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# Osmoregulatory responses to cadmium in reference and historically metal contaminated *Gammarus fossarum* (Crustacea, Amphipoda) populations

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## H I G H L I G H T S

- Osmoregulatory responses to Cd were studied in 3 *Gammarus* populations.
- One population lives in a Cd-contaminated river, the others inhabit Cd-free sites.
- High inter-individual variations in osmoregulation were noted in 2 populations.
- Gills of Cd-impacted animals showed histopathology, ion transporters overexpression.
- No obvious correlation was found between osmoregulatory responses and sampling sites.

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## A B S T R A C T

In order to better understand the variable sensitivities of crustaceans to metals, we investigated the impact of cadmium exposure in 3 populations of *Gammarus fossarum* from different rivers of France. The first population lives in a Cd-contaminated river from a geochemical background, while the others inhabit Cd-free sites. Osmoregulation, a relevant biomarker to evaluate crustacean health following metal contamination, was used as a proxy to evaluate the intra- and inter-population sensitivities to Cd. Specimens from each population were experimentally exposed to 9 µg Cd<sup>2+</sup>/L Cd for 7 days and hemolymph osmolality (HO) was then individually measured. In exposed populations, high inter-individual variations in HO values were noted, resulting in their separation into non-impacted and slightly or highly Cd-impacted (with lower HO) animals. In gills of impacted organisms, deep histopathological alterations and protein overexpression of Na<sup>+</sup>/K<sup>+</sup>-ATPase and V-H<sup>+</sup>-ATPase were observed through histology and immunolocalization, while non-impacted animals showed profiles comparable to controls. Moreover, the osmoregulatory processes in the population living in the Cd-contaminated site were impacted by acute Cd exposure in the laboratory as much as for one of the two populations originating from Cd-free sites. The observed changes did not reveal any obvious adaptive osmoregulatory phenomena at the population scale, but they may be due to differences in fitness between individuals and between populations in relation to the features of their respective environments, unrelated with the presence of the metal.

### Keywords:

Crustacean  
Cadmium  
Na<sup>+</sup>/K<sup>+</sup>-ATPase  
V-H<sup>+</sup>-ATPase  
Histology  
Immunolocalization

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**Abbreviations:** HO, hemolymph osmolality; NKA, Na<sup>+</sup>/K<sup>+</sup>-ATPase; VHA, V-H<sup>+</sup>-ATPase; NI, non-impacted; SI, slightly impacted; HI, highly impacted; Ard, Ardillats; TdP, Tour du Pin; Bas, Bastide.

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## 1. Introduction

The aquatic environment is subjected to increasing contamination by metals that, in addition to geochemical backgrounds, is mainly due to past and present mining activities and to the

continuous industrialization of watersheds since the industrial revolution. In France, some cadmium-contaminated streams show mean annual concentrations of up to 22 µg/L (e.g. Riou Mort, France; Morin et al., 2008). In crustaceans, water-borne cadmium is known to induce a decline of the overall performance in feeding rate, locomotor and ventilation activities, metabolic rate and mating (Lawrence and Poulter, 1998; Maltby et al., 2002; Wallace and Estephan, 2004; Wu and Chen, 2005; Mills et al., 2006). Cadmium is also known to severely impact osmoregulation in crustaceans (review in Charmantier et al., 2009) and among them in amphipods (Felten et al., 2008; Issartel et al., 2010). Osmoregulation is a vital physiological mechanism conditioning the maintenance of the overall metabolism and plays a major role in the distribution of aquatic organisms. In freshwater crustaceans, body fluids are strongly regulated hyperosmotic to the external environment, thus against water invasion and ion loss. Compensatory active uptake of ions from the water is performed through the gills into body fluids. Ion transport is performed by specific transmembrane enzymes and ion channels, highly expressed in specific cells of the gill epithelium, the ionocytes. Two major ionocyte enzymes involved in osmoregulation play a key role in ion uptake: the basolaterally located Na<sup>+</sup>/K<sup>+</sup>-ATPase (NKA) and, particularly in fresh water, the apically located V-H<sup>+</sup>-ATPase (VHA) (review in Charmantier et al., 2009; McNamara and Faria, 2012).

The freshwater amphipod crustacean *Gammarus fossarum* (Koch, 1836) is commonly found in West Palearctica streams (Karaman and Pinkster, 1977). Previous studies have revealed that *G. fossarum* sampled from a reference population (River Bourbre, France) and exposed to 15 µg Cd/L for 3 and 7 days showed a decrease in hemolymph osmolality (Felten et al., 2008; Issartel et al., 2010), resulting from a strong alteration of the gill tissues (collapse of the gill epithelium resulting in the disappearance of the hemolymph canals, hyperplasia), along with a disturbed expression of NKA (Issartel et al., 2010). Interestingly, though exposed groups showed significant differences with controls, a high inter-individual variability was noted in response to Cd exposure, with different levels of impact at the sub-individual scale suggesting the existence of a high inter-individual sensitivity to Cd.

In the case study of Cd impact in *Gammarus fossarum*, we identified one population (Ardillats: Ard) that reproduces and spends its entire life cycle in a French stream chronically exposed to Cd due to a geochemical background and low water hardness (Vigneron et al., 2015). Taking this opportunity to further assess the response of crustacean populations to Cd environmental contamination, the present study aimed at investigating the different sensitivities to sub-lethal Cd stress in populations of the amphipod *G. fossarum*, and explore the importance of pre-exposure of populations in the field using osmoregulation, which allows to accurately determine the physiological status of contaminated organisms at the individual scale. For this, we investigated the osmoregulatory responses to a sub-lethal Cd exposure in individuals sampled from three populations of *Gammarus fossarum* of southeastern France: first the population (Ard) identified as chronically exposed to Cd due to geochemical background (Vigneron et al., 2015); and secondly two populations inhabiting Cd-free sites, one in the River Bourbre flowing in a weakly and sporadically cultivated area (station called Tour du Pin: TdP) which is the population used in our previous studies (Felten et al., 2008; Issartel et al., 2010), and one in a brook spring in a pristine environment (Bastide: Bas). After sampling in the field, all animals were exposed in the laboratory in their river water to Cd concentrations yielding to 9 µg Cd<sup>2+</sup>/L for 7 days. Hemolymph osmolality measurements, histological observations of the gills, and immunolocalization of NKA and VHA were performed as a proxy to highlight potential difference in sensitivities to Cd.

## 2. Material and methods

### 2.1. Description of the sampling sites

Adult gammarids were collected from three different sites. The first population, denoted Ardillats (Ard), is located within an upstream location of the River Ardères, Rhône, France (46°11'11"N; 04°31'14"E). This site is characterized by a bioavailable Cd source coming from a natural geochemical source (Vigneron et al., 2015), ensuring that the Ard organisms have been long-term exposed to Cd. As already used in our previous studies on osmoregulatory disruption by Cd in *Gammarus* (Felten et al., 2008; Issartel et al., 2010), adult gammarids from the Tour du Pin (TdP) were collected within an upstream location of the River Bourbre, Isère, France (45°34'10"N; 05°27'34"E). Gammarids from a third population, called Bastide (Bas), were collected within a brook spring, upstream of a tributary of the Amous River, Gard, France (44°05'59"N; 03°58'55"E).

### 2.2. Water physicochemical properties of the sampling sites

Water physicochemical parameters of the three sites (Table 1) were determined in April 2012, and May 2013 (the latter date corresponds to the collection of water and organisms for Cd toxicity tests) following French standard operating procedures (AFNOR). For both sampling dates, we concluded that the Ard site is a soft water environment (low level of Ca<sup>2+</sup> and conductivity) in comparison to the two other sites (Table 1).

The characterization of metallic contamination of the three study sites was made according to the active biomonitoring protocol developed at Irstea (Besse et al., 2013). Following previous studies aiming to document metal effects on invertebrates in natural waters (Luoma et al., 2010; Rainbow et al., 2012), the choice of accumulated concentrations as contamination indicators was made in order to allow a reliable assessment of field exposure of the three populations. This would not have been achieved by measuring dissolved Cd concentrations in water samples, because this measurement does not integrate the phenomenon of bioavailability, notably the strong effect of water hardness on Cd bioaccumulation process, a parameter which presents marked differences between the three study sites. The active monitoring protocol involved the measurement of metallic concentrations accumulated in transplanted male gammarids from the Bourbre River population after 7-day *in situ* exposures to the study sites, followed by their comparison with threshold values allowing to evaluate their level of

**Table 1**  
Physicochemical properties of water from the 3 sites in April 2012 and May 2013.

	April 2012			May 2013		
	Bastide	Tour du Pin	Ardillats	Bastide	Tour du Pin	Ardillats
Conductivity (µS/cm)	505	560	105	400	445	95
NO <sub>2</sub> <sup>-</sup> (mg/L)	<0.02	0.12	0.02	0.03	<0.02	0.02
NO <sub>3</sub> <sup>-</sup> (mg/L)	<1.0	17.0	8.2	<1.0	15.0	9.2
PO <sub>4</sub> <sup>3-</sup> (mg/L)	<0.03	0.40	0.21	0.04	0.21	0.13
HCO <sub>3</sub> <sup>-</sup> (mg/L)	325	310	<30	250	250	<30
Cl <sup>-</sup> (mg/L)	5	16	11	6	10	9
SO <sub>4</sub> <sup>2-</sup> (mg/L)	13	14	4	13	9	5
Mg <sup>2+</sup> (mg/L)	30.8	3	1.9	40.8	2.5	2.3
Ca <sup>2+</sup> (mg/L)	66	115	9	23	90	7
Na <sup>+</sup> (mg/L)	2.9	7	8	3.4	5.1	6.6
K <sup>+</sup> (mg/L)	<1.0	2	2	<1.0	2.3	1
pH (14°C)	8.4	8.3	8	8.5	8.5	7.7

contamination vs national low level. The complete methodology and full results of the characterization of metallic bioavailable contamination of the Ard and TdP sites are detailed in [Vigneron et al. \(2015\)](#). In addition to these data, a simultaneous bioaccumulation assay performed in April 2012 revealed the low level of bioavailable Cd contamination in Bas site, at  $0.32 \pm 0.09 \mu\text{g/g}$  (dry weight) of accumulated Cd, to be compared to  $0.18 \pm 0.04 \mu\text{g/g}$  DW in Tour du Pin and  $1.43 \pm 0.10 \mu\text{g/g}$  DW in Ard. Hence our experimental design aimed to compare the osmoregulatory responses to Cd exposure between gammarids from one Cd chronically exposed population (Ard) and gammarids naïve towards Cd stress from two other distinct populations (TdP and Bas).

### 2.3. Organisms sampling and rearing

Specimens of *G. fossarum* were sampled with a net in the three different streams in May 2013. The organisms were transported in plastic vessels to the laboratory, where they were maintained at  $12^\circ\text{C}$  in aerated non filtered water from their respective sites for 7 days before being used in experiments (water changed every 3 days, photoperiod of 14 h of light and 10 h of darkness). Animals were fed *ad libitum* on alder leaves (*Alnus glutinosa*). Males in similar intermolt C stage with 10 mm body size were selected for the study.

### 2.4. Cadmium exposure

These experiments were conducted at the Ecophysiology experimental facility of the University of Montpellier, where the animals had been transported. To avoid any modification of the measured osmoregulatory biomarkers unrelated to Cd exposure, each population was exposed to Cd in water sampled from its river of origin. As Cd bioavailability is related to water hardness, total dissolved Cd concentration was adjusted for water from each site to provide a similar  $\text{Cd}^{2+}$  exposure ( $\text{CdCl}_2$ , Sigma-Aldrich, St Quentin Fallavier, France). For this, we used a speciation modeling approach (Minteq software) based on water physicochemical parameters recorded for the three sites (Table 1) to predict the total dissolved Cd concentration that had to be prepared (Table 2). The  $9 \mu\text{g Cd}^{2+}/\text{L}$  experimental concentration was chosen according to our previous results, corresponding to 60% of the highest Cd level for which no mortality was obtained in our reference population (TdP) for an exposure period of 7 d ([Issartel et al., 2010](#)).

Continuous exposure was performed (500 mL tanks were renewed four times a day: flow rate = 1.34 mL/min), using the method described and validated by [Felten et al. \(2008\)](#) and [Pellet et al. \(2009\)](#). Control and contaminated waters from each site, respectively stored in 30 L containers, were continuously aerated, and distributed to each 500 mL experimental tank with peristaltic pumps. Four replicates of 30 gammarids were exposed at  $12^\circ\text{C}$

during 7 d to the Cd solutions in 500 mL tanks. Four replicates of 30 gammarids (time-matched controls, Tc) from each population were also placed in the same conditions but without cadmium during 7 d. During the entire experiment, animals were fed *ad libitum* on disks of alder leaves. No death occurred during the experiment.

### 2.5. Hemolymph sampling and osmolality measurement

The animals from each site ( $N = 120$  for each population) were used for osmolality measurements. Each animal was gently dried between two sheets of filter paper, before one leg was cut using Wecker micro-scissors. The resulting drop of hemolymph was sampled using a hand-made glass micropipette and it was immediately transferred into mineral oil to avoid evaporation. The hemolymph osmolality (HO) was measured on 30 nL samples with a Kalber–Clifton nano-osmometer (Clifton Technical Physics, Hartford, NY, USA) using a 300 mOsm/kg internal standard. The rearing water osmolality was measured on a 3300 Advanced Instruments micro-osmometer (Norwood, Massachusetts, USA) using among others the same standard.

In order to highlight potential correlations between the capacity to maintain HO, gill histology and ionic transporter localization and protein expression, specimens exposed (E) to Cd were divided into 2 groups according to their respective HO values (Fig. 2): non-impacted (NI), with HO values similar to controls, i.e. ranging from 310 to 280 mOsm/Kg and impacted (I), with HO values below or equal to 270 mOsm/Kg. In impacted individuals, two sub-groups were separated: slightly impacted (SI) with HO values ranging from 270 to 250 mOsm/Kg, and highly impacted (HI) with HO ranging from 240 to the lowest measured HO values (230 mOsm/Kg).

### 2.6. Histology

As described by [Issartel et al. \(2010\)](#), the putative relation between HO values and histological features of gammarids exposed to cadmium was carefully investigated at the individual level as an important inter-individual variation was observed in this species. Several categories of animals were differentiated according to the value of their hemolymph osmolality (see Results). The histology procedure was realized as described by [Issartel et al. \(2010\)](#). Briefly, following hemolymph sampling, individuals were immediately fixed in a Bouin solution for 24 h. Individuals ( $N = 3-4$  for each category) were then embedded in paraffin, cross-sectioned at  $4\text{-}\mu\text{m}$  thickness on a Minot Leitz Wetzlar microtome, stained with Masson's trichrome and examined with a light microscope (Leica, Diaplan).

### 2.7. Immunocytochemistry

The preparation of samples for immunocytochemistry was

**Table 2**  
Model predictions of Cd speciation used to set identical dissolved  $\text{Cd}^{2+}$  concentrations ( $8.96 \mu\text{g/L}$ ) in experimental waters of the 3 sites.

Tour du Pin			Bastide			Ardillats		
Species name	$\mu\text{g/L}$	%	Species name	$\mu\text{g/L}$	%	Species name	$\mu\text{g/L}$	%
$\text{Cd}^{2+}$	8.96	59.737	$\text{Cd}^{2+}$	8.96	53.949	$\text{Cd}^{2+}$	8.96	92.999
$\text{CdOH}^+$	0.05	0.331	$\text{CdOH}^+$	0.06	0.381	$\text{CdOH}^+$	0.03	0.302
$\text{CdCl}^+$	0.25	1.689	$\text{Cd}(\text{OH})^2$ (aq)	0.00	0.012	$\text{CdCl}^+$	0.22	2.234
$\text{CdSO}_4$ (aq)	0.10	0.656	$\text{CdCl}^+$	0.08	0.485	$\text{CdSO}_4$ (aq)	0.05	0.536
$\text{CdNO}_2^+$	0.00	0.011	$\text{CdSO}_4$ (aq)	0.10	0.579	$\text{CdNO}_2^+$	0.00	0.044
$\text{CdNO}_3^+$	0.01	0.047	$\text{CdHPO}_4$ (aq)	0.00	0.012	$\text{CdHPO}_4$ (aq)	0.05	0.471
$\text{CdHPO}_4$ (aq)	0.04	0.265	$\text{CdHCO}_3^+$	0.80	4.8	$\text{CdHCO}_3^+$	0.08	0.789
$\text{CdHCO}_3^+$	0.75	4.977	$\text{CdCO}_3$ (aq)	6.32	38.032	$\text{CdCO}_3$ (aq)	0.25	2.612
$\text{CdCO}_3$ (aq)	4.68	31.177	$\text{Cd}(\text{CO}_3)_2^2-$	0.29	1.747	–	–	–
$\text{Cd}(\text{CO}_3)_2^2-$	0.16	1.099	–	–	–	–	–	
Total dissolved concentration	15.00		Total dissolved concentration	16.61		Total dissolved concentration	9.63	

performed as described by [Boudour-Boucheke et al. \(2014\)](#). Briefly, after a fixation in Bouin's solution for 24 h, gammarids were embedded in Paraplast (Sigma), 4- $\mu\text{m}$ -cross-sectioned on Leitz Wetzlar microtome and collected on poly-L-lysine-coated slides. Slides were then dewaxed and rehydrated through a descending series of ethanol baths.

Immunolocalization of NKA and VHA was performed using a rabbit polyclonal primary antibody (Santa Cruz Biotechnology<sup>®</sup>, Inc., USA) at 200  $\mu\text{g}/\text{mL}$  and a Guinea pig polyclonal antibody at 1/250 dilution in 0.5% SM-PBS ([Wieczorek et al., 1999](#)), respectively. Following washes, the slides were incubated for 1 h in the dark with a Rhodamine<sup>®</sup> donkey secondary anti-rabbit antibody for the NKA-marked slides, and an AlexaFluor<sup>®</sup> Goat anti-Guinea pig (Invitrogen Molecular Probes, Oregon, USA) for the VHA-marked slides. Control slides were exposed to the same conditions without primary antibody. After being mounted, the slides were observed with a Leitz Diaplan microscope, equipped for fluorescence and with a Leica DC300F camera and FW4000 software (Leica Microsystems, Reuil-Malmaison, France). For co-immunolocalization of NKA and VHA, a few observations were realized using a Leica TCS confocal microscope.

## 2.8. Semi-quantification of fluorescence

All treated pictures were taken using a 40 $\times$  objective with an exposure time of 460 ms in order to ensure the same conditions. The semi-quantification of the fluorescence was performed as described by [Burgess et al. \(2010\)](#) and [McCloy et al. \(2014\)](#) using the corrected total cell fluorescence method using the formula:  $\text{CTCF} = \text{integrated density} - (\text{selected area} \times \text{mean fluorescence of background readings})$ , with integrated density representing the sum of the grey values of all the pixels in the selected area relative to the number of pixels. In order to standardize the area of fluorescence measurement, CTCF was assessed in a 600 pixels (100  $\mu\text{m}$ ) rectangle length  $\times$  gill width. Each value represents the mean CTCF

of 7–9 gills from 3 individuals. CTCF measurement was performed using Image J software (Wayne Rasband, US National Institutes of Health, Bethesda, Maryland, USA).

## 2.9. Scanning electron microscopy

Branchial chambers of control individuals from Tour du Pin were fixed in Bouin solution, before being washed in 70° ethanol and dehydrated in ascendant ethanol series. Samples were then placed in hexamethyldisilazane (HMDS) solution for 30s. After drying at ambient temperature, samples were coated with colloidal platinum under partial airspace. Samples were then observed with a FEI Quanta 200 scanning electron microscope equipped with a Low Field Detector (LFD), using a 15 kV accelerating voltage and a working distance of 10 nm.

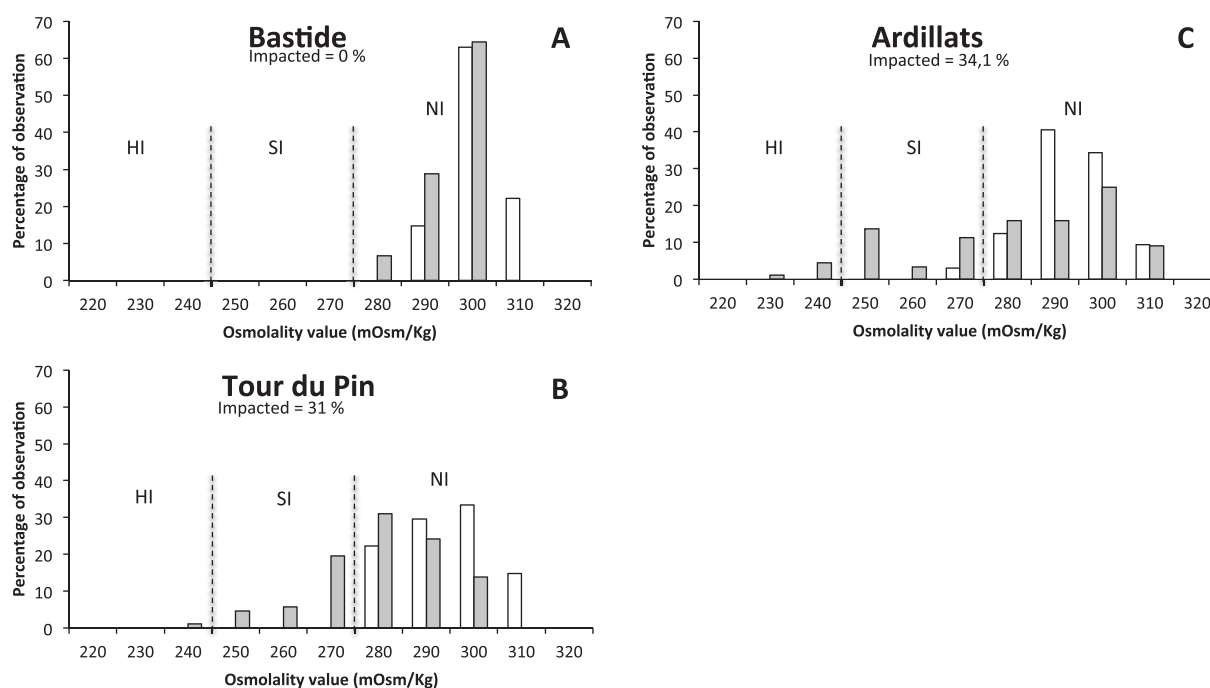
## 2.10. Statistical analysis

Differences in hemolymph osmolalities between controls and exposed organisms were assessed performing a one-way ANOVA. When significant differences were found, a Fisher post hoc test was performed. Differences in HO values distributions between control and exposed organisms were analyzed using a Kolmogorov-Smirnov test. Significance between mean fluorescence (CTCF) of controls and the different categories of exposed animals was analyzed using a Kruskal-Wallis test, followed by a Dunn post hoc test. Statistical analysis was performed using XlStat (Addinsoft, 2010).

## 3. Results

### 3.1. Impact of cadmium on hemolymph osmolality

There was no difference between HO mean values of controls (C) and of exposed (E) specimens in the 3 populations. However, high

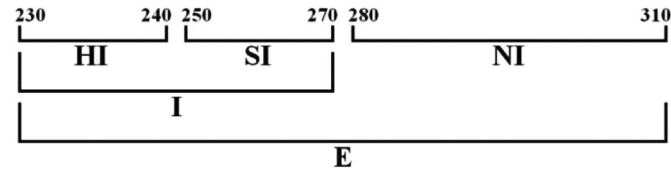


**Fig. 1.** *Gammarus fossarum*. Distribution of values of hemolymph osmolality in control (open bars) and Cd-exposed (grey and black bars) specimens showing the percent of non impacted (NI), slightly impacted (SI) and highly impacted (HI) individuals. Organisms originate from three field populations: Bastide (A), Tour du Pin (B) and Ardillats (C), the latter inhabiting an environment characterized by a marked bioavailable Cd contamination.



inter-individual variations of HO were observed in Cd-exposed gammarids which are translated by shifts in HO distribution between controls and exposed conditions (Fig. 1). Both Ard (Fig. 1C) and TdP (Fig. 1B) exposed organisms showed significantly different HO distributions compared to controls ( $p = 0.001$  and  $p < 0.001$ , respectively) whereas exposed and controls distributions in Bas specimens (Fig. 1A) were not different ( $p = 0,236$ ).

In the Bas population, all Cd-exposed gammarids showed HO values in the same range as controls, and were thus all NI individuals (Fig. 1A). In the TdP population, 31% of the exposed organisms had HO values below the range of the controls; the



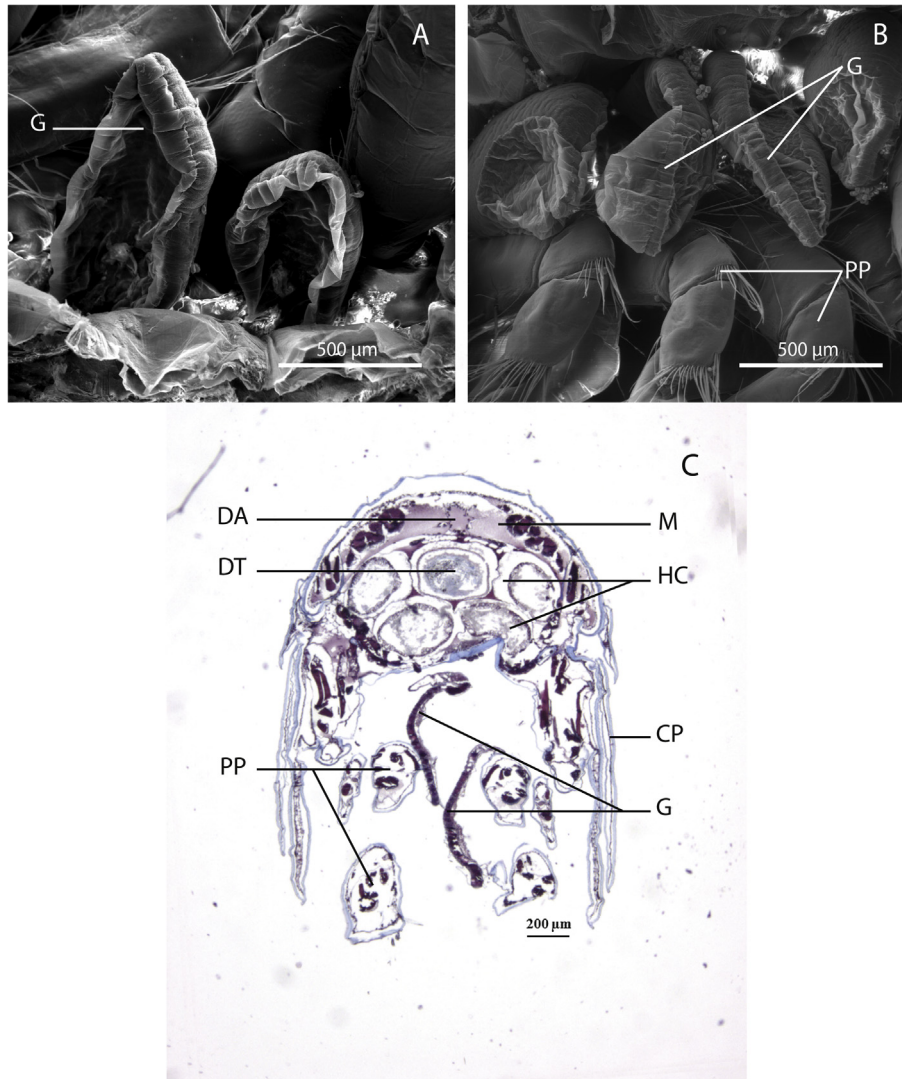
**Fig. 2.** *Gammarus fossarum*. Categories of organisms based on their hemolymph osmolality (HO in mOsm/Kg) following exposure to Cd. E: Animals exposed to Cd; NI: Non-impacted; I: Impacted; SI Slightly impacted; HI: Highly impacted.

distribution of the HO values showed that among exposed specimens, 30% of the organisms were SI, and only 1% were HI (Fig. 1B). In the Ard population, 34% of the organisms exposed to Cd presented impacted HO values; 28% of exposed individuals were slightly impacted, and 6% had highly impacted HO values (Fig. 1C).

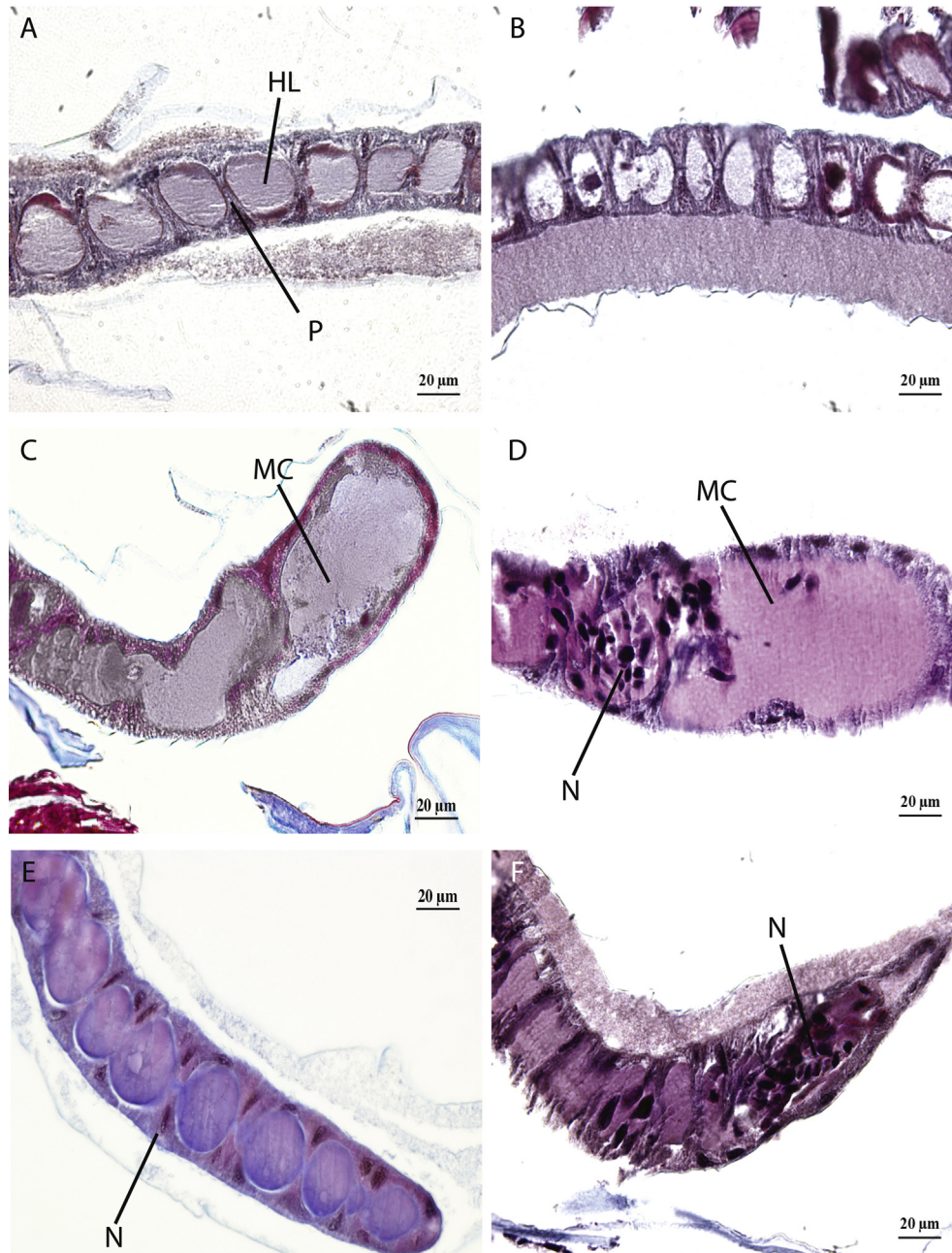
### 3.2. Effect of cadmium on gill histology, and on localization and expression of NKA and VHA

#### 3.2.1. Gill structure

Gills of *G. fossarum* are typically flattened leaf-shaped single structures (Fig. 3A, B), situated in the pereon region under coxal plates (Fig. 3C). A single layer of epithelial cells limits them on both sides. The abutting basal cell bodies of the opposing epithelial layers delimit the hemolymph spaces together with apical expansions that form an interface beneath the cuticle with other interposed cells (Fig. 4A). At the tips of the gills, large expansions form marginal canals that allow hemolymph to return to the efferent vessel (Fig. 4C). According to the section plane, pillars and marginal canals may appear thinner and smaller, respectively (Fig. 4E). In the Bas population, no modification of the gill histological structure



**Fig. 3.** *Gammarus fossarum*. SEM observations showing the gill external morphology (A, B) and transverse histological section showing the gill position below the coxal plates (C). CP: coxal plate; DA: dorsal artery; DT: digestive tract; HC: hepatopancreatic caeca; G: gills; M: muscles; PP: pereopods.



**Fig. 4.** *Gammarus fossarum* (population: Ardillats). Histological observations of gill structure in control (A, C, E), and Cd-exposed specimens, “NI, non impacted” (B); “SI, slightly impacted” (D); “HI, highly impacted” (F). HL: hemolymph lacuna; MC: marginal canal; P: pilaster; N: nucleus. For NI, SI and HI details, see Material and Methods section 5.

was observed compared to controls. However, in both the TdP and Ard populations, histopathological damages were observed in gills after Cd exposure. As they were similar, the following description of the observed histological modifications is thus valid for these two populations. In non-impacted (NI) specimens, no difference with controls was observed (Fig. 4B). In contrast, in slightly and highly impacted (SI and HI) individuals (Fig. 4D and F, respectively), a general histological disorder was observed, with cell proliferation (hyperplasia) and increased pigmentation of nuclei.

### 3.2.2. Localization of NKA and VHA

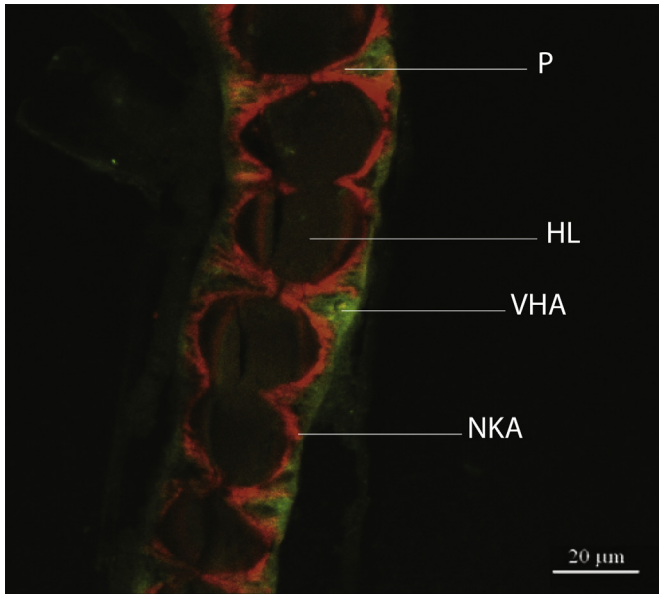
The co-immunolocalization of  $\text{Na}^+/\text{K}^+$ -ATPase (red fluorescence) and  $\text{V-H}^+$ -ATPase (green fluorescence) in a control *G. fossarum* from the Ard population is illustrated in Fig. 5. NKA

immunostaining is observed at the baso-lateral side of the cells and in the deep infoldings of the basal membrane that reach the apical area, whereas VHA is located at the apical and sub-apical pole of cells. The presence of both ionic transporters is revealed by regular and continuous staining along the gill, indicating that all cells of the gill epithelium are involved in ion transport and thus correspond to ionocytes.

### 3.2.3. Impact of cadmium on the localization and semi-quantification of fluorescence of NKA and VHA

The different levels of fluorescence intensity of both ionic transporters observed in each of the four groups of gammarids (C, NI, SI and HI) from the Ard population are illustrated in Fig. 6. There was no difference of localization and expression in both





**Fig. 5.** *Gammarus fossarum* (population: Ardillats). Co-immunolocalization of  $\text{Na}^+/\text{K}^+$  ATPase (red fluorescence) and V-type  $\text{H}^+$  ATPase (green fluorescence) in a control individual, viewed in confocal microscopy. NKA:  $\text{Na}^+/\text{K}^+$ -ATPase, VHA: V-type  $\text{H}^+$ -ATPase, P: pillar, HL: hemolymph lacuna. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

VHA and NKA between controls of the three populations. The cellular localization of VHA and NKA in gills was unchanged following Cd exposure in the three populations. Immunolocalization of NKA and VHA was not visually different between controls (Fig. 6A and B) and NI (Fig. 6C and D), whereas a higher fluorescence level was noticed in HI for both transporters NKA (Fig. 6E) and VHA (Fig. 6F). Intensity of NKA fluorescence returned to control level in HI (Fig. 6G) in contrast to VHA fluorescence (Fig. 6H), which was higher than in controls. The semi-quantification of NKA and VHA fluorescence in the 3 populations is illustrated in Fig. 7. In the Bas population, no difference in fluorescence intensity was observed between controls and NI for both enzymes (Fig. 7A, B). In the TdP population, NKA fluorescence (Fig. 7C) was not affected in NI and SI groups, but it was significantly higher by 150% in the HI group ( $p < 0.05$ ). VHA fluorescence (Fig. 7D) of NI individuals was not different from controls, but it was 5 times significantly higher than in controls in SI and HI organisms ( $p < 0.05$ ). In the Ardillats population, NKA fluorescence (Fig. 7E) was statistically not different from the controls in the NI and HI groups, but it was 3 times higher in SI individuals than in controls ( $p < 0.05$ ). VHA fluorescence (Fig. 7F) was unchanged in NI, but it was 3 times higher and 2.5 times higher in SI and HI organisms, respectively ( $p < 0.05$ ).

## 4. Discussion

### 4.1. Hemolymph osmolality

Hemolymph osmolality is a highly regulated physiological parameter in osmoregulating crustaceans, particularly in freshwater species. Deviations of the HO from its standard value could have dramatic consequences on the integrity of cells and of their metabolism and ultimately on the survival of organisms (review in Charmantier et al., 2009). In the present work, Cd exposure did not induce an overall homogenous decrease of HO of exposed organisms in any of the tested populations. But, as previously observed in this species (Felten et al., 2008; Issartel et al., 2010), Cd induced

high inter-individual variations in HO values, highlighting the existence of different groups of sensitivity among organisms of the same population. Interestingly, the distribution of HO values and the proportion of impacted organisms following Cd exposure differed among the three tested populations.

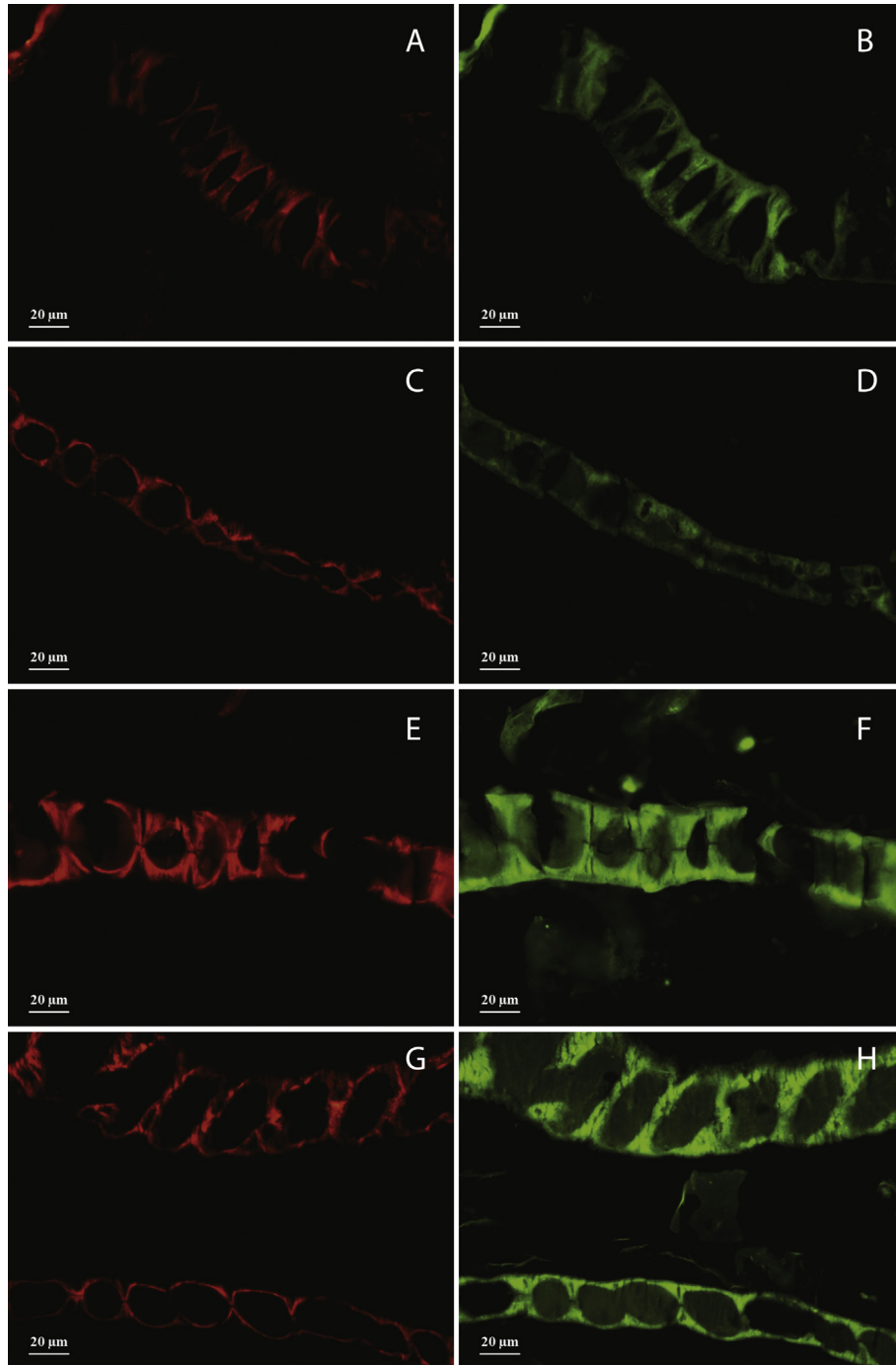
In organisms from Bastide - first Cd-free site -, as Cd exposure did not affect HO, all exposed individuals were thus considered as non-impacted. In contrast, individuals sampled at Tour du Pin - second Cd free site -, Cd exposure resulted in 31% of impacted organisms (i.e. with a significantly lower mean HO than in controls); they mainly included slightly impacted organisms (SI: HO = [270–250] mOsm/kg). In organisms originating from Ardillats, the site with Cd geochemical background (Vigneron et al., 2015), experimental Cd exposure induced HO values lower than controls in 34% of exposed individuals. Among these impacted organisms, a few displayed very low HO values (HI specimens), close to the lethal HO threshold known in this species ( $< 210$  mOsm/kg) (Felten et al., 2008; Issartel et al., 2010). In our previous study, the exposure of *G. fossarum* from the Tour du Pin population to Cd for 7 days resulted in high inter-individual variability with HO values ranging from 280 to 210 mOsm/kg, for a mean HO of 244 mOsm/kg (Issartel et al., 2010). The HO profiles reported in the present study are in accordance with these previous results, and they confirm the high inter-individual variability of responses to Cd in this population. As variability due to sex, size/age and molt cycle was avoided by selecting only males of a determined size range and intermolt stage in both studies, the observed heterogeneity of HO values in responses to contamination may be due to a high genetic and/or phenotypic variability within and between populations (Vigneron et al., 2015, 2016).

These results show that there is no obvious relationship between the respective levels of contamination of the different natural populations and the intra- and inter-population sensitivities to Cd, as both reference populations TdP and Bas were differently affected by an experimental Cd exposure, whereas TdP and the naturally Cd-exposed population Ard show similar HO ranges profiles.

### 4.2. Gill histology

In order to study the impact of cadmium in *G. fossarum* tissues and to decipher the cellular basis of HO alteration among the three populations, the structure of gills from non-impacted, slightly impacted and highly impacted organisms was then investigated. Gills of *G. fossarum* are characterized by a typically flattened shape, and are constituted by two single layer epithelia separated by pillar cells, delimiting hemolymphatic space in which hemolymph flows. In individuals from Bas, Cd exposure did not induce HO disturbance, and interestingly, no alteration of the gill structure was observed in this population. In contrast, histological observations of exposed organisms from the TdP and Ard populations revealed that Cd similarly induced histopathological damages in gills of individuals in which HO levels decreased, but not in NI. In SI and HI gills of specimens from both populations, hyperplasia (i.e. abnormal cell proliferation) and increased nuclear pigmentation were noticed at the tips of the gills. These typical histopathological effects of Cd confirmed the observations previously conducted on gills of *G. fossarum* (Issartel et al., 2010) and in other crustaceans such as the shrimps *Penaeus japonicus* (Soegiato et al., 1999) and *P. duorarum* (Couch, 1977), the crayfish *Procambarus clarkii* (Torreblanca et al., 1989) or the freshwater crab *Sinopotamon henanense* (Wang et al., 2013). Cadmium is thought to enter aquatic organisms from the water through gills via calcium channels of the apical plasma membrane of cells (Verbost et al., 1989; Wang et al., 2013). In addition to the consequent disturbance in  $\text{Ca}^{2+}$

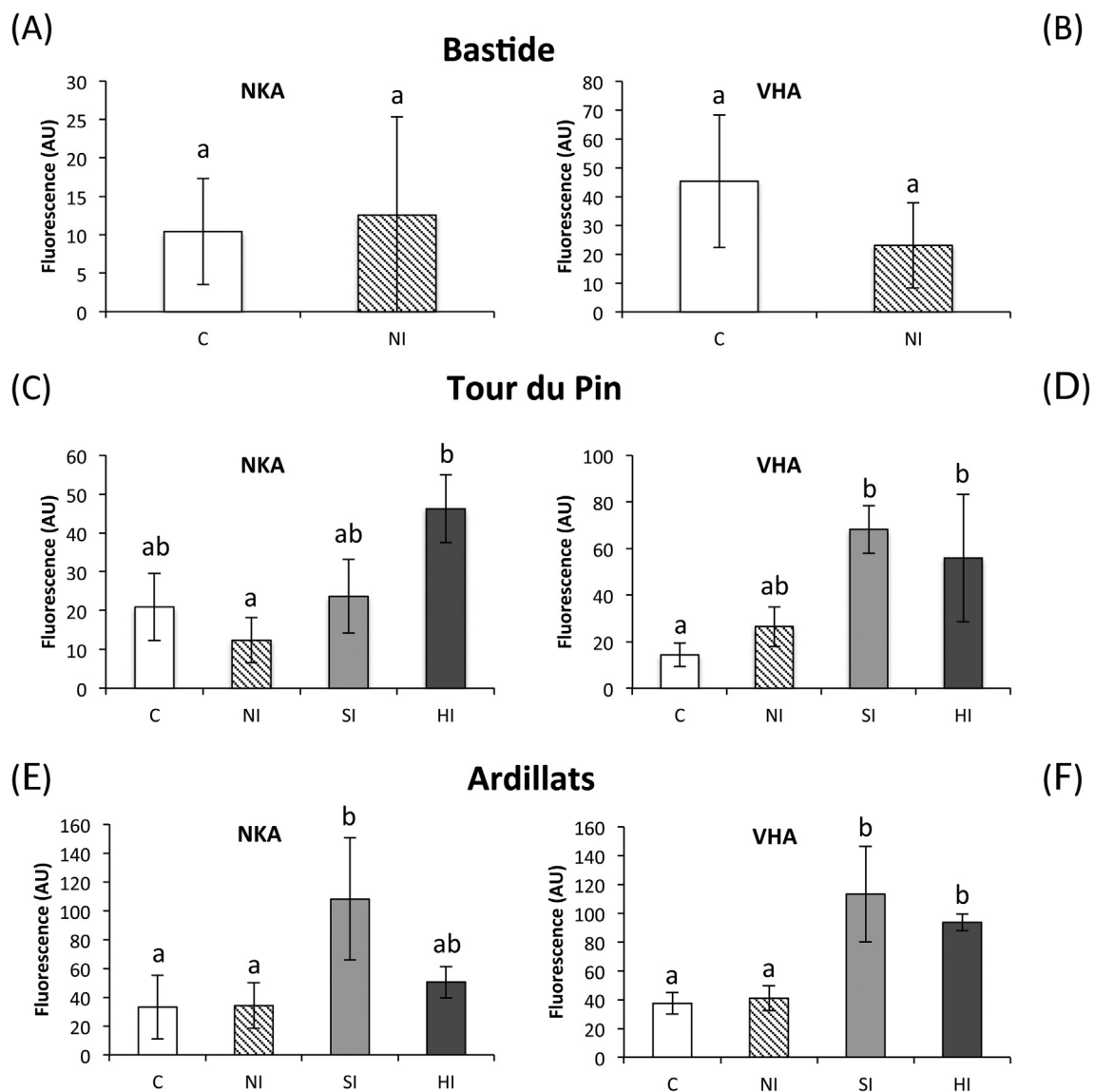




**Fig. 6.** *Gammarus fossarum* (population: Ardillats). Immunolocalization of  $\text{Na}^+/\text{K}^+$ -ATPase (red fluorescence) and V-type  $\text{H}^+$ -ATPase (green fluorescence) in a control (A, B) and Cd-exposed specimens: “NI, non impacted” (C, D); “SI, slightly impacted” (E, F); “HI, highly impacted” (G, H). All pictures were recorded using the same camera settings. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

homeostasis, once Cd is inside the cell, the metal is also known to induce a massive production of reactive oxygen species (ROS) by altering mitochondrial properties and antioxidant system (Bertin and Averbeck, 2006), possibly triggering cell death mechanisms such as apoptosis and necrosis. For instance, in the freshwater crab *Sinopotamon henanense* exposed to 14.5 mgCd/L for 24 h, a concomitant increase of the ROS  $\text{H}_2\text{O}_2$  and malondialdehyde (a sub-product of lipid peroxidation by ROS) was measured in the gills, followed by the appearance of apoptotic and necrotic cells after a 48 h and 72 h exposure, respectively (Wang et al., 2013). In our experiment,

gammarids were exposed to a much lower Cd dose but for a longer experimental period, and comparable phenomena may have occurred in gill cells, resulting in the observed histopathological alterations. Metals and particularly Cd are also known to induce alterations of the cytoskeletal organization (Li et al., 1993; Silvestre et al., 2006). In *G. fossarum* gills, a disruption of the microtubules of gill cells by Cd could affect the cell structure and hemolymph flow within the gill, as it was suggested for copper in *Carcinus maenas* (Lawson et al., 1995). In this study, the observed tissular disorganization of gill epithelium coincides with lower HO levels of SI and



**Fig. 7.** Na<sup>+</sup>/K<sup>+</sup>-ATPase and V-type H<sup>+</sup>-ATPase fluorescence semi-quantification (arbitrary units) in control and Cd-exposed specimens of Bastide (A, B), Tour du Pin (C, D) and Ardillats (E, F) populations. C: Controls; NI: non impacted; SI: slightly impacted; HI: highly impacted. Values are means ± SD. A different letter represents a significant difference (P < 0.05).

HI in both impacted populations. The altered ability to osmoregulate in these groups may originate from cell death and cytoskeletal disruption resulting in an increased ion loss in freshwater, and ultimately in a decrease of HO. However, hyper-osmoregulators as *G. fossarum* actively uptake ions from their environment via specific ionic transporters located in the epithelial cells of gills, which we studied using immunolocalization and semi-quantification of NKA and VHA.

#### 4.3. Immunolocalization and semi-quantification of NKA and VHA

Co-immunolocalization of NKA and VHA in *G. fossarum* gills confirmed their respective positions in crustacean ionocytes, with baso-lateral NKA and apical or sub-apical VHA. Similar localizations of VHA have been reported in other freshwater crustaceans e.g. the crabs *Uca formosensis* (Tsai and Lin, 2007) and *Dilocarcinus pagei* (Weihrach et al., 2004), the shrimp *Macrobrachium amazonicum* (Boudour-Boucheker et al., 2014), and the copepod *Eurytemora affinis* (Johnson et al., 2014). The respective localizations of NKA and

VHA reinforce the hypothesis of their synergic action to compensate ionic efflux (Beyenbach, 2001; Weihrach et al., 2001, 2004; Charmantier et al., 2009; McNamara and Faria, 2012). In ionocytes of freshwater hyper-osmoregulators, basal secretion of Na<sup>+</sup> by NKA from the cytoplasm to the hemolymph induces an apical uptake of Na<sup>+</sup> from the external medium, which is facilitated by the increased electronegativity on the cytosolic side of the apical membrane resulting from apical H<sup>+</sup> secretion by VHA. The resulting increased electronegativity of the inner side of the apical membrane facilitates Na<sup>+</sup> entry into the cell through apical sodium channels or still unknown Na/H exchangers, feeding the basal NKA (reviews in Charmantier et al., 2009; McNamara and Faria, 2012).

In accordance with physiological observations, Cd exposure resulted in drastically different fluorescence patterns of ion transporters between the Bas (pristine) population and the TdP and especially the Ard (chronically Cd-exposed) populations. In the Bas population, in which neither impacted HO nor histopathology were observed, no difference in VHA and NKA protein abundance was noticed in non impacted (NI) individuals

compared to controls. In contrast, in the TdP population, both NKA and VHA protein abundance showed important variations in impacted specimens. NKA protein expression was higher in highly impacted specimens, and VHA was strongly over-expressed in both slightly impacted (SI) and highly impacted (HI) groups. In the Ard population, exposed individuals also showed an increased presence of VHA in SI and HI; NKA increased only in SI but not in the most severely impacted organisms HI. In summary, NKA and VHA protein abundance in gills varied in the slightly and highly impacted organisms from both responsive populations TdP and Ard. The observed increased abundance of these two key enzymes may represent a mechanism aiming at increasing the efficiency of ion uptake to compensate the higher ion loss resulting from Cd-induced disruption of the gill structure. Cd and other metals are known to influence NKA enzymatic activity in crustaceans (review in Lignot et al., 2000). For example, a 6.5 µg Cd/L exposure for 10 d induced a 50% decrease in NKA activity in the amphipod *Hyalella curvispina* (Giusto and Ferrari, 2014). By contrast, Felten et al. (2008) measured a significant increase of the gill NKA activity in *Gammarus* exposed to 15 µg Cd/L for 7 d. In aquatic organisms, NKA enzymatic activity is controlled by expression level and phosphorylation (Péqueux, 1995; Lucu and Flik, 1999; Towle et al., 2001; Lovett et al., 2006). Cd is known to bind to proteins and to alter their conformation and activity (Bertin and Averbeck, 2006). Organisms may respond to altered NKA conformation by Cd that is leading to a decrease of its activity, by increasing the expression of NKA in gills, or by increasing the pool of active – phosphorylated – enzymes to maintain hemolymph osmolality. In this study, the higher abundance of NKA and VHA measured in Cd-impacted organisms is in accordance with an adaptive increase of enzyme concentration, and it is in agreement with the higher NKA activity previously measured in Cd-exposed gammarids (Felten et al., 2008). In addition to the increase in NKA measured in HI (from TdP individuals) and SI (from Ard individuals), the present study showed that VHA was over-expressed in slightly and highly impacted individuals from both populations. These results confirm that NKA and VHA cooperate in ion uptake and that VHA plays a crucial role in the maintenance of ionic homeostasis in freshwater hyperosmoregulators.

## 5. Conclusion

All 3 tested populations showed similar physiological profiles under control conditions: no influence of a chronic Cd exposure in its natural habitat was found in Ard compared to reference (metal-free) populations Bas and TdP. Organisms from Ard may cope with high Cd level by higher “basal” expressions of metallothioneins, and/or antioxidant enzymes. However, after Cd exposure in laboratory conditions, the response profiles of exposed organisms differed between the TdP and Bas reference populations and between Bas and the Cd-exposed population Ard. The first reference population living in the pristine Cd-free site (Bastide) was not impacted by Cd exposure in laboratory conditions, whereas organisms from the other reference Cd free site (Tour du Pin) and from the river contaminated by Cd geochemical background (Ardillats) showed high inter-individual variations in HO values both in control and Cd conditions, with slightly and highly Cd-impacted organisms. In gills of the impacted organisms of both affected populations, deep histopathological alterations and a protein over-expression of NKA and VHA were observed, while non-impacted animals showed profiles comparable to controls. These different patterns of responses between populations revealed no obvious adaptive phenomena in relation to the Cd levels of their respective biotopes. However, the strong impact of Cd exposure in Ard organisms may reflect the saturation of their

defense mechanisms that were already challenged by the chronic Cd level in their natural habitat. Furthermore, Ard individuals evolve in a river showing a slightly lower conductivity compared to the other sites, that may affect their capacity to respond to environmental stressors, as observed in a freshwater population of the amphipod *Gammarus lacustris* compared to saline counterparts (Vereshchagina et al., 2016).

As a result, the difference of profiles following Cd exposure could be explained by different levels of fitness and differences in energy allocation profiles between populations in response to the features of their natural environment.

The present study confirmed that osmoregulation represents a relevant biomarker to assess crustacean physiological status. By depicting for the first time in amphipods the co-localization of the two most important ionic transporters in gills, we gave new information regarding the impact of metal contamination on osmoregulation in freshwater crustaceans. Furthermore, by measuring the inter-individual responses, the impact of toxicological phenomena was highlighted whereas it would have been hidden by a global conventional approach. Finally, we have demonstrated that a common exposure to a contaminant in a same species could lead to drastically different responses according to the population tested. Consequently, studying the inter-population variation of vulnerability to chemical stressors in relation to life history or to pre-exposure of populations in the field is mandatory in order to yield robust hazard assessment of environmental contaminations.

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