only a few studies have evaluated the lethal and sublethal effects of inorganic Hg on *Gammarus* sp. Therefore, this study aimed at evaluating the effects of inorganic Hg ($HgCl_2$) on the expression of 17 genes involved in crucial biological functions or mechanisms for organisms, namely respiration, osmoregulation, apoptosis, immune and endocrine system, and antioxidative and antitoxic defence systems. The study was performed in males of the freshwater amphipod *Gammarus pulex* exposed to two environmentally relevant concentrations (50 and 500 ng/L) at two temperature regime fluctuations (16 °C and 20 °C +/−2 °C) for 7 and 21 days. Results showed that *G. pulex* mortality was dependent on Hg concentration and temperature; the higher the concentration and temperature, the higher the mortality rate. In addition, the Integrated Biomarker Response emphasized that $HgCl_2$ toxicity was dependent on the concentration, time and temperature of exposure. Overall, antioxidant and antitoxic defences, as well as the endocrine and immune systems, were the biological functions most impacted by Hg exposure (based on the concentration, duration, and temperature tested). Conversely, osmoregulation was the least affected biological function. The results also demonstrated a possible adaptation of *G. pulex* after 21 days at 500 ng/L, regardless of the exposure temperature. This study allowed us to show that Hg deregulates many crucial biological functions after a short exposure, but that during a long exposure, an adaptation phenomenon could occur, regardless of temperature.

1. Introduction

Mercury (Hg) is a global contaminant resulting of both natural processes (i.e. erosion) and human activities, such as disposal of electronic products, pharmaceuticals, textile and paint industries, fossil fuel combustion and mining (Wu et al., 2015; Outridge et al., 2018; Zhang et al., 2018). Since 1991, Hg has been listed third in the Substance Priority List published every two years by the Agency for Toxic Substances and Disease Registry (ATSDR), due to its numerous toxic effects on human health. The release of anthropogenic Hg is also regulated by the Minamata Convention on Mercury to protect human health and the environment from its adverse effects. In fact, long-term exposure to Hg can result in toxic effects on the skin, cardiovascular, pulmonary, urinary, gastrointestinal, and neurological systems (Kim et al., 2016; Eagles-Smith et al., 2018). As it is widely known that fish is a major source of Hg in the human diet and that its accumulation poses a high risk to human health, many toxicological investigations have been conducted to understand the impact of Hg on aquatic organisms (Rice et al., 2014), including biomagnification in food webs from different aquatic environments (McIntyre and Beauchamp, 2007; Chen et al., 2009; Tom et al., 2010; Kidd et al., 2011; Bisi et al., 2012; Lavoie et al., 2013; Le et al., 2017).

In aquatic ecosystems, Hg exists in two main forms: inorganic (Hg^{2+} and Hg^{+}) and organic (e.g. methylmercury, MeHg) compounds. Since

* Correspondence to: University of Liège, Laboratory of Animal Ecology and Ecotoxicology (LEAE) - Freshwater and OceaniC sciences Unit of reSearch (FOCUS), Chemistry Institute, Bât. B6C, 11 allée du 6 Août, B-4000 Sart-Tilman, Belgium.
E-mail address: eric.gismondi@uliege.be (E. Gismondi).

https://doi.org/10.1016/j.ecoenv.2020.111815
Received 14 September 2020; Received in revised form 8 December 2020; Accepted 14 December 2020

MeHg is considered to be the most toxic form (Hempel et al., 1995), many studies have focused on its sublethal toxic effects; however, aquatic organisms are also exposed to inorganic Hg, which accounts for the majority of total Hg in freshwaters (Watras et al., 1998). For example, Tsui and Wang (2004) demonstrated in *Daphnia magna* that MeHg bioaccumulates more through the food web while inorganic Hg accumulates more through water uptake, confirming that the toxicity of inorganic Hg should not be overlooked. In fact, due to its high affinity for sulfhydryl groups, such as reduced glutathione and N-acetylcysteine, inorganic Hg has been shown to disturb cellular redox balance in many in-vitro and rats investigations (Stohs and Bagchi, 1995), mainly by disturbing antioxidant defenses, promoting the formation of reactive oxygen species (ROS) (Lund et al., 1993), which is characteristic of oxidative stress. Similar results have been demonstrated in aquatic vertebrates (e.g. fish), showing that Hg has an impact on antitoxic and antioxidant defense systems by modulating the gene expression or activity of enzymes such as superoxide dismutase (SOD), catalase (Cat), or glutathione peroxidase (GPx) (Huang et al., 2010; Monteiro et al., 2010; Zhen et al., 2014; Naïja et al., 2016; Zhang et al., 2016; Lu et al., 2017). In addition, exposure to Hg can affect many important biological functions such as osmoregulation which controls water and salt balance through the Na+/K+ATPase pump (Handayani et al., 2020), the immune system by reducing, for example, phagocytosis or immunoglobulin levels in fish (Sanchez-Dardon et al., 1997; Sweet and Zelikoff, 2001), thereby increasing the susceptibility of fish to infectious agents. Hg also has a high affinity with the thioredoxin system that prevents oxidative stress, but it is involved in other functions, namely, cell proliferation, control and activity of many transcription factors, and regulation of the cell signaling pathway (Branco and Carvalho, 2019). Finally, Hg exposure can cause endocrine disruption because it is able to inhibit many sites on the reproductive axis of fish, including the hypothalamus, pituitary gland and gonads (e.g. reductions in gonad size, circulating reproductive steroids, gamete production – Crump and Trudeau, 2009). In addition, although most toxicity studies of Hg in aquatic invertebrates (e.g. crustaceans) have focused on bioaccumulation and lethal toxicity, it has been reported that Hg is able to disturb biological functions similar to those of vertebrates. For example, Hg disrupts antitoxic and antioxidant defense systems, inducing oxidative stress (Elumalai et al., 2007; MERPD et al., 2010; Zhao et al., 2010; Singaram et al., 2013; Roos-Muñoz et al., 2019), reduces immune system by decreasing phagocytosis (Kaoud et al., 2011; Singaram et al., 2013), reduces feeding rate (Bundschuh et al., 2011), or causes DNA damage in hemocytes and spermatozoa (Di Donato et al., 2016).

Among freshwater invertebrate species, *Gammarus* spp. is a suitable organism for assessing large-scale pollution risks, as they are widely distributed throughout the northern hemisphere, and commonly found in European rivers (Jażdżewski, 1980; Hou and Sket, 2016). The use of gammarids in ecotoxicology is also due to their central role in aquatic ecosystems since they are a food source for many organisms (fish, bird) and play an essential role in the cycle of organic matter (detritivores). In addition, *Gammarus* sp. are known to be sensitive to many pollutants and can easily be used in laboratory and field investigations (Kunz et al., 2010). Consequently, many ecotoxicological studies have been conducted using *Gammarus* sp. as a model species for assessing the toxicity of organic and inorganic pollutants (Gismondi et al., 2017; Gismondi, 2018; Gouveia et al., 2018; Jaegers and Gismondi, 2020; Lebrun and Gismondi, 2020). However, despite the fact that Hg is a highly toxic compound in aquatic environments, only a few studies have evaluated the sublethal effects of inorganic Hg on *Gammarus* sp. In fact, Costa (1980) found a decrease in heart pulsation in *G. pulex* exposed to $HgCl_2$ and Di Donato et al. (2016) observed genotoxicity of $HgCl_2$ (from 100 ng/L to 1 μg/L) in hemocytes and spermatozoa of *Gammarus elvirae*.

This study was devoted to assessing the impacts of Hg on the health of the freshwater amphipod *G. pulex*. First, we analyzed the effects of $HgCl_2$ on the survival of *G. pulex*. Then, to obtain an overview of the effects of Hg on the organism, we analyzed the expression of genes mainly involved in several crucial biological mechanisms and functions, namely respiration (hemocyanin), osmoregulation (Na/K ATPase), apoptosis (caspase 3), immune system (prophenoloxidase), endocrine system (molt-inhibiting hormone, ecdysone receptor, farnesoic acid O-methyltransferase, methyl farnesoate epoxidase), oxidative (glutathione-S-transferase, selenium-dependent glutathione peroxidase, catalase, Cu/Zn superoxide dismutase, Mn superoxide dismutase) and antitoxic (HSP70, HSP90, thioredoxin, thioredoxin reductase) defense systems, whose effects of Hg have already been observed in other organisms (see above). Moreover, as it is now well established that chemical toxicity can be temperature-dependent (Noyes et al., 2009; Hooper et al., 2013) and that constant temperature regimes are not realistic for many aquatic ecosystems, where water temperatures fluctuates daily (Willming and Maul, 2016; Verheyen and Stoks, 2019), the toxicity of Hg was evaluated at two different temperature (16 °C and 20 °C) subject to a daily fluctuation of +/−2 °C. Finally, to further assess the toxicity of Hg, dose- and time-dependent toxicity was evaluated by exposing *G. pulex* to 50 and 500 ng/L $HgCl_2$ for 7 days and 21 days.

2. Materials and methods

2.1. Experimental animals and acclimation

Adult males *G. pulex* were sampled in the Blanc-Gravier stream (50°34'60" N and 5°34'60" E, Colonster, Belgium) using a pond net in November 2019 and February 2020 for experiment I and II, respectively (see Fig. 1). As the moult stage may be a confounding factor, only males in the intermoult stage were kept (Trevisan et al., 2014). At each sampling period, gammarids were sorted directly on site using the gnathopod size, which are longer in males than females. The animals were then transferred to the laboratory where they were maintained for 7 days at the same temperature as in experiments I and II (16 °C ± 2 °C, T_1 or 20 °C ± 2 °C, T_2 - Fig. 1), in large aerated aquaria filled with artificial freshwater (Rasmussen et al., 2016) according to the US EPA method (Weber, 1991). These temperatures were chosen to be consistent with the freshwater temperatures generally measured in spring/autumn (16 °C) or summer (20 °C) in European rivers, as well as the optimal metabolic temperature of *Gammarus* sp. which is about 16 °C. *G. pulex* were fed ad libitum with alder leaves, and medium was renewed once.

2.2. Exposure conditions

The study was carried out using 50 and 500 ng/L $HgCl_2$, nominal concentrations chosen to surround the Environmental Quality Standard for Hg(II) (70 ng/L- Directive, 2013/39/EU), and a control condition (artificial freshwater). All exposure conditions were performed in triplicate using a thermostatic chamber to adjust the daily fluctuation temperature (Fig. 1). As only one temperature variation could be used in the thermostatic chamber at a time, the study was divided into two experiments: experiment I at 16 °C ± 2 °C (T_1) and experiment II at 20 °C ± 2 °C (T_2).

The contamination was conducted in aquaria whose walls were previously saturated for five days with corresponding exposure solutions, to avoid Hg adsorption during the gammarids exposure. For each exposure temperature, three aquaria of 35 *G. pulex* were exposed to each $HgCl_2$ concentration and the control, for maximum 21 days, with a photoperiod of 12 h light and 12 h dark. During the exposures, *G. pulex* were fed with alder leaves discs and dried chironomids the day before the medium change, which were renewed every three days. This helped to limit contamination through food. Besides, to investigate the mortality rate throughout the 21d-exposure and to avoid cannibalism, dead animals were counted daily and removed from the aquaria.

For both experiments, eight *G. pulex* were randomly sampled in each of the three exposure conditions, after 7 and 21 days. Then, gammarids were individually frozen in liquid nitrogen and stored at −80 °C until RNA extraction and gene expression analysis.

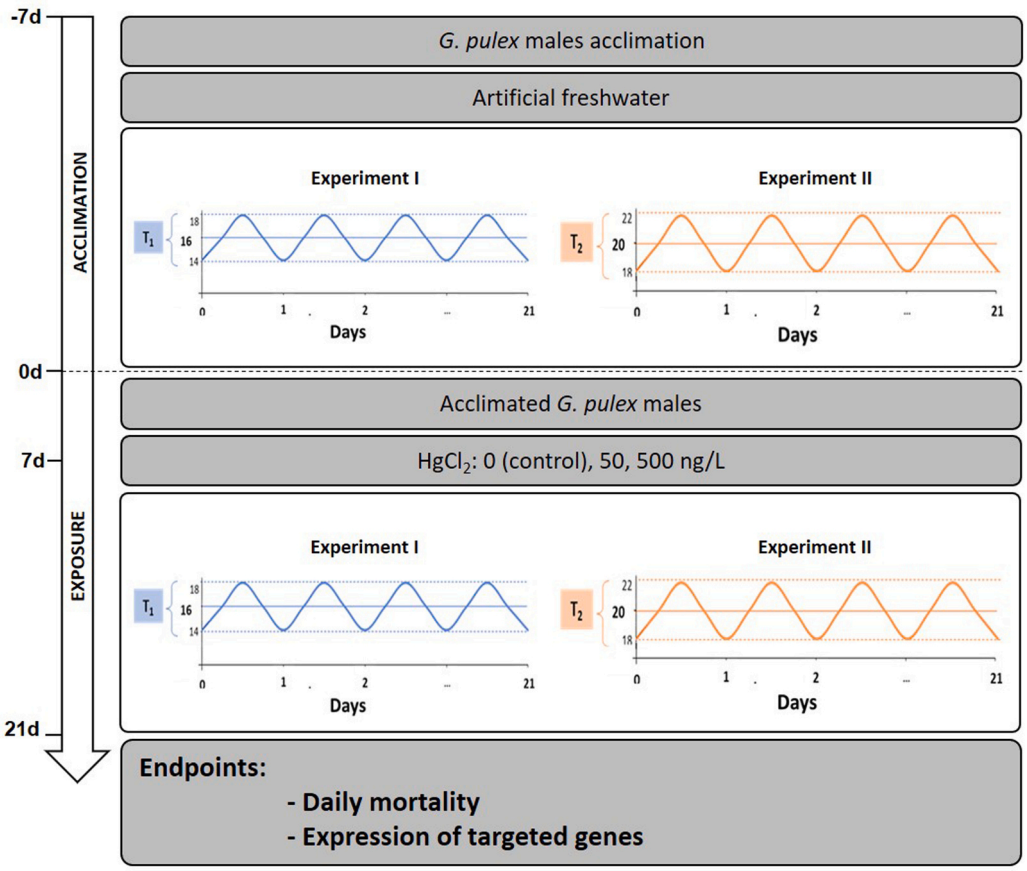


Fig. 1. Experimental design of *Gammarus pulex* exposed for 7 and 21 days to $HgCl_2$ 50 and 500 ng/L at two temperature variations (T_1 16 °C ± 2 °C; T_2 20 °C ± 2 °C).

2.3. Semi-quantitative real-time polymerase chain reaction (qPCR)

Total RNA from each sample was extracted using the NucleoSpin RNA kit (Macherey Nagel, Germany). The RNA integrity was verified on a 1.5% agarose gel electrophoresis and the concentrations were measured using a NanoDrop ND-1000 spectrometer (NanoDrop, USA). All A260/280 and A260/230 ratios were between 1.9 and 2.2. Subsenquently, reverse transcriptions were performed on 300 ng total RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) with random hexamers and following the manufacturer's protocol.

All gene sequences were obtained from previous works (Gismondi and Thomé, 2016; Gismondi, 2018) and the primer pairs were designed using Primer3 Plus software (Untergasser et al., 2007). Two genes were used as reference genes: ribosomal protein 3 (RSP3) and elongation factor 1α (EF1a) (Table 1). Optimal primer concentrations were evaluated for three concentrations (150, 300, and 600 nM). Dilutions of a cDNA mixture (mixture of 1 μL of each sample) between 1:2 and 1:64 were also tested to estimate the qPCR efficiency of primers. Two no template controls were performed as negative control to assess external contamination, resulting from a nonspecific increase of the fluorescence signal.

After optimization, qPCR analyses were performed with a CFX96 Touch® Real-Time PCR Detection System, using a reactional mixture containing: 2 μL of diluted cDNA, 5 μL of iTaq® Universal SYBR Green Supermix (BioRad, Netherlands), 2 μL of primer mix 150 nM and 1 μL of ultrapure water. The qPCR program consisted of 3-min initial denaturation at 95 °C, followed by 40 denaturation cycles at 95 °C for 15 s, annealing/polymerization at 59 °C for 45 s. After amplification, a melting curve of the amplified products (65–95 °C, 0.5 °C/min) was performed to ensure that no artefact amplification occurred. In addition to the analyzed samples, a no template control was performed for each pair primer to ensure the absence of dimer product. The relative transcription expression levels of each gene of interest were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), considering the PCR efficiency, and normalized to the respective control condition.

2.4. Integrated biomarker response (IBRv2)

The Integrated Biomarker Response index version 2 (IBRv2) was calculated as described by Sanchez et al. (2013). For each gene of interest, the mean (X_0) relative expression was calculated for the control group. Then, a log transformation (Y_i) was applied to reduce variance, and calculated as the ratio of individual biomarker data (X_i) to X_0: $Yi = \log (X_i/X_0)$. In a next step, Y_i was standardized according to the equation $Z_i = (Y_i - \mu)/\sigma$, where μ is the general mean and σ the standard deviation of Y_i. Next, a biomarker deviation index (A) was defined as the difference between the mean of standardized biomarker response (Z_i) and mean of biomarker data of control group (Z_0), as follow: $A = Z_i - Z_0$. Finally, the IBRv2 was calculated as the sum of the absolute value A for each biomarker in each $HgCl_2$ exposure condition.

2.5. Statistical analysis

For each exposure temperature and time, a heat map using a hierarchical clustering analysis was performed to explore the relationship between exposure conditions and variations in gene expressions. Besides, two separate Principal Component Analysis (PCA) were used to visualize and describe the relationship between gene expression and exposure condition for each exposure temperature.

Relative gene expressions were expressed as $\log_2$ of fold changes. The

Table 1
Specific primer pairs used for RT-qPCR analyses on *Gammarus pulex* exposed for 7 and 21 days to $HgCl_2$ 50 and 500 ng/L and at two temperature variations.

Biological functions	Genes	Primer	Efficiency	Sequences (5'-3')
Respiration	Hemocyanin (Hc)	Hc_F	103.1%	AAAGGATGAACGAGGGCGAG
		Hc_R		GGAGTTGGTGAACAAGTGTGG
Osmoregulation	Na/K ATPase	NaKTPase_F	101.3%	ATTCTGCGACTACCTCTTGCC
		NaKTPase_R		AGAGAGATGAGACCCACGAAAC
Apoptosis	Caspase 3 (Casp3)	Casp3_F	103.9%	TCTATGAAGCATCCTCGGCG
		Casp3_R		TTGCAGACCTCACTATCCTCC
Immune system	Prophenoloxidase (ProPO)	ProPO_F	99.2%	TTCCAGCAATACAAGGCGACC
		ProPO_R		CTGTTCCAGCCAGTGTGAAG
Endocrine system	Molt-inhibiting hormone (MIH)	MIH_F	98.9%	TACTCCGACTGGTACTTCCAC
		MIH_R		CAAGCAGCTCCGATCTTTGAC
	Ecdysteroid receptor (EcR)	EcR_F	97.0%	GAGGATCTCTGCCTCGTGT
		EcR_R		GCTGCCGAACTTGCACTGATA
	Farnesoic acid *O*-methyltransferase (Famet)	Famet_F	98.4%	ACGGCGATACCTTCACTTTCAC
		Famet_R		TTGACCATCCGCCAATGAAGAC
	Methyl farnesoate epoxidase (Cyp15A1)	Cyp15A1_F	100.3%	CCACCACTTCCGCAACCTC
		Cyp15A1_R		TCTCTGCCCAGCCAACACC
Antioxidant defences	Glutathione-S-transferase (GST)	GST_F	100.0%	CGAGGAGCTGGAACCCATC
		GST_R		TACTCAACCCCGCTACCAAAC
	Selenium-dependant glutathione peroxidase (SeGPx)	SeGPx_F	101.8%	CATCAGGAGAACACCACCACC
		SeGPx_R		GTCCCCGAGAGGCTGAAAG
	Catalase (Cat)	Cat_F	99.0%	CATCCACACACAGAAACGCAAG
		Cat_R		CTCGGTCCGTAAACAGGAAG
	Cu/Zn superoxide dismutase (CuSOD)	CuSOD_F	98.6%	GTGGACCGGATAGCGTACAG
		CuSOD_R		CGCCGTACAGGGTCACATAG
	Mn superoxide dismutase (MnSOD)	MnSOD_F	98.9%	TGTCTGGGAGCACGCCTAC
		MnSOD_R		AGCGAGATCATATCTGGCAGC
Antitoxic defences	HSP70	HSP70_F	100.4%	CAACTATTCCTACCAAGCAGACC
		HSP70_R		ATTCCAGTAAGCTCAAACTTGCC
	HSP90	HSP90_F	97.3%	CTGGAGTCATCACCATCATCAC
		HSP90_R		TGGGGTCGAGGAGGATGAG
	Thioredoxin (THx)	THx_F	96.4%	GGTCCCTGCAAATTATTGGCTC
		THx_R		TGACAGATGGAACAGCAGACAC
	Thioredoxin reductase (THx-red)	THx-red_F	102.8%	GATGACCTGTTCTCGCTGCC
		THx-red_R		GCCACTTTGTTTGCCATTTGCTG
Reference genes	Ribosomal small protein 3	RSP3_F	100.6%	TGCCCAACACAAACAGGTCA
		RSP3_R		GCGAATGTCGTCAGCAATGG
	Elongation factor 1α	EF1a_F	100.1%	GTCGCTTGCTGAGTTATCACG
		EF1a_R		GATGAGATGACCGGTGGTGG

dffects on molecular responses were analyzed on log_2 transformed normalized relative quantities. For each exposure temperature and time, ANOVA tests followed by a Fisher LSD post hoc test was performed to analyze the Hg exposure effects on molecular responses, as compared to respective control group. All statistical analyses were carried out with the XLSTAT 2019.1.2 Software (Addinsoft, France) and a probability value lower than 0.05 was considered significant.

3. Results

3.1. G. pulex mortality

The mortality rate of *G. pulex* exposed to $HgCl_2$ was estimated after 7 days and 21 days of exposure (Fig. 2). The results showed that mortality increased with time and concentration of exposure. For example, the exposure to Hg 50 ng/L at 16 °C caused significantly higher mortality after 21 days of exposure (55.6%) than after 7 days (20%). In addition, although no significant differences were observed, the mortality rate tended to be higher for exposure at 20 °C compared to 16 °C, regardless of the time and condition of exposures (i.e. control or Hg exposures)..

3.2. Hierarchical clustering and PCA analyses

For each temperature and time of exposure, a heat map was created

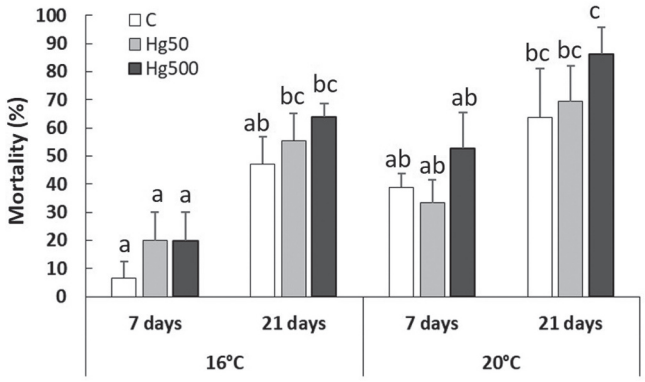


Fig. 2. Mortality rate (%) of *Gammarus pulex* exposed for 7 and 21 days to $HgCl_2$ 50 and 500 ng/L at two temperature variations (16 °C ± 2 °C; 20 °C ± 2 °C).

using hierarchical clustering to determine the relationship between exposure condition according to molecular responses (Fig. 3). The clustering analysis revealed significant grouping depending the exposure time, but similar groupings for both exposure temperatures. After 7 days of exposure, regardless of temperature, *G. pulex* exposed to $HgCl_2$

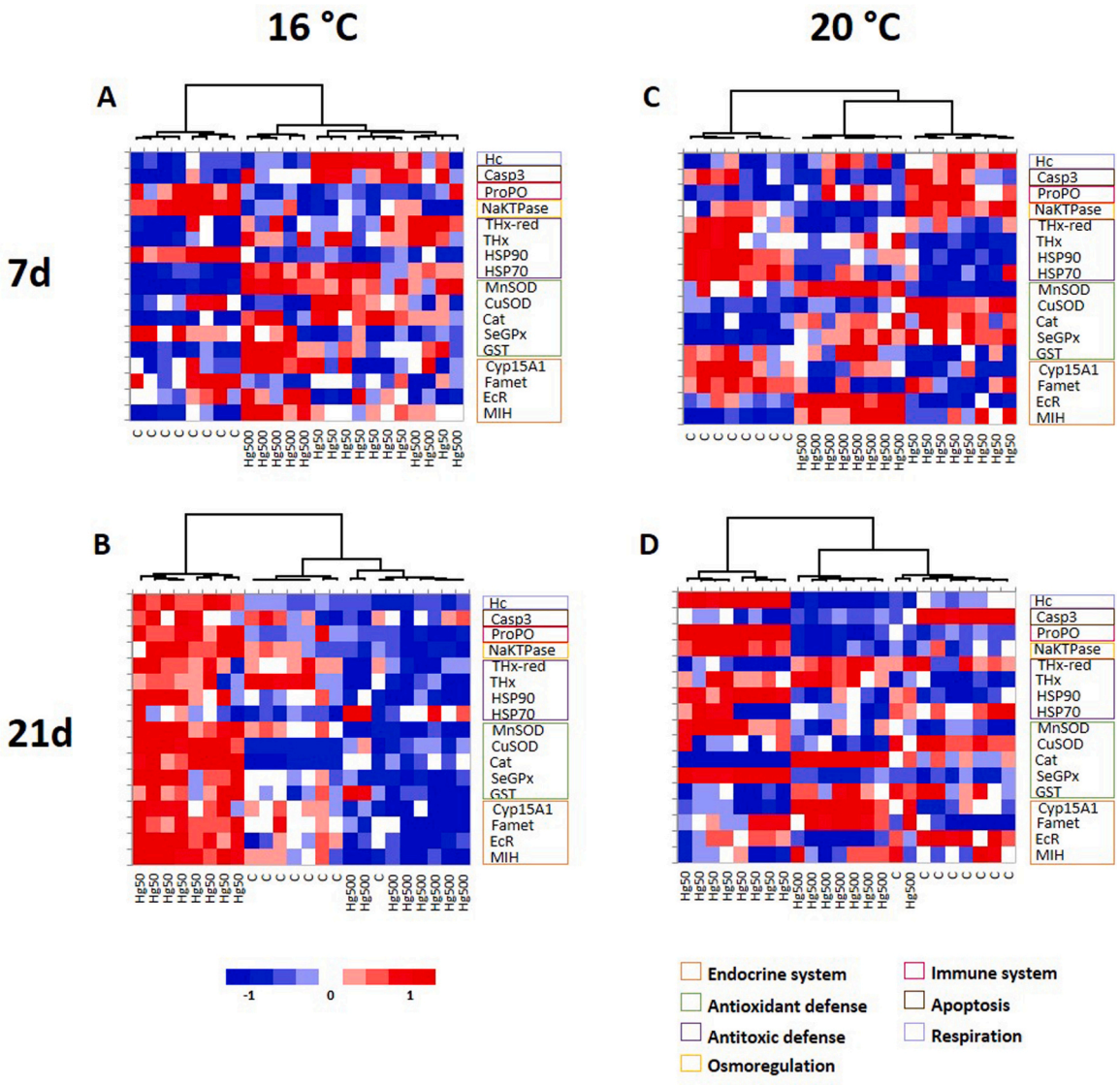


Fig. 3. Heat map of hierarchical clustering of *Gammarus pulex* exposed for 7 and 21 days to $HgCl_2$ 50 and 500 ng/L at two temperature variations (16 °C ± 2 °C; 20 °C ± 2 °C) for each biological mechanism and function.

50 and 500 ng/L were clustered (Fig. 3 A,C); whereas after 21 days of exposure, *G. pulex* exposed to $HgCl_2$ 500 ng/L were grouped with control group (Fig. 3 B,D).

Principal Component Analysis realized for experiments I and II confirmed the clustering of exposure conditions and highlighted the correlation with molecular responses (Fig. 4). PCA obtained for the exposure at 16 °C revealed grouping by treatment with 31.3% of variance explained by F_1 and 24.7% by F_2. After the 7d-exposure, control *G. pulex* were strongly positively correlated to HSP90, ProPO, Famet and negatively correlated to HSP70 and Casp3; whereas the opposite result was observed for the 21d-control group (C-21d). The clustering of *G. pulex* exposed for 7d to the two $HgCl_2$ concentrations (Hg50–7d and Hg500–7d) observed on the heat map was confirmed by the superposition of these two groups on PCA. Besides, these groups were strongly positively correlated to genes involved in antioxidant and antitoxic defenses (i.e. CuSOD, MnSOD, Cat, THx, THx-red) and endocrine systems (i.e. MIH, Cyp15A1). This positive correlation was even stronger after 21 days of exposure to 50 ng/L, but completely reversed at 500 ng/L. All exposure groups were positively correlated to HSP70 and Casp3, and negatively correlated to HPS90, ProPO and Famet.

Similarly, PCA resulting from the exposure to 20 °C revealed grouping by treatment with 29.1% of variance explained by F_1 and 22.8% by F_2. The 7d-control group was positively correlated to Cyp15A1, GST, THx, and HSP90 while the Hg50–7d and Hg500–7d groups were both positively correlated to oxidative defense genes (i.e. Cat and CuSOD) and endocrine system genes (i.e. EcR, MIH). After 21d of exposure, the control group was positively correlated to HSP70, Famet and Casp3, all involved in different biological functions. Besides, the molecular responses of *G. pulex* exposed to 50 ng/L were highly positively correlated to Na/K-ATPase, Hc, ProPO and SeGPx genes, while when exposed to 500 ng/L, positive correlations with Cyp15A1, GST, THx, and HSP90 genes were observed.

3.3. Molecular responses

To assess the sublethal effects of $HgCl_2$, the relative expressions of several genes involved in different biological functions and mechanisms were evaluated considering temperature, time and concentrations of exposure. Although gene expression analysis didn't reveal specific gene variations, results revealed that all biological function studied were significantly impacted by $HgCl_2$ exposure (Table 2). Among these, osmoregulation was the least affected by $HgCl_2$, as only *G. pulex* exposed for 21 days at 16 °C and Hg 500 ng/L showed a significant variation in Na/K ATPase expression compared to the respective control. On the

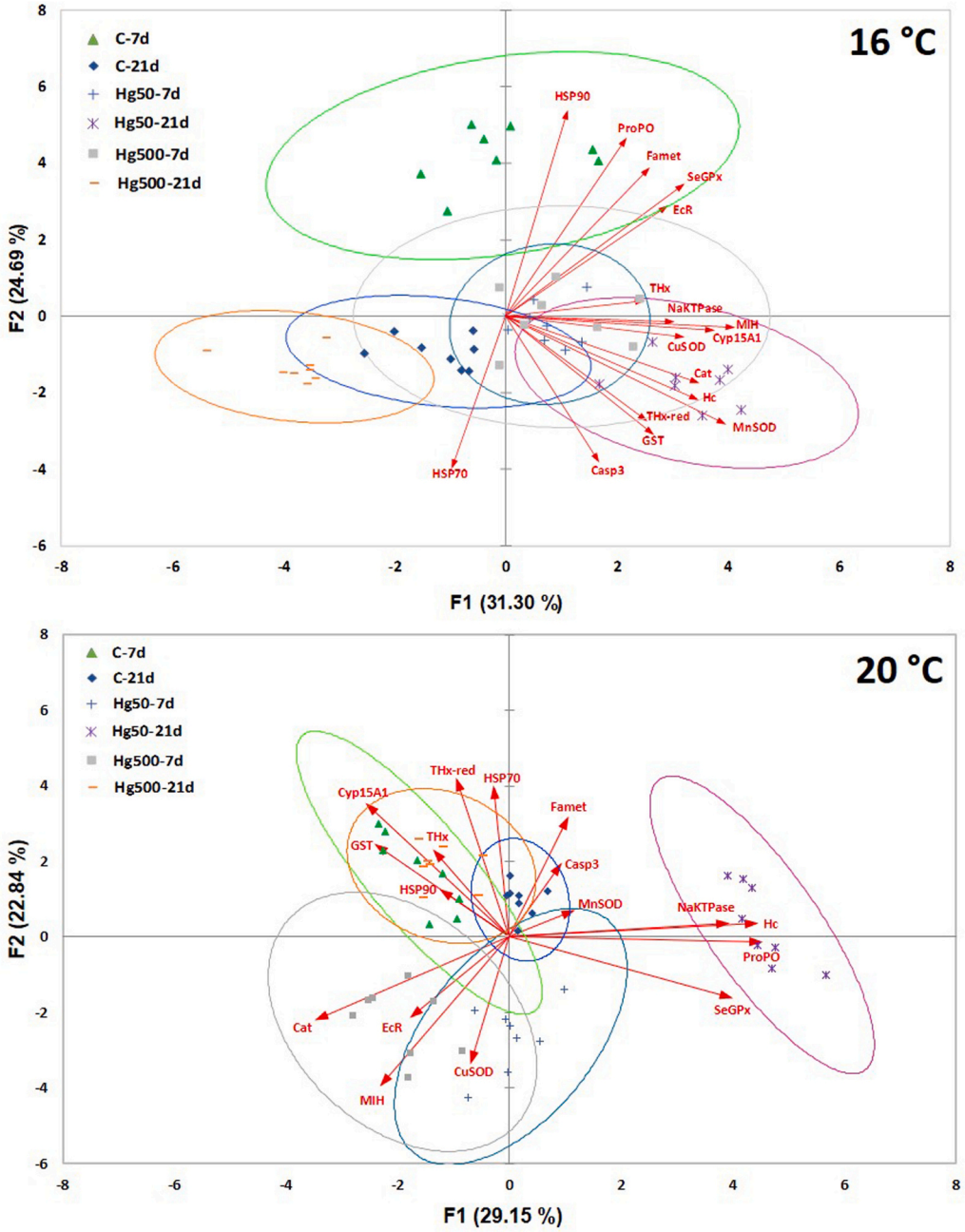


Fig. 4. Principal Component Analysis (PCA) of *Gammarus pulex* exposed for 7 and 21 days to $HgCl_2$ 50 and 500 ng/L at two temperature variations (16 °C ± 2 °C; 20 °C ± 2 °C) for each biological mechanism and function.

contrary, genes involved in antioxidant and antitoxic defense systems were the most affected by $HgCl_2$ exposures. Overall, oxidative defense genes were between 1.5- to 4.6-fold overexpressed in *G. pulex* exposed to $HgCl_2$ as compared to control group. Cat gene was the most impacted by Hg exposure, as its expression was significantly altered under all temperature and exposure conditions (i.e. from 1.5- to 3.5-fold overexpressed in all exposure conditions, except for a 1.7-fold under-expression after 21d of exposure to 50 ng/L at 20 °C). CuSOD and MnSOD were mainly affected by $HgCl_2$ when exposures were carried out at 16 °C; CuSOD being overexpressed after 21d of exposure (4.6 and 1.9-fold change, respectively) and MnSOD being mainly overexpressed after 7d of exposure (average 3-fold change). The expression of the GST gene was from 1.6- to 2-fold overexpressed after 7d of exposure at 16 °C and no change was measured for the other exposure conditions. On the contrary, changes in SeGPx expression were mainly measured after 7d of exposure at 20 °C (Table 2).

Genes involved in antitoxic defenses also showed different patterns according to exposure time, temperature and $HgCl_2$ concentration. After 7d of exposure at 16 °C, both the HSP70 and THx were significantly overexpressed (average 2.5 and 1.7-fold, respectively) and the HSP90 gene was 3-fold under-expressed, while after 21d of exposure, HSP90 was 1.7-fold overexpressed and THx expression was twice as low. Conversely, exposure at 20 °C resulted in significant under-expression of all antitoxic defense genes after 7d of exposure, regardless of $HgCl_2$ concentration; while slight over-expressions were measured for HSP90 and THx, after 21d-exposure to 50 and 500 ng/L, respectively.

The results obtained for endocrine system genes showed that MIH and EcR genes were mainly over-expressed after 7d-exposure at 20 °C (1.5- to 2-fold changes), while at 16 °C, the variation in expression was only observed after 21d of exposure (1.5-fold change). Besides, $HgCl_2$

Table 2
Relative gene expression (log_2 fold-change) of each biological function and mechanism of *Gammarus pulex* exposed for 7 and 21 days to $HgCl_2$ 50 and 500 ng/L at two temperature variations (16°C ± 2°C; 20°C ± 2°C). Statistical analyses reveal significant under- or over-expressions compared to the respective control group.

	16 °C				20 °C			
	7d		21d		7d		21d	
[Hg] (ng/L)	50	500	50	500	50	500	50	500
Endocrine system								
MIH		0.8	0.6	-0.8	0.6	1.0		
EcR			0.6			0.7		-1.0
Famet		-1.2		-0.9		-1.0	0.7	0.9
Cyp15A1				-0.8	-1.0			0.7
Antioxidant defence								
GST	0.7	1.0						
Se-GPx			0.7		1.1	0.9	1.5	
Cat	1.0	0.7	1.8	0.8	0.8	0.8	-1.7	0.8
CuSOD		-1.0	2.2	0.9	1.0			
MnSOD	1.6	1.7	0.6	-1.4	-0.8		0.6	
Antitoxic defence								
HSP70	1.3	1.3		0.8	-0.8	-0.8		
HSP90	-1.8	-1.7	0.7		-1.5	-0.8	0.6	
THx	0.7	0.8		-1.3	-1.4			0.6
THx-red				-0.7	-0.7	-1.4		
Immune system								
ProPO	-1.0	-1.0	0.8		0.8		1.5	-0.8
Apoptosis								
Casp3	0.9	0.6					-0.8	-0.8
Osmoregulation								
Na/K ATPase				-0.7				
Respiration								
Hc	0.9		2.0		0.6		1.2	-1.2

Red: significant over-expression (FC ≥ 1.5, log_2 ≥ 0.6)
Blue: significant under-expression (FC ≤ 0.6, log_2 ≤ -0.7)
White: no significant effect on relative gene expression

exposure halved the expressions of Famet and Cyp15A1, except after 21d of exposure at 20 °C where Famet and Cyp15A1 were from 1.6 to 1.9-fold over-expressed.

ProPO gene was primarily over-expressed when *G. pulex* were exposed to $HgCl_2$ 50 ng/L, except after 7d of exposure at 16 °C where its expression was under-expressed by both $HgCl_2$ concentrations. Conversely, Casp3 gene was from 1.5- to 2-fold over-expressed after 7d of exposure at 16 °C, but 1.7-fold under-expressed after 21d of exposure at 20 °C. Finally, Hc over-expression was measured after exposure at $HgCl_2$ 50 ng/L, regardless of the time and temperature of exposure.

3.4. Integrated biomarker responses

All 17 biomarker genes were integrated into star plots for each $HgCl_2$ concentration considering time and temperature of exposure, and IBR indices were calculated to assess the global toxic effect of $HgCl_2$ in each exposure situation (Fig. 5). The IBR results confirmed that the Hg toxicity was dependent on the concentration, time and temperature of exposure. At 16 °C, the IBR_{Hg500} values were higher than the IBR_{Hg50} values regardless of the time of exposure, however both the IBR_{Hg50} and IBR_{Hg500} values were lower after 21d of exposure than after 7d of exposure. On the contrary, at 20 °C, the IBR_{Hg500} values were lower than the IBR_{Hg50} values regardless of the time of exposure. Moreover, the IBR_{Hg50} values were similar after both exposure times, while a lower IBR_{Hg500} value was estimated after 21d of exposure compared to the 7d-exposure.

4. Discussion

4.1. Mortality and sub-lethal effects of Hg

The present work focused on assessing of the toxicity of inorganic Hg ($HgCl_2$) in the freshwater amphipod *G. pulex*, by measuring the relative expression of several genes involved in seven biological mechanisms and functions, e.g. respiration, osmoregulation, apoptosis, immune system, endocrine system, and antitoxic and antioxidant defense systems. In addition, the effects of Hg were evaluated at two exposure times (7 days and 21 days) and at two different temperatures subject to daily fluctuations (16 °C ± 2 °C and 20 °C ± 2 °C).

4.1.1. Mortality

Although statistical analysis did not reveal any significant differences, the results showed that mortality tended to increase with exposure temperature and Hg concentration. Indeed, it is not surprising to highlight the effect of temperature on mortality (i.e. a higher mortality rate in the control group exposed to 20 °C than in the group exposed to 16 °C), as it is well established that the survival rate of ectothermic organisms is affected by temperature (Issartel et al., 2005; Maazouzi

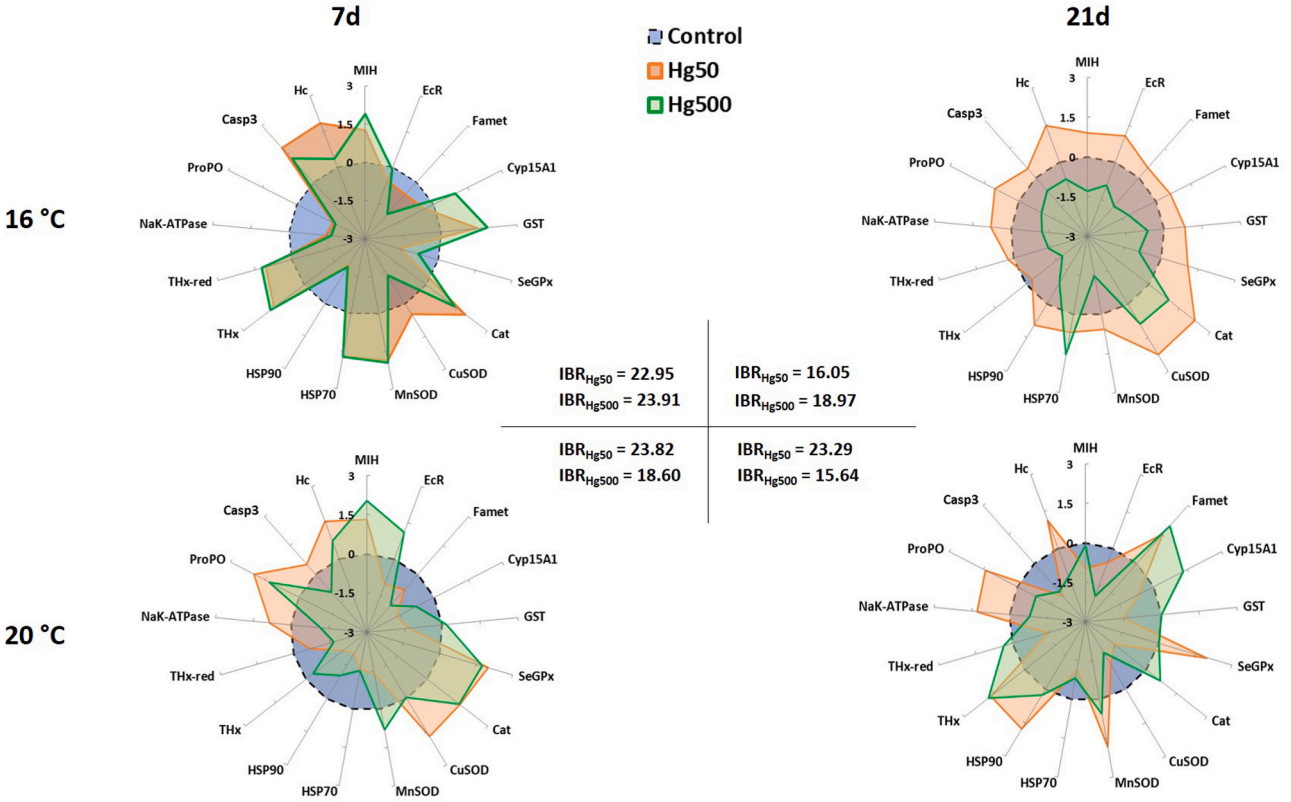


Fig. 5. Radar plots and Integrated Biomarker Response (IBR) of *Gammarus pulex* exposed for 7 and 21 days to $HgCl_2$ 50 and 500 ng/L at two temperature variation (16 °C ± 2 °C; 20 °C ± 2 °C).

et al., 2011). This result could be related to the decrease in oxygen concentration when temperature increases, which leads crustaceans to hyperventilate (Hervant et al., 1997; Wijnhoven et al., 2003; Maazouzi et al., 2011), which represents a significant energy cost (Foucreau et al., 2014). In addition, it has also been shown that the oxygen consumption of crustaceans increases with increasing temperature (Issartel et al., 2005; González et al., 2010; Maazouzi et al., 2011), which probably leads to an increase in the production of reactive oxygen species, known to be harmful to the organism. Nevertheless, the high mortality measured in the control groups remains surprising, considering that all precautions were taken according to the US EPA method (food, synthetic water,. - Weber, 1991). Although no hypothesis could be put forward to explain this result, it is consistent with the mortality measured by Henry et al. (2017) in their control groups of *G. pulex*, exposed according to the same experimental protocol (e.g. acclimatization, feeding, synthetic water, renewal). Indeed, after 8 days of exposure (maximum exposure time of their study), the authors measured mortality of about 20% and 30% at 15 °C and 20 °C, respectively.

As for the effect of temperature, the mortality rate was influenced by the Hg exposure concentration because the higher the Hg concentration, the higher the mortality rate (although no significant difference was demonstrated). Similar results were observed in the amphipod *Hyalella azteca* (Borgmann et al., 1993) or the crab *Eriocheir sinensis* (Zhao et al., 2010). These results are probably related to the dose-dependent bioaccumulation of Hg in organisms, and thus to possible sublethal toxic effects caused by Hg as observed in other organisms (e.g. oxidative stress, DNA damage - Ali et al., 2000; Di Donato et al., 2016; Roos-Munoz et al., 2019).

Finally, regardless of the Hg concentration, the mortality rate was higher at 20 °C than at 16 °C, which is consistent with previous studies on *Gammarus* sp. considering the influence of temperature on the toxicity of other trace elements. For example, mortality of *G. pulex* exposed to copper, zinc or lead was greater at 25 °C than at 15 °C (Bat et al., 2000). A similar result was observed in *Gammarus fossarum* exposed to cadmium and arsenic at 5, 10 and 15 °C (Vellinger et al., 2012). Increasing temperature is known to increase activity and oxygen consumption in *G. pulex* (Maazouzi et al., 2011), which could therefore be responsible for increased production of reactive oxygen species, leading to toxicity and mortality. In addition, it has been observed that an increase in temperature leads to an increased accumulation of Hg in Nile tilapia, *Oreochromis niloticus* (Waheed et al., 2020), and that Hg can interact with mitochondria, causing the production of hydrogen peroxide (Lund et al., 1993). Therefore, it can be assumed that the highest mortality of *G. pulex* observed at 20 °C is the consequence of both high oxygen consumption and high Hg accumulation, both of which cause significant production of ROS, which are harmful to organisms. This also corroborates the findings of Heit and Fingerman (1977) who found that Hg toxicity in crayfish was less pronounced during cold periods than during warm periods, probably due to a lower metabolism.

4.1.2. Antioxidant and antitoxic defense system-related genes

The sublethal effects of Hg on antioxidant enzyme genes were investigated after 7 days and 21 days of exposure at both temperatures. In general, a gene overexpression was observed under several conditions, suggesting that oxidative stress occurred during Hg exposure. Among the antioxidant enzymes, Cat was the most over-expressed, considering all experimental conditions. Cat is an enzyme common to almost all living organisms, known to breakdown hydrogen peroxide (H_2O_2) into oxygen and water, and therefore protects the cell from oxidative stress (Kirkman et al., 1987). Its overexpression in Hg conditions, compared to control, suggests a H_2O_2 production (Lund et al., 1993). Although this result is consistent with the increase of catalase activity in the hepatopancreas of the crab *E. sinensis* exposed to Hg 0.01 mg/L (Zhao et al., 2010), it is in contrast to the fact that $HgCl_2$ caused a significant depletion of catalase activity in the crab *Scylla*

serrata exposed to Hg 1 and 10 μg/L (Singaram et al., 2013) or in mouse liver (Sharma et al., 2007). The assumption of ROS production under $HgCl_2$ exposure is accentuated with the increase in both SOD genes, mainly at 16 °C. SOD are the first line of cellular defense against ROS toxicity, and similar results has been observed in flounder, zebrafish, or crab exposed to Hg (Huang et al., 2010; Zhang et al., 2016; Ren et al., 2019). MnSOD was the first form of SOD to show overexpression (from 7d of exposure at 16 °C), probably due to its localization in the mitochondria which are the main site of ROS production, compared to CuSOD localized in the cytosol and mainly overexpressed after 21d of exposure at 16 °C.

SeGPx was up-regulated in *G. pulex* exposed to Hg, mainly after 7d of exposure at 20 °C. SeGPx activity accounts for most of the total GPx activity in Gammarids (i.e. ~80% - Sroda and Cossu-Leguille, 2011) and plays an important role in protecting membranes from lipoperoxidation by stopping the radical chain propagation. Indeed, GPx decomposes hydrogen peroxide into water by using glutathione as substrate (van der Oost et al., 2003), which corroborates the high affinity of $HgCl_2$ to sulfhydryl groups such as glutathione (Stohs and Bagchi, 1995). An increase in GPx activity or mRNA expression has also been observed in other organisms exposed to $HgCl_2$ such as aquatic vertebrates (Monteiro et al., 2010; Naïja et al., 2016; Zhang et al., 2016; Ren et al., 2019) or invertebrates (Zhao et al., 2010; Rabeh et al., 2019). However, an over-expression of SeGPx remains surprising considering that the activity of SeGPx depends of the Se concentration in organisms, and that this could be reduced, because in vertebrates, Se is known to prevent Hg toxicity by complexing to form insoluble and less toxic HgSe (Watanabe, 2002). Finally, the overexpression of GST, especially at 7 days of exposure at 16 °C, supports the hypothesis of activation of detoxification processes. It is well known that GST intervenes mainly in phase II detoxification of organic compounds, transforming them into compounds that are more soluble in water and easier to eliminate. However, previous investigations have shown an increase in GST activity in several crustaceans exposed to Hg (Elumalai et al., 2007; Yamuna et al., 2012; Singaram et al., 2013), suggesting that increased GST in organisms exposed to Hg may be an adaptive response to oxidative stress.

The thioredoxin system (thioredoxin - THx, thioredoxin reductase - THx-red) is involved in the maintenance of redox homeostasis, but also plays a key role in several essential biological functions such as DNA synthesis and cell proliferation, angiogenesis, control and activity of many transcription factors, cells protection against apoptosis and regulation of the cell signalling pathway (Lu and Holmgren, 2014). Exposure to $HgCl_2$ mainly resulted in THx and THx-red under-expression in *G. pulex*, which could be explained by the interaction between Hg and the thioredoxin system. This finding is corroborated by Branco et al., (2012, 2014) who observed an inhibition of THx-red activity in HepG2 cells and in *Diplodus cervinus* exposed to $HgCl_2$. Besides, this inhibition and THx under-expression could explain the concomitant THx under-expression which is the substrate of the enzyme. Since the thioredoxin system is upstream of many important pathways (e.g. protein repair and folding, DNA synthesis, apoptosis, H_2O_2 reduction - Holmgren, 1989; Zhang et al., 1997; Branco and Carvalho, 2019), inhibition or under-expression of THx and THx-red may be a major step in the toxic effects of xenobiotics, such as Hg, and could probably be related to the high mortality of *G. pulex* observed in exposure conditions (20 °C compared to 16 °C).

Heat-shock proteins (HSPs), such as HSP70 and HSP90, have important roles in countering toxic effects on cell's proteins, including chaperoning proteins during synthesis, folding, assembly, and degradation. Their role is linked to the proteasome to remove non-functional proteins, and avoid adverse effects (Santoro, 2000; Reeg et al., 2016). Oxidative stress caused by $HgCl_2$ exposure in *G. pulex* can lead to protein misfolding compared to their native conformation, resulting in an increase of HSP70 as observed in the present study. Similar variations in HSP70 were observed in several organisms exposed to $HgCl_2$. For example, over-expressions were measured in *O. niloticus* (Waheed et al., 2020), *Pelteobagrus fulvidraco* (Sun et al., 2018), *Folsomia candida* (Liu et al., 2010) or *Mytilus galloprovincialis* (Franzellitti and Fabbri, 2005) but no change was observed in the copepod *Pseudodiaptomus annandalei* (Bai and Wang, 2020). In contrast, HSP90 was mainly under-expressed suggesting induction of apoptosis due to its direct or indirect role in caspase activation (Lanneau et al., 2008). This assumption is corroborated by the concomitant over-expression of Casp3, known to be involved in the apoptosis pathway, being the last molecule activated before cell nucleus disruption (Porter and Jänicke, 1999), which is also consistent with the overexpression of genes related to the antitoxic and antioxidant defence system (GST, Cat, MnSOD, THx). Finally, after a longer time of exposure, HSP70, HSP90 and the THx system genes were less modulated in the two temperatures tested, suggesting an adaptive response of *G. pulex*.

4.1.3. Endocrine system related genes

Although Hg toxicity on the endocrine system has already been observed in aquatic vertebrates (e.g. pro-adipogenic properties in rainbow trout preadipocytes – (Tinant et al., 2020); inhibition of aromatase activity – (Hinfray et al., 2006); decreased gonadotropin-releasing hormone in beluga and zebrafish - Gharaei et al., 2010; Richter et al., 2011), no studies have been conducted to assess the impact of Hg on the endocrine system of invertebrates such as crustaceans. However, Weis (1978) and Callahan and Weis (1983) observed that Hg delayed limb regeneration and ecdysis in three crab species, *Uca pugilator*, *U. pugnax* and *U. minax*, suggesting that Hg also has an impact on the endocrine system related to molting and growth of crustaceans. These studies support our findings showing over-expression of the molt-inhibition hormone (MIH) which control the release of ecdysteroid hormones (Hyne, 2011). However, it is surprising that over-expression of the ecdysteroid receptor (EcR) was also measured. This could be explained by an induction of ecdysteroid synthesis (e.g. 20-hydroxyecdysone, 20HE) early in exposure (no change observed at 7 days) which could lead to feedback control of 20HE (Gismondi, 2018) and thus to an increase of MIH and EcR after a long exposure (21 days). Similarly, the observed delay in limb regeneration (Weis, 1978) could be a consequence of the under-expression of Famet and Cyp15A1, both of which are enzymes involved in the methylfarnesoate pathway, the growth hormone of crustaceans (Hyne, 2011). Consequently, changes in the expressions of genes involved in the ecdysteroids and methylfarnesoate pathways in *G. pulex* exposed to Hg suggest that Hg may interact with endocrine organs (i.e. X-organ, Y-organ or mandibular organ – Hyne, 2011), resulting in a disruption of molting, known to be related to amphipod reproduction and growth, and thus having a potential adverse effect on population fitness.

4.1.4. Other genes

The present study evaluated the Hg effect on the Na/K ATPase, ProPO and Hc genes, involved in osmoregulation, the immune system and respiration, respectively. The Hg toxicity to the invertebrate immune system has already been observed in several studies (Sauvé et al., 2002; Gagnaire et al., 2004; Sreenivasula Reddy et al., 2011). For instance, in crustaceans, Kaoud and Singaram et al., (2011, 2013) both observed a decrease in phagocytosis activity in the decapod *Macrobrachium rosenbergii* and the crab *S. serrata*, respectively, after Hg exposure.

Besides, the ProPO cascade, comprising the inactive form (ProPO) and the active form (phenoloxidase, PO), is a general defense system that provides immunity against pathogens found in many organisms (Cerenius and Söderhäll, 2004). Singaram et al. (2013) have pointed out that Hg caused an increase in PO activity in *S. serrata,* which contrasts with our results showing an under-expression of ProPO after 7 days of exposure. This discrepancy could be due to the fact that *S. serrata* was exposed for a longer time (14 days) to Hg, but therefore could be related to the increase in PO activity, observed by Singaram et al. (2013), and to the over-expression of ProPO measured in our study after a longer

exposure period (21 days). However, over- or under-expression of the ProPO gene does not mean that Hg decreases or increases the effectiveness of *G. pulex*'s immune system, but only indicates that immune system maintenance is impaired. Only the evaluation of the activity of PO would give more information on the effect of Hg on the effectiveness of the immune system of *G. pulex*.

The ubiquitous Na/K ATPase plays a key role in organisms, mainly in osmoregulation but also in maintaining resting potential, transport, and regulation of cellular volume. It is also a signal transducer/integrator for regulating ROS, as well as intracellular calcium. Many studies have demonstrated the inhibition of Na/K ATPase in aquatic organisms exposed to Hg (Bouquegneau, 1977; Verma et al., 1983; Jagoe et al., 1996; Poopal et al., 2013), due to binding of Hg to a protein-specific cysteine (Wang and Horisberger, 1996; Bhattacharya et al., 1997). However, no significant change in Na/K ATPase gene expression was observed in *G. pulex*, under any exposure condition, suggesting that there is no change in pump activity. This could likely be explained by a very low concentration of Hg impacting the Na/K ATPase system, as observed in the crab *E. sinensis*, where no effect was measured on Na/K ATPase activity in organisms exposed at Hg concentrations below 0.01 mg/L (Zhao et al., 2011).

Hemocyanin (Hc), on the other hand, is the protein necessary for oxygen transport in arthropods and mollusks (Bellelli et al., 1985). To date, studies conducted on crustaceans have shown that Hg reduces oxygen consumption, as shown in shrimp *Pandalus borealis* (St-Amand et al., 1999), *Farfantepenaeus brasiliensis* (Barbieri et al., 2005) and *Exopalaemon carinicauda* (Zhang et al., 2017), and the crab *U. annulipus* and *U. triangularis* (Devi and Rao, 1989); probably because it can eliminate copper atoms from the active site of Hc, which is essential for oxygen transport (Bellelli et al., 1985). However, the increases in Hc gene expression observed in our study could be explained by an increase in protein synthesis as an adaptation mechanism of *G. pulex* to compensate for the possible commitment in oxygen consumption.

4.2. Influence of temperature and time of exposure on Hg toxicity

It has been well known for many decades that an increase in temperature can influence the susceptibility of organisms exposed to contaminants, such as Hg, either by altering bioaccumulation or by influencing the biochemical performance of organisms (Heugens et al., 2001; Sokolova and Lannig, 2008). In general, it has often been observed than an increase in temperature leads to higher accumulation of Hg in exposed organisms (Tsui and Wang, 2004; Dijkstra et al., 2013; Pack et al., 2014; Waheed et al., 2020). However, it cannot be concluded that the increase accumulation of Hg has led to higher effects, as detoxification and excretion processes may also have been stimulated (Cairns et al., 1975). Our IBR data revealed that an increase in temperature resulted in higher Hg toxicity at the lowest concentration (50 ng/L), but the opposite was observed at the highest concentration (500 ng/L), regardless of the time of exposure. *G. pulex* exposed to Hg for 7 days to 20 °C showed similar patterns of antioxidant defence gene expression as *G. pulex* exposed to 16 °C, suggesting that increased temperature didn't increase oxidative stress caused by Hg. This finding is in agreement with Freitas et al. (2017) who observed similar antioxidant capacities in *M. galloprovincialis* exposed to Hg at 17 °C and 20 °C, but disagrees with Verlecar et al. (2007) who showed that temperature increase amplified oxidative damage caused by Hg in *Perna viridis*. The absence of higher Hg effects after a 7days exposure at 20 °C, compared to 16 °C for the highest concentration, was also evidenced by the generalised under-expression of all genes related to the antitoxic defence system (HSP, THx) and apoptosis, which is however surprising considering that heat stress is known to induce HSP. Nevertheless, over-expression of immune and antioxidant system genes (Cat, SeGPx), enzymes responsible for the conversion of H_2O_2 to water and oxygen, was observed in the same experimental group. Our results could be explained by the period of maintenance of *G. pulex* in the laboratory at 20 °C after sampling, as it has been shown that a period of acclimatization can reduce thermal sensitivity and improve tolerance to heat stress (Semsar-kazerouni and Verberk, 2018).

In addition, studies conducted in bivalves have observed that an increase in exposure temperature resulted in a loss of metabolic and biochemical functions, as well as a decrease in the electron transport system activity preventing the bioaccumulation of Hg and thus reducing the effects of Hg (Coppola et al., 2017, 2018; Morosetti et al., 2020). However, this does not appear to be the case in our study, as it cannot be ignored that exposure to Hg at 20 °C resulted in higher mortality of *G. pulex* than at 16 °C. The IBR results also revealed that 21d-exposure has less effect than 7d-exposure, at both temperatures. Additionally, induction of all genes, except THx, was observed for *G. pulex* exposed to the lowest Hg concentration (50 ng/L) for 21 days at 16 °C, compared to the same temperature group exposed for 7 days, which may explain the lower IBR values. However, since higher mortality rates were observed at 21 days, these findings may be the consequence of gene expression analysis performed on the still alive organisms that may be the most resistant and/or adapted amphipods. This would also likely explain the overlap between these individuals and those in the control group in the PCA analysis, attesting a weak effect of Hg. Indeed, many studies have shown a weakening of individuals exposed over the long term or during repeated toxic pressures (Russo et al., 2018). Nevertheless, the acquisition of tolerance or adaptation to pesticides through physiological acclimatization or genetic adaptation has also been observed (Becker and Liess, 2015, 2017; Weston et al., 2013). In the present study, genetic adaptation is the least possible hypothesis since the exposure was only carried out for 21 days, which is too short for the appearance of a mutation, but it cannot be excluded that the individuals remaining alive at the end of exposure (and used for gene expression) already possessed genetics favourable to resist pollutants. A physiological adaptation leading to better tolerance seems the most likely hypothesis, but further analysis (e.g. antitoxic defence enzyme activities or Na/K ATPase pump) is needed to confirm this hypothesis.

5. Conclusion

The novelty presented herein contributes to the knowledge of the lethal and sublethal effects of environmental relevant inorganic Hg concentrations on freshwater amphipods at two temperatures. Our results showed that higher exposure temperatures increased *G. pulex* mortality. Moreover, a higher temperature resulted in an increase in the Integrated Biomarker Response values for *G. pulex* exposed to 50 ng/L $HgCl_2$ for 7 and 21 days, but not to those exposed to 500 ng/L, suggesting possible adaptation of *G. pulex*, especially after 21 days of exposure.

Overall, genes related to antioxidant and antitoxic defence system, as well as genes of the endocrine and immune system genes were more affected by exposure, regardless of the concentration, time and temperature tested. In contrast, osmoregulation was the least affected biological function. Other genes, involved in respiration and apoptosis were also over-expressed after exposure. Finally, the present study confirms the importance of using combined biomarkers to identify the harmful effects of low concentrations of Hg in different biological functions of this widely distributed amphipod. Nonetheless, it would be interesting to evaluate the expression of genes as a function of the specific tissue in order to deepen this study and the understanding of the effects of Hg, since genes are not expressed identically depending on the tissue under consideration.

CRediT authorship contribution statement

Eric Gismondi: Conceptualization, Methodology, Supervision, Formal analysis, Funding, Writing - original draft, Writing - review & editing. **Madson de Melo**: Methodology, Writing - original draft, Writing - review & editing. **Krishna Das**: Writing - original draft.

Declaration of Competing Interest

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

Acknowledgements

We thank the three reviewers' comments that led to improve the manuscript. This project was funded by the FSR2018 obtained from the FOCUS research unit (ULièfBelgium). KD is a F.R.S.-FNRS Senior Research Associate. We thank also Dr Helen Poynton for her help and comments that improve the manuscript.

References

Ali, M.B., Vajpayee, P., Tripathi, R.D., Rai, U.N., Kumar, A., Singh, N., Behl, H.M., Singh, S.P., 2000. Mercury bioaccumulation induces oxidative stress and toxicity to submerged macrophyte *Potamogeton crispus* L. Bull. Environ. Contam. Toxicol. 65 (5), 573–582.

Bai, Z., Wang, M., 2020. Warmer temperature increases mercury toxicity in a marine copepod. Ecotoxicol. Environ. Saf. 201, 110861 https://doi.org/10.1016/j.ecoenv.2020.110861.

Barbieri, E., Passos, E.A., Garcia, C.A.B., 2005. Use of metabolism to evaluate the sublethal toxicity of mercury on *Farfantepaneus brasiliensis* larvae (Latreille 1817, Crustacean). J. Shellfish Res. 24, 1229–1233. https://doi.org/10.2983/0730-8000.

Bat, L., Akbulut, M., Ulha, M., HŸseyin, S.H., 2000. Effect of temperature on the toxicity of zinc, copper and lead to the freshwater amphipod *Gammarus pulex pulex* (L., 1758). Turk. J. Zool. 24, 409–416.

Becker, J.M., Liess, M., 2015. Biotic interactions govern genetic adaptation to toxicants. Proc. Royal Soc. B, 282,1806, 20150071.

Becker, J.M., Liess, M., 2017. Species diversity hinders adaptation to toxicants. Environ. Sci. Technol. 51 (17), 10195–10202.

Bellelli, A., Zolla, L., Giardina, B., Costantini, S., Cau, A., Brunori, M., 1985. Hemocyanin from *Palinurus elephas*: general properties and effects of heavy metals. Biochim. Biophys. Acta BBA Protein Struct. Mol. 830, 325–331. https://doi.org/10.1016/0167-4838(85)90290-0.

Bhattacharya, S., Bose, S., Mukhopadhyay, B., Sarkar, D., Das, D., Bandyopadhyay, J., Bose, R., Majumdar, C., Mondal, S., Sen, S., 1997. Specific binding of inorganic mercury to Na+- K+-ATPase in rat liver plasma membrane and signal transduction. BioMetals 10, 157–162. https://doi.org/10.1023/a:1018391409426.

Bisi, T.L., Lepoint, G., Azevedo, A.D.F., Dorneles, P.R., Flach, L., Das, K., Malm, O., Lailson-Brito, J., 2012. Trophic relationships and mercury biomagnification in Brazilian tropical coastal food webs. Ecol. Indic. 18, 291–302. https://doi.org/10.1016/j.ecolind.2011.11.015.

Borgmann, U., Norwood, W.P., Clarke, C., 1993. Accumulation, regulation and toxicity of copper, zinc, lead and mercury in *Hyalella azteca*. Hydrobiologia 259 (2), 79–89.

Bouquegneau, J.M., 1977. ATPase activity in mercury intoxicated eels. Experientia 33, 941–943. https://doi.org/10.1007/BF01951295.

Branco, V., Carvalho, C., 2019. The thioredoxin system as a target for mercury compounds. Biochim. Et. Biophys. Acta BBA Gen. Subj. 1863, 129255 https://doi.org/10.1016/j.bbagen.2018.11.007.

Branco, V., Ramos, P., Canário, J., Lu, J., Holmgren, A., Carvalho, C., 2012. Biomarkers of adverse response to mercury: histopathology versus thioredoxin reductase activity. J. Biomed. Biotechnol. 2012, 1–9. https://doi.org/10.1155/2012/359879.

Branco, V., Godinho-Santos, A., Gonçalves, J., Lu, J., Holmgren, A., Carvalho, C., 2014. Mitochondrial thioredoxin reductase inhibition, selenium status, and Nrf-2 activation are determinant factors modulating the toxicity of mercury compounds. Free Radic. Biol. Med. 73, 95–105. https://doi.org/10.1155/2012/359879.

Bundschuh, M., Zubrod, J.P., Seitz, F., Newman, M.C., Schulz, R., 2011. Mercury-contaminated sediments affect amphipod feeding. Arch. Environ. Contam. Toxicol. 60, 437–443. https://doi.org/10.1007/s00244-010-9566-6.

Cairns, J., Heath, A.G., Parker, B.C., 1975. Temperature influence on chemical toxicity to aquatic organisms. J. Water Pollut. Control Fed. 47, 267–280.

Callahan, P., Weis, J.S., 1983. Methylmercury effects on regeneration and ecdysis in fiddler crabs (*Uca pugilator*, *U. pugnax*) after short-term and chronic pre-exposure. Arch. Environ. Contam. Toxicol. 12, 707–714. https://doi.org/10.1007/BF01060755.

Cerenius, L., Söderhäll, K., 2004. The prophenoloxidase-activating system in invertebrates. Immunol. Rev. 198, 116–126. https://doi.org/10.1111/j.0105-2896.2004.00116.x.

Chen, C.Y., Dionne, M., Mayes, B.M., Ward, D.M., Sturup, S., Jackson, B.P., 2009. Mercury bioavailability and bioaccumulation in estuarine food webs in the Gulf of Maine. Environ. Sci. Technol. 43, 1804–1810. https://doi.org/10.1021/es8017122.

Coppola, F., Almeida, Â., Henriques, B., Soares, A.M.V.M., Figueira, E., Pereira, E., Freitas, R., 2017. Biochemical impacts of Hg in Mytilus galloprovincialis under present and predicted warming scenarios. Sci. Total. Environ. 601–602, 1129–1138. https://doi.org/10.1016/j.scitotenv.2017.05.201.

Coppola, F., Henriques, B., Soares, A.M.V.M., Figueira, E., Pereira, E., Freitas, R., 2018. Influence of temperature rise on the recovery capacity of *Mytilus galloprovincialis* exposed to mercury pollution. Ecol. Indic. 93, 1060–1069. https://doi.org/10.1016/j.ecolind.2018.05.077.

Costa, H., 1980. The effects of some heavy metal pollutants on the heart beat of *Gammarus pulex* (L.). Crustac 170–181.

Crump, K.L., Trudeau, V.L., 2009. Mercury-induced reproductive impairment in fish. Environ. Toxicol. Chem. 28 (5), 895–907.

Devi, V.U., Rao, Y.P., 1989. Heavy metal toxicity to fiddler crabs, *Uca annulipes* Latreille and *Uca triangularis* (Milne Edwards): respiration on exposure to copper, mercury, cadmium, and zinc. Bull. Environ. Contam. Toxicol. 43, 165–172. https://doi.org/10.1007/BF01702254.

Di Donato, G., De Matthaeis, E., Ronci, L., Setini, A., 2016. Genotoxicity biomarkers in the amphipod Gammarus elvirae exposed in vivo to mercury and lead, and basal levels of DNA damage in two cell types. Chem. Ecol. 32, 843–857. https://doi.org/10.1080/02757540.2016.1201078.

Dijkstra, J.A., Buckman, K.L., Ward, D., Evans, D.W., Dionne, M., Chen, C.Y., 2013. Experimental and natural warming elevates mercury concentrations in estuarine fish. PLoS One 8, e58401. https://doi.org/10.1371/journal.pone.0058401.

Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards.

Eagles-Smith, C.A., Silbergeld, E.K., Basu, N., Bustamante, P., Diaz-Barriga, F., Hopkins, W.A., Kidd, K.A., Nyland, J.F., 2018. Modulators of mercury risk to wildlife and humans in the context of rapid global change. Ambio 47, 170–197. https://doi.org/10.1007/s13280-017-1011-x.

Elumalai, M., Antunes, C., Guilhermino, L., 2007. Enzymatic biomarkers in the crab *Carcinus maenas* from the Minho River estuary (NW Portugal) exposed to zinc and mercury. Chemosphere 66, 1249–1255. https://doi.org/10.1016/j.chemosphere.2006.07.030.

Foucreau, N., Cottin, D., Piscart, C., Hervant, F., 2014. Physiological and metabolic responses to rising temperature in *Gammarus pulex* (Crustacea) populations living under continental or Mediterranean climates. Comp. Biochem. Physiol. Part A 168, 69–75.

Franzellitti, S., Fabbri, E., 2005. Differential HSP70 gene expression in the Mediterranean mussel exposed to various stressors. Biochem. Biophys. Res. Commun. 336, 1157–1163. https://doi.org/10.1016/j.bbrc.2005.08.244.

Freitas, R., Coppola, F., Henriques, B., Wrona, F., Figueira, E., Pereira, E., Soares, A.M.V.M., 2017. Does pre-exposure to warming conditions increase Mytilus galloprovincialis tolerance to Hg contamination? Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 203, 1–11. https://doi.org/10.1016/j.cbpc.2017.09.010.

Gagnaire, B., Thomas-Guyon, H., Renault, T., 2004. In vitro effects of cadmium and mercury on Pacific oyster, *Crassostrea gigas* (Thunberg), haemocytes. Fish Shellfish Immunol. 16, 501–512. https://doi.org/10.1016/j.fsi.2003.08.007.

Gharaei, A., Mahboudi, F., Esmaili-Sari, A., Edalat, R., Adeli, A., Keyvanshokooh, S., 2010. Molecular cloning of cDNA of mammalian and chicken II gonadotropin-releasing hormones (mGnRHs and cGnRH-II) in the beluga (*Huso huso*) and the disruptive effect of methylmercury on gene expression. Fish Physiol. Biochem. 36, 803–817. https://doi.org/10.1007/s10695-009-9356-0.

Gismondi, E., 2018. Identification of molt-inhibiting hormone and ecdysteroid receptor cDNA sequences in *Gammarus pulex*, and variations after endocrine disruptor exposures. Ecotoxicol. Environ. Saf. 158, 9–17. https://doi.org/10.1016/j.ecoenv.2018.04.017.

Gismondi, E., Thomé, J.P., 2016. Transcriptome of the freshwater amphipod *Gammarus pulex* hepatopancreas. Genom. Data 8, 91–92. https://doi.org/10.1016/j.gdata.2016.04.002.

Gismondi, E., Thomé, J.P., Urien, N., Uher, E., Baiwir, D., Mazzucchelli, G., De Pauw, E., Fechner, L.C., Lebrun, J.D., 2017. Ecotoxicoproteomic assessment of the functional alterations caused by chronic metallic exposures in gammarids. Environ. Pollut. 225, 428–438. https://doi.org/10.1016/j.envpol.2017.03.006.

González, R.A., Díaz, F., Licea, A., Re, A.D., Sánchez, L.N., García-Esquivel, Z., 2010. Thermal preference, tolerance and oxygen consumption of adult white shrimp *Litopenaeus vannamei* (Boone) exposed to different acclimation temperatures. J. Therm. Biol. 35 (5), 218–224.

Gouveia, D., Bonneton, F., Almunia, C., Armengaud, J., Quéau, H., Degli-Esposti, D., Geffard, O., Chaumot, A., 2018. Identification, expression, and endocrine-disruption of three ecdysone-responsive genes in the sentinel species *Gammarus fossarum*. Sci. Rep. 8, 1–13. https://doi.org/10.1038/s41598-018-22235-7.

Handayani, K.S., Irawan, B., Soegianto, A., 2020. Short-term mercury exposure in tilapia (*Oreochromis niloticus*) at different salinities: impact on serum osmoregulation, hematological parameters, and Na+/K+-ATPase level. Heliyon 6 (7), e04404.

Heit, M., Fingerman, M., 1977. The influences of size, sex and temperature on the toxicity of mercury to two species of crayfishes. Bull. Environ. Contam. Toxicol. 18, 572–580. https://doi.org/10.1007/BF01684003.

Hempel, M., Chau, Y.K., Dutka, B.J., McInnis, R., Kwan, K.K., Liu, D., 1995. Toxicity of organomercury compounds: Bioassay results as a basis for risk assessment. Analyst 120, 721–724. https://doi.org/10.1039/an9952000721.

Henry, Y., Piscart, C., Charles, S., Colinet, H., 2017. Combined effect of temperature and ammonia on molecular response and survival of the freshwater crustacean *Gammarus pulex*. Ecotoxicol. Environ. Saf. 137, 42–48.

Hervant, F., Mathieu, J., Barré, H., Simon, K., Pinon, C., 1997. Comparative study on the behavioral, ventilatory, and respiratory responses of hypogean and epigean crustaceans to long-term starvation and subsequent feeding. Comp. Biochem. Physiol. Part A 118 (4), 1277–1283.

Heugens, E.H.W., Hendriks, A.J., Dekker, T., Van Straalen, N.M., Admiraal, W., Heugens, E.H.W., 2001. A review of the effects of multiple stressors on aquatic organisms and analysis of uncertainty factors for use in risk assessment. Crit. Rev. Toxicol. 31, 247–284. https://doi.org/10.1080/20014091111695.

Hinfray, N., Porcher, J.M., Brion, F., 2006. Inhibition of rainbow trout (*Oncorhynchus mykiss*) P450 aromatase activities in brain and ovarian microsomes by various environmental substances. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 144, 252–262. https://doi.org/10.1016/j.cbpc.2006.09.002.

Holmgren, A., 1989. Thioredoxin and glutaredoxin systems. J. Biol. Chem. 264, 13963–13966.

Hooper, M.J., Ankley, G.T., Cristol, D.A., Maryoung, L.A., Noyes, P.D., Pinkerton, K.E., 2013. Interactions between chemical and climate stressors: a role for mechanistic toxicology in assessing climate change risks. Environ. Toxicol. Chem. 32, 32–48. https://doi.org/10.1002/etc.2043.

Hou, Z., Sket, B., 2016. A review of Gammaridae (Crustacea: amphipoda): the family extent, its evolutionary history, and taxonomic redefinition of genera. Zool. J. Linn. Soc. 176, 323–348. https://doi.org/10.1111/zoj.12318.

Huang, W., Cao, L., Liu, J., Lin, L., Dou, S., 2010. Short-term mercury exposure affecting the development and antioxidant biomarkers of Japanese flounder embryos and larvae. Ecotoxicol. Environ. Saf. 73, 1875–1883. https://doi.org/10.1016/j.ecoenv.2010.08.012.

Hyne, R.V., 2011. Review of the reproductive biology of amphipods and their endocrine regulation: identification of mechanistic pathways for reproductive toxicants. Environ. Toxicol. Chem. 30, 2647–2657. https://doi.org/10.1002/etc.673.

Issartel, J., Hervant, F., Voituron, Y., Renault, D., Vernon, P., 2005. Behavioural, ventilatory and respiratory responses of epigean and hypogean crustaceans to different temperatures. Comp. Biochem. Physiol. Part A 141 (1), 1–7.

Jaegers, J., Gismondi, E., 2020. Gammarid exposure to pyriproxyfen and/or cadmium: what effects on the methylfarnesoate signalling pathway? Environ. Sci. Pollut. Res. 27, 31330–31338. https://doi.org/10.1007/s11356-020-09419-3.

Jagoe, C.H., Shaw-Allen, P.L., Brundage, S., 1996. Gill Na+-ATPase activity in largemouth bass (*Micropterus salmoides*) from three reservoirs with different levels of mercury contamination. Aquat. Toxicol. 36, 161–176. https://doi.org/10.1016/S0166-445X(96)00814-4.

Jażdżewski, K., 1980. Range extensions of some Gammaridean species in European Inland Waters caused by human activity. Crustac Suppl. 84–107.

Kaoud, H.A., Zaki, M.M., Ismail, M.M., 2011. Effect of exposure to mercury on health in tropical *Macrobrachium rosenbergii*. Life Sci. J. 8, 154–163.

Kidd, K., Clayden, M., Jardine, T., 2011. Environmental chemistry and toxicology of mercury: liu/toxicology of mercury!bioaccumulation and biomagnification of mercury through food webs. Chem. Toxicol. Mercury 453–499. https://doi.org/10.1002/9781118146644.ch14.

Kim, K.H., Kabir, E., Jahan, S.A., 2016. A review on the distribution of Hg in the environment and its human health impacts. J. Hazard. Mater. 306, 376–385. https://doi.org/10.1016/j.jhazmat.2015.11.031.

Kirkman, H.N., Galianoe, S., Gaetanie, G.F., 1987. The function of catalase-bound NADPH. J. Biol. Chem. 262, 660–666.

Kunz, P.Y., Kienle, C., Gerhardt, A., 2010. Gammarus spp. in Aquatic Ecotoxicology and Water Quality Assessment: Toward Integrated Multilevel Tests. In: Whitacre, D.M. (Ed.), Vol. 205. Springer, New York, pp. 1–76. https://doi.org/10.1007/978-1-4419-5623-1_1.

Lanneau, D., Brunet, M., Frisan, E., Solary, E., Fontenay, M., Garrido, C., 2008. Heat shock proteins: essential proteins for apoptosis regulation: apoptosis review series. J. Cell. Mol. Med. 12, 743–761. https://doi.org/10.1111/j.1582-4934.2008.00273.x.

Lavoie, R.A., Jardine, T.D., Chumchal, M.M., Kidd, K.A., Campbell, L.M., 2013. Biomagnification of mercury in aquatic food webs: a worldwide meta-analysis. Environ. Sci. Technol. 47, 13385–13394. https://doi.org/10.1021/es403103t.

Le, D.Q., Tanaka, K., Dung, L.V., Siau, Y.F., Lachs, L., Kadir, S.T.M.S.A., Sano, Y., Shirai, K., 2017. Biomagnification of total mercury in the mangrove lagoon foodweb in east coast of Peninsula, Malaysia. Reg. Stud. Mar. Sci. 16, 49–55. https://doi.org/10.1016/j.rsma.2017.08.006.

Lebrun, J.D., Gismondi, E., 2020. Behavioural and biochemical alterations in gammarids as induced by chronic metallic exposures (Cd, Cu and Pb): implications for freshwater biomonitoring. Chemosphere 257, 127253. https://doi.org/10.1016/j.chemosphere.2020.127253.

Liu, Y.R., Zheng, Y.M., Zhang, L.M., Luan, Y.X., He, J.Z., 2010. Effects of mercury on reproduction, avoidance, and heat shock protein gene expression of the soil springtail *Folsomia candida*. Environ. Toxicol. Chem. 29, 654–659. https://doi.org/10.1002/etc.59.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262.

Lu, J., Holmgren, A., 2014. The thioredoxin antioxidant system. Free Radic. Biol. Med. 66, 75–87. https://doi.org/10.1016/j.freeradbiomed.2013.07.036.

Lu, X., Xiang, Y., Yang, G., Zhang, L., Wang, H., Zhong, S., 2017. Transcriptomic characterization of zebrafish larvae in response to mercury exposure. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 192, 40–49. https://doi.org/10.1016/j.cbpc.2016.12.006.

Lund, B.O., Miller, D.M., Woods, J.S., 1993. Studies on Hg(II)-induced H_2O_2 formation and oxidative stress in vivo and in vitro in rat kidney mitochondria. Biochem. Pharmacol. 45, 2017–2024. https://doi.org/10.1016/0006-2952(93)90012-l.

Maazouzi, C., Piscart, C., Legier, F., Hervant, F., 2011. Ecophysiological responses to temperature of the "killer shrimp" *Dikerogammarus villosus*: Is the invader really stronger than the native *Gammarus pulex*? Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 159, 268–274. https://doi.org/10.1016/j.cbpa.2011.03.019.

McIntyre, J.K., Beauchamp, D.A., 2007. Age and trophic position dominate bioaccumulation of mercury and organochlorines in the food web of Lake Washington. Sci. Total. Environ. 372, 571–584. https://doi.org/10.1016/j.scitotenv.2006.10.035.

MERPD, Consortium, Abdoullaye, D., Acevedo, I., Adebayo, A.A., Behrmann-Godel, J., Benjamin, R.C., Bock, D.G., Born, C., Brouat, C., Caccone, A., Cao, L.-Z., Casado-Amezúa, P., Catanéo, J., Correa-Ramirez, M.M., Cristescu, M.E., Dobigny, G., Egbosimba, E.E., Etchberger, L.K., Fan, B., Fields, P.D., Forcioli, D., Furla, P., GARCIA de, L.E.O.N.F.J., García-Jiménez, R., Gauthier, P., Gergs, R., González, C., Granjon, L., Gutiérrez-Rodríguez, C., Havill, N.P., Helsen, P., Hether, T.D., Hoffman, E.A., Hu, X., Ingvarsson, P.K., Ishizaki, S., Ji, H., Ji, X.S., Jimenez, M.L., Kapil, R., Karban, R., Keller, S.R., Kubota, S., Li, S., Li, W., Lim, D.D., Lin, H., Liu, X., Luo, Y., Machordom, A., Martin, A.P., Matthysen, E., Mazzella, M.N., McGEOCH, M.A., Meng, Z., Nishizawa, M., O'brien, P., Ohara, M., Ornelas, J.F., Ortu, M.F., Pedersen, A.B., Preston, L., Ren, Q., Rothhaupt, K.-O., Sackett, L.C., Sang, Q., Sawyer, G.M., Shiojiri, K., Taylor, D.R., Van DONGEN, S., Van VUUREN, B.J., Vandewoestijne, S., Wang, H., Wang, J.T., Wang, L., Xu, X.-L., Yang, G., Yang, Y., Zeng, Y.Q., Zhang, Q.-W., Zhang, Y., Zhao, Y., Zhou, Y., 2010. Permanent genetic resources added to molecular ecology resources database 1 August 2009–30 September 2009. Mol. Ecol. Resour. 10, 232–236. https://doi.org/10.1111/j.1755-0998.2009.02796.x.

Monteiro, D.A., Rantin, F.T., Kalinin, A.L., 2010. Inorganic mercury exposure: toxicological effects, oxidative stress biomarkers and bioaccumulation in the tropical freshwater fish matrinxã, *Brycon amazonicus* (Spix and Agassiz, 1829). Ecotoxicology 19, 105–123. https://doi.org/10.1007/s10646-009-0395-1.

Morosetti, B., Freitas, R., Pereira, E., Hamza, H., Andrade, M., Coppola, F., Maggioni, D., Della Torre, C., 2020. Will temperature rise change the biochemical alterations induced in *Mytilus galloprovincialis* by cerium oxide nanoparticles and mercury? Environ. Res. 188, 109778 https://doi.org/10.1016/j.envres.2020.109778.

Naïja, A., Marchand, J., Kestemont, P., Haouas, Z., Blust, R., Chénais, B., Helal, A.N., 2016. Mercury accumulation and its effects on molecular, physiological, and histopathological responses in the peacock blenny *Salaria pavo*. Environ. Sci. Pollut. Res. 23, 22099–22115. https://doi.org/10.1007/s11356-016-7401-y.

Noyes, P.D., McElwee, M.K., Miller, H.D., Clark, B.W., Van Tiem, L.A., Walcott, K.C., Erwin, K.N., Levin, E.D., 2009. The toxicology of climate change: environmental contaminants in a warming world. Environ. Int. 35, 971–986. https://doi.org/10.1016/j.envint.2009.02.006.

Outridge, P.M., Mason, R.P., Wang, F., Guerrero, S., Heimbürger-Boavida, L.E., 2018. Updated global and oceanic mercury budgets for the united nations global mercury assessment 2018. Environ. Sci. Technol. 52, 11466–11477. https://doi.org/10.1021/acs.est.8b01246.

Pack, E.C., Kim, C.H., Lee, S.H., Lim, C.H., Sung, D.G., Kim, M.H., Park, K.H., Hong, S.S., Lim, K.M., Choi, D.W., Kim, S.W., 2014. Effects of environmental temperature change on mercury absorption in aquatic organisms with respect to climate warming. J. Toxicol. Environ. Heal Part A 77, 1477–1490 https://doi:0.1080/15287394.2014.955892.

Poopal, R.K., Ramesh, M., Dinesh, B., 2013. Short-term mercury exposure on Na+/K+-ATPase activity and ionoregulation in gill and brain of an Indian major carp, *Cirrhinus mrigala*. J. Trace Elem. Med. Biol. 27, 70–75. https://doi.org/10.1016/j.jtemb.2012.06.002.

Porter, A.G., Jänicke, R.U., 1999. Emerging roles of caspase-3 in apoptosis. Cell Death Differ. 6, 99–104. https://doi.org/10.1038/sj.cdd.4400476.

Rabeh, I., Telahigue, K., Bejaoui, S., Hajji, T., Chouba, L., EL Cafsi, M., Soudani, N., 2019. Effects of mercury graded doses on redox status, metallothionein levels and genotoxicity in the intestine of sea cucumber *Holothuria forskali*. Chem. Ecol. 35, 204–218. https://doi.org/10.1080/02757540.2018.1546292.

Rasmussen, J.J., Cedergreen, N., Kronvang, B., Andersen, M.B.B., Nørum, U., Kretschmann, A., Strobel, B.W., Hansen, H.C.B., 2016. Suspended particles only marginally reduce pyrethroid toxicity to the freshwater invertebrate *Gammarus pulex* (L.) during pulse exposure. Ecotoxicology 25, 510–520. https://doi.org/10.1007/s10646-016-1609-y.

Reeg, S., Jung, T., Castro, J.P., Davies, K.J.A., Henze, A., Grune, T., 2016. The molecular chaperone Hsp70 promotes the proteolytic removal of oxidatively damaged proteins by the proteasome. Free Radic. Biol. Med. 99, 153–166. https://doi.org/10.1016/j.freeradbiomed.2016.08.002.

Ren, Z., Liu, J., Huang, W., Cao, L., Cui, W., Dou, S., 2019. Antioxidant defenses and immune responses of flounder *Paralichthys olivaceus* larvae under methylmercury exposure. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 225, 108589 https://doi.org/10.1016/j.cbpc.2019.108589.

Rice, K.M., Walker, E.M., Wu, M., Gillette, C., Blough, E.R., 2014. Environmental mercury and its toxic effects. J. Prev. Med. Public Heal. 47, 74–83. https://doi.org/10.3961/jpmph.2014.47.2.74.

Richter, C.A., Garcia-Reyero, N., Martyniuk, C., Knoebl, I., Pope, M., Wright-Osment, M.K., Denslow, N.D., Tillitt, D.E., 2011. Gene expression changes in female zebrafish (Danio rerio) brain in response to acute exposure to methylmercury. Environ. Toxicol. Chem. 30, 301–308. https://doi.org/10.1002/etc.409.

Roos-Muñoz, S., Abad-Rosales, S.M., Aguilar-Juárez, M., Frías-Espericueta, M.G., Voltolina, D., 2019. Acute toxicity of mercury and nervous tissue damage in postlarvae and juveniles of *Litopenaeus vannamei*. Thalass. . Int. J. Mar. Sci. 35, 57–63. https://doi.org/10.1007/s41208-018-0085-y.

Russo, R., Becker, J.M., Liess, M., 2018. Sequential exposure to low levels of pesticides and temperature stress increase toxicological sensitivity of crustaceans. Sci. Total Environ. 610, 563–569.

Sanchez, W., Burgeot, T., Porcher, J.M., 2013. A novel "integrated biomarker response" calculation based on reference deviation concept. Environ. Sci. Pollut. Res. 20, 2721–2725. https://doi.org/10.1007/s11356-012-1359-1.

Sanchez-Dardon, J., Voccia, I., Hontela, A., Anderson, P., Brousseau, P., Blakely, B., Boermans, H., Fournier, M., 1997. FP 13 Immunotoxicity of cadmium, zinc and mercury after in vivo exposure, alone or in mixture in rainbow trout (Oncorhynchus mykiss). Dev. Comp. Immunol. 21 (21), 133.

Santoro, M.G., 2000. Heat shock factors and the control of the stress response. Biochem. Pharmacol. 59, 55–63. https://doi.org/10.1016/s0006-2952(99)00299-3.
Sauvé, S., Brousseau, P., Pellerin, J., Morin, Y., 2002. Phagocytic activity of marine and freshwater bivalves: in vitro exposure of hemocytes to metals (Ag, Cd, Hg and Zn). Aquat. Toxicol. 58, 189–200. https://doi.org/10.1016/s0166-445x(01)00232-6.
Semsar-kazerouni, M., Verberk, W.C.E.P., 2018. It's about time: linkages between heat tolerance, thermal acclimation and metabolic rate at different temporal scales in the freshwater amphipod *Gammarus fossarum* Koch, 1836. J. Therm. Biol. 75, 31–37. https://doi.org/10.1016/j.jtherbio.2018.04.016.
Sharma, M.K., Sharma, A., Kumar, A., Kumar, M., 2007. Spirulina fusiformis provides protection against mercuric chloride induced oxidative stress in Swiss albino mice. Food Chem. Toxicol. 45, 2412–2419. https://doi.org/10.1016/j.fct.2007.06.023.
Singaram, G., Harikrishnan, T., Chen, F.Y., Bo, J., Giesy, J.P., 2013. Modulation of immune-associated parameters and antioxidant responses in the crab (*Scylla serrata*) exposed to mercury. Chemosphere 90, 917–928. https://doi.org/10.1016/j.chemosphere.2012.06.031.
Sokolova, I., Lannig, G., 2008. Interactive effects of metal pollution and temperature on metabolism in aquatic ectotherms: implications of global climate change. Clim. Res. 37, 181–201. https://doi.org/10.3354/cr00764.
Sreenivasula Reddy, P., Ramachandra Reddy, P., Sainath, S.B., 2011. Cadmium and mercury-induced hyperglycemia in the fresh water crab, Oziotelphusa senex senex: involvement of neuroendocrine system. Ecotoxicol. Environ. Saf. 74, 279–283. https://doi.org/10.1016/j.ecoenv.2010.10.031.
Sroda, S., Cossu-Leguille, C., 2011. Seasonal variability of antioxidant biomarkers and energy reserves in the freshwater gammarid *Gammarus roeseli*. Chemosphere 83, 538–544. https://doi.org/10.1016/j.chemosphere.2010.12.023.
St-Amand, L., Gagnon, R., Packard, T.T., Savenkoff, C., 1999. Effects of inorganic mercury on the respiration and the swimming activity of shrimp larvae, *Pandalus borealis*. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 122, 33–43. https://doi.org/10.1016/S0742-8413(98)10071-3.
Stohs, S.J., Bagchi, D., 1995. Oxidative mechanisms in the toxicity of metal ions. Free Radic. Biol. Med. 18, 321–336. https://doi.org/10.1016/0891-5849(94)00159-H.
Sun, Y., Li, Y., Rao, J., Liu, Z., Chen, Q., 2018. Effects of inorganic mercury exposure on histological structure, antioxidant status and immune response of immune organs in yellow catfish (*Pelteobagrus fulvidraco)*. J. Appl. Toxicol. 38, 843–854. https://doi.org/10.1002/jat.3592.
Sweet, L.I., Zelikoff, J.T., 2001. Toxicology and immunotoxicology of mercury: a comparative review in fish and humans. J. Toxicol. Environ. Health B Crit. Rev. 4 (2), 161–205.
Tinant, G., Neefs, I., Das, K., Rees, J.F., Larondelle, Y., Debier, C., 2020. Methylmercury displays pro-adipogenic properties in rainbow trout preadipocytes. Chemosphere, 127917.
Tom, K.R., Newman, M.C., Schmerfeld, J., 2010. Modeling mercury biomagnification (South River, Virginia, USA) to inform river management decision making. Environ. Toxicol. Chem. 29, 1013–1020. https://doi.org/10.1002/etc.117.
Trevisan, M., Leroy, D., Decloux, N., Thomé, J.P., Compère, P., 2014. Moult-related changes in the integument, midgut, and digestive gland in the freshwater amphipod *Gammarus pulex*. J. Crustace Biol. 34 (5), 539–551.
Tsui, M.T.K., Wang, W.X., 2004. Uptake and elimination routes of inorganic mercury and methylmercury in *Daphnia magna*. Environ. Sci. Technol. 38, 808–816. https://doi.org/10.1021/es034638x.
Untergasser, A., Rao, H., Bisseling, T., Geurts, R., 2007. Primer3Plus, an enhanced web interface to Primer3 nucleic acids research. Nucleic Acids Res. 35, 1–4. https://doi.org/10.1093/nar/gkm306.
van der Oost, R., Beyer, J., Vermeulen, N.P., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57–149. https://doi.org/10.1016/s1382-6689(02)00126-6.
Vellinger, C., Parant, M., Rousselle, P., Immel, F., Wagner, P., Usseglio-Polatera, P., 2012. Comparison of arsenate and cadmium toxicity in a freshwater amphipod (*Gammarus pulex)*. Environ. Pollut. 160, 66–73. https://doi.org/10.1016/j.envpol.2011.09.002.
Verheyen, J., Stoks, R., 2019. Current and future daily temperature fluctuations make a pesticide more toxic: contrasting effects on life history and physiology. Environ. Pollut. 248, 209–218. https://doi.org/10.1016/j.envpol.2019.02.022.
Verlecar, X.N., Jena, K.B., Chainy, G.B.N., 2007. Biochemical markers of oxidative stress in *Perna viridis* exposed to mercury and temperature. Chem. Biol. Interact. 167, 219–226. https://doi.org/10.1016/j.cbi.2007.01.018.
Verma, S.R., Jain, M., Tonk, I.P., 1983. In vivo effect of mercuric chloride on tissue ATPases of *Notopterus notopterus*. Toxicol. Lett. 16, 305–309. https://doi.org/10.1016/0378-4274(83)90191-1.
Waheed, R., El Asely, A.M., Bakery, H., El-Shawarby, R., Abuo-Salem, M., Abdel-Aleem, N., Malhat, F., Khafaga, A., Abdeen, A., 2020. Thermal stress accelerates mercury chloride toxicity in *Oreochromis niloticus* via up-regulation of mercury bioaccumulation and HSP70 mRNA expression. Sci. Total Environ. 718, 137326 https://doi.org/10.1016/j.scitotenv.2020.137326.
Wang, X., Horisberger, J.D., 1996. Mercury binding site on Na+/K(+)-ATPase: a cysteine in the first transmembrane segment. Mol. Pharmacol. 50.
Watanabe, C., 2002. Modification of mercury toxicity by selenium: practical importance? Tohoku J. Exp. Med. 196, 71–77. https://doi.org/10.1620/tjem.196.71.
Watras, C.J., Back, R.C., Halvorsen, S., Hudson, R.J.M., Morrison, K.A., Wente, S.P., 1998. Bioaccumulation of mercury in pelagic freshwater food webs. Sci. Total. Environ. 219, 183–208. https://doi.org/10.1016/s0048-9697(98)00228-9.
Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms (p. 197). In: Weber, C.I. (Ed.), 1991. Environmental Monitoring Systems Laboratory, Office of Research and Development, US Environmental Protection Agency, Washington, DC.
Weis, J.S., 1978. Interactions of methylmercury, cadmium, and salinity on regeneration in the fiddler crabs *Uca pugilator*, *U. pugnax* and *U. minax*. Mar. Biol. 49, 119–124. https://doi.org/10.1007/BF00387111.
Weston, D.P., Poynton, H.C., Wellborn, G.A., Lydy, M.J., Blalock, B.J., Sepulveda, M.S., Colbourne, J.K., 2013. Multiple origins of pyrethroid insecticide resistance across the species complex of a nontarget aquatic crustacean, *Hyalella azteca*. Proc. Natl. Acad. Sci. U.S.A. 110 (41), 16532–16537.
Wijnhoven, S., Riel, Van, van der Velde, G, M.C., 2003. Exotic and indigenous freshwater gammarid species: physiological tolerance to water temperature in relation to ionic content of the water. Aquat. Ecol. 37 (2), 151–158.
Willming, M.M., Maul, J.D., 2016. Direct and indirect toxicity of the fungicide pyraclostrobin to *Hyalella azteca* and effects on leaf processing under realistic daily temperature regimes. Environ. Pollut. 211, 435–442. https://doi.org/10.1016/j.envpol.2015.11.029.
Wu, Q., Leung, J.Y.S., Geng, X., Chen, S., Huang, X., Li, H., Huang, Z., Zhu, L., Chen, J., Lu, Y., 2015. Heavy metal contamination of soil and water in the vicinity of an abandoned e-waste recycling site: Implications for dissemination of heavy metals. Sci. Total Environ. 506–507, 217–225. https://doi.org/10.1016/j.scitotenv.2014.10.121.
Yamuna, A., Bhavan, Saravana, Geraldine, P, P., 2012. Glutathione S-transferase and metallothionein levels in the freshwater prawn *Macrobrachium malcolmsonii* exposed to mercury. J. Environ. Biol. 33, 133–137.
Zhang, C., Yu, K., Li, F., Xiang, J., 2017. Acute toxic effects of zinc and mercury on survival, standard metabolism, and metal accumulation in juvenile ridgetail white prawn, *Exopalaemon carinicauda*. Ecotoxicol. Environ. Saf. 145, 549–556. https://doi.org/10.1016/j.ecoenv.2017.07.075.
Zhang, P., Liu, B., Kang, S.W., Seo, M.S., Rhee, S.G., Obeid, L.M., 1997. Thioredoxin peroxidase is a novel inhibitor of apoptosis with a mechanism distinct from that of Bcl-2. J. Biol. Chem. 272, 30615–30618. https://doi.org/10.1074/jbc.272.49.30615.
Zhang, Q.F., Li, Y.W., Liu, Z.H., Chen, Q.L., 2016. Exposure to mercuric chloride induces developmental damage, oxidative stress and immunotoxicity in zebrafish embryos-larvae. Aquat. Toxicol. 181, 76–85. https://doi.org/10.1016/j.aquatox.2016.10.029.
Zhang, Y., Wang, M., Huang, B., Akhtar, M.S., Hu, W., Xie, E., 2018. Soil mercury accumulation, spatial distribution and its source identification in an industrial area of the Yangtze Delta, China. Ecotoxicol. Environ. Saf. 163, 230–237. https://doi.org/10.1016/j.ecoenv.2018.07.055.
Zhao, Y., Wang, X., Qin, Y., Zheng, B., 2010. Mercury (Hg^{2+}) effect on enzyme activities and hepatopancreas histostructures of juvenile Chinese mitten crab *Eriocheir sinensis*. Chin. J. Oceanol. Limnol. 28, 427–434. https://doi.org/10.1007/s00343-010-9030-2.
Zhao, Y., Wang, X., Qin, Y., Zheng, B., Zhang, L., 2011. Gill ATPase activities and microstructures of juvenile Chinese mitten crab, *Eriocheir sinensis*, exposed to ambient mercury. Adv. Mater. Res. 183–185, 1543–1548. https://doi.org/10.4028/www.scientific.net/amr.183-185.1543.
Zhen, H., Wen, M., Yang, Y., Can, Z., Hui, G., Li, X., Deli, L., 2014. Toxic effects of $HgCl_2$ on activities of SOD, AchE and relative expression of SOD, AChE, CYP1A1 of zebrafish. Ecotoxicology 23, 1842–1845. https://doi.org/10.1007/s10646-014-1350-3.