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Chloride and sulphate toxicity to *Hydropsyche exocellata* (Trichoptera, Hydropsychidae): Exploring intraspecific variation and sub-lethal endpoints

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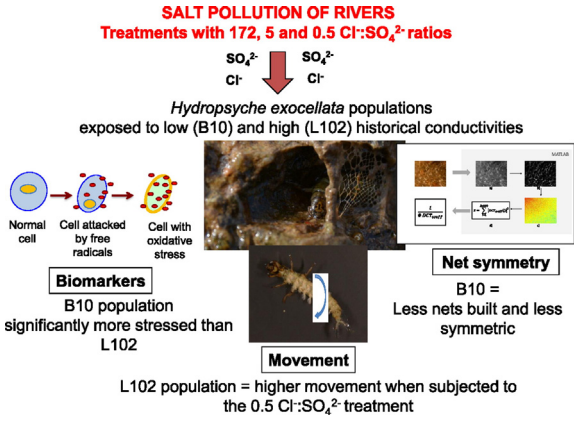
HIGHLIGHTS

- We assessed Cl- and SO42- toxicity to two populations of *H. exocellata*.
- The populations came from streams with different background conductivities.
- We measured different sub-lethal endpoints.
- Overall we registered weak responses to Cl- and SO42- toxicity.
- Some endpoints differed significantly between populations.

GRAPHICAL ABSTRACT



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ABSTRACT

The rivers and streams of the world are becoming saltier due to human activities. In spite of the potential damage that salt pollution can cause on freshwater ecosystems, this is an issue that is currently poorly managed. Here we explored intraspecific differences in the sensitivity of freshwater fauna to two major ions (Cl^- and SO_4^{2-}) using the net-spinning caddisfly *Hydropsyche exocellata* Dufour 1841 (Trichoptera, Hydropsychidae) as a model organism. We exposed *H. exocellata* to saline solutions (reaching a conductivity of 2.5 mS cm^{-1}) with Cl^-:SO_4^{2-} ratios similar to those occurring in effluents coming from the meat, mining and paper industries, which release dissolved salts to rivers and streams in Spain. We used two different populations, coming from low and high conductivity streams. To assess toxicity, we measured sub-lethal endpoints: locomotion, symmetry of the food-

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capturing nets and oxidative stress biomarkers. According to biomarkers and net building, the population historically exposed to lower conductivities (B10) showed higher levels of stress than the population historically exposed to higher conductivities (L102). However, the differences between populations were not strong. For example, net symmetry was lower in the B10 than in the L102 only 48 h after treatment was applied, and biomarkers showed a variety of responses, with no discernable pattern. Also, treatment effects were rather weak, i.e. only some endpoints, and in most cases only in the B10 population, showed a significant response to treatment. The lack of consistent differences between populations and treatments could be related to the high salt tolerance of *H. exocellata*, since both populations were collected from streams with relatively high conductivities. The sub-lethal effects tested in this study can offer an interesting and promising tool to monitor freshwater salinization by combining physiological and behavioural bioindicators.

1. Introduction

Globally, rivers and streams are getting saltier because of a wide variety of human activities (e.g. agriculture, resource extraction) and climate change (e.g. seawater intrusion in freshwater coastal areas through sea-level rise) (Cañedo-Argüelles et al., 2013). Not all sources of salt pollution have the same ionic composition (Cormier et al., 2013), and not all have the same toxic effects on freshwater organisms (Clements and Kotalik, 2016; Dunlop et al., 2015; Kunz et al., 2013). In this paper, we focus on two anions that tend to be dominant in mining and industrial effluents: Cl^- and SO_4^{2-}; testing them in the form of Na_2SO_4 and NaCl. According to Mount et al. (1997), Cl^- should be more toxic than SO_4^{2-} to freshwater fauna, but toxicity can change when both ions interact with each other. For example, in the same study the combination of NaCl and Na_2SO_4 had a lower LC50 (lethal concentration to 50% of a sample population) than each salt separately. Also, various studies (Soucek and Kennedy, 2005; Soucek, 2007) reported that adding chloride at low concentrations had a protective effect against sulphate toxicity to freshwater crustaceans, whereas increasing chloride from approximately 33 to 500 mg L^{-1} resulted in lower sulphate LC50s (Soucek, 2007). Thus, it is not clear how NaCl and Na_2SO_4 toxicity could vary along a range of concentrations when both salts are combined.

The effects of salt pollution also vary widely depending on the ability of the species to regulate ion concentrations within their body (Komnick, 1977; Bradley, 2008; Kefford et al., 2016). For example, Ephemeroptera and Plecoptera are among the most sensitive insect species (Cormier et al., 2013; Kefford et al., 2011; Pond, 2010), whereas other insects (e.g. Diptera: Ephydridae) can withstand salinities well beyond the salinity of seawater (Millán et al., 2011; Short et al., 1991). However, little is known about how salt sensitivity could vary among populations of the same species, e.g. depending on their history of exposure to different salt concentrations. Dunlop et al. (2008) and Kefford et al. (2003) found very limited inter-population differences in the short-term lethal salinity tolerance of aquatic invertebrates in Eastern Australia. On the contrary, Clements and Kotalik (2016) reported significant differences in the response to salt pollution of aquatic invertebrate communities from streams with different background salinity. Also, Gillis (2011) reported intraspecific differences in half maximal effective concentration (EC50) of NaCl between populations of the same species, but the author could not determine if they were directly related to intraspecific differences in salt sensitivity.

We explored intraspecific differences in the sensitivity of freshwater fauna to Cl^- and SO_4^{2-} using the net-spinning caddisfly *Hydropsyche exocellata* Dufour 1841 (Trichoptera, Hydropsychidae) as a model organism. This species is widespread in western Europe (Bonada et al., 2004b) and it plays a key role in streams by capturing suspended particles (Wallace et al., 1977) and by serving as food for fish (Cadwallader, 1975; Elliott, 1967). Its wide distribution can be partly attributed to its tolerance to pollution (Bonada et al., 2004a) and salinity (Gallardo-Mayenco, 1994; Piscart et al., 2005). Thus, toxic effects observed in this species would mean a threat for a great proportion of the aquatic invertebrate species, which are generally less tolerant to salt pollution. Moreover, there are several sub-lethal endpoints (i.e. those reflecting stress without mortality) that have been already tested in this genus, such as locomotion (Gerhardt, 1996; Macedo-Sousa et al., 2008), the symmetry of the nets they build to capture food particles (Besch et al., 1979; Petersen and Petersen, 1984; Tessier et al., 2000), the fluctuating asymmetry levels of their legs (Bonada et al., 2005) or biomarker responses (Barata et al., 2005). Sub-lethal effects are important to consider because they can warn of pollution before the risk of damage to the ecosystem is too high (Gerhardt, 1996; Ren et al., 2007). However, most studies on salinization of rivers and streams have focused on lethal effects on aquatic life (Cañedo-Argüelles et al., 2013), with very few exploring sub-lethal endpoints (Cañedo-Argüelles et al., 2015; Hassell et al., 2006; Kefford et al., 2006; Paradise et al., 2009; Pond et al., 2014; Zalizniak et al., 2009).

We reproduced the Cl^- and SO_4^{2-} concentrations of effluents originated from meat, mining and paper industries to assess the toxicity of these ions to *H. exocellata*. We selected these industries because of being widespread in Europe and contributing to freshwater salinization (Braukmann and Böhme, 2011; Maheshwari and Rani, 2012; Massé and Masse, 2000). We used two populations of *H. exocellata* historically exposed to different salt concentrations and evaluated three different endpoints that have been reported to be signals of toxicity for *Hydropsyche* larvae: locomotion, symmetry of the food-capturing nets and oxidative stress biomarkers. Our hypotheses were that: i) salt pollution (i.e. increased conductivity) would have a significant effect on all the measured sub-lethal endpoints; ii) the different effluents would have different toxicities according to their different Cl^- and SO_4^{2-} concentrations; iii) the population historically exposed to higher salt concentrations would be more resistant to salt addition.

2. Methods

2.1. Study site and collection of individuals

We collected *H. exocellata* larvae from two different streams (B10 = La Garriga; L10 = Pont de Vilomara) located in the Besós and Llobregat river basins (Catalonia, Spain), respectively (Prat and Rieradevall, 2006). Both basins have similar climatic and morphological conditions, although the former is more siliceous whereas the latter is more calcareous (Robles et al., 2002). Our study sites are impacted by human activities, i.e. both have moderate ecological status according to the Water Framework Directive (Prat and Rieradevall, 2006; Prat et al., 2014), but they have different conductivities (Prat and Rieradevall, 2006). A field survey in 1979 (Prat et al., 1982) reported the following conductivities and ion concentrations for B10 and L102 respectively: conductivity = 1590 vs. 3150 μS cm^{-1}; Cl^- = 214.50 vs. 547.18 mg L^{-1}; SO_4^{2} = 1.89 vs. 4.16 mg L^{-1}. Thus, we had two different populations of *H. exocellata*, with one (L102) exposed to historically higher conductivities than the other (B10). Historical salt exposure was analysed by looking at the conductivities of both streams during the period 2007–2014 (twice a year, in spring and summer). The data belonged to water monitoring campaigns conducted by the Freshwater Ecology and Management group of the University of Barcelona, and they are mostly available in their website (FEM, 2016).

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We collected (collection date = 03.03.2015) 4th instar *H. exocellata* individuals (length range 15–20 mm) at each site by carefully removing them from the river cobbles using forceps. Then, we placed them in a portable refrigerator containing river water and transported them to the laboratory.

2.2. Habitat design

We recreated field conditions in the laboratory by using plastic trays with river water that was pumped (using EHEIM® compact 300 pumps) to generate stable flow conditions (Fig. 2). The individuals were separated from the pump by a net to prevent the individuals from being drawn into the pump. We created artificial habitats by adhering sand to polystyrene blocks. *H. exocellata* needs structure to build their nets and we needed access to easily photograph those nets. For example, placing rocks in the trays was ideal for *H. exocellata* because these form the natural habitat in which they build their nets, but nets built between rocks were nearly impossible to photograph without damaging them. Both needs were met by systematically inserting several rows of toothpicks into polystyrene and then looping sewing thread down each row like a fence (Fig. 2). *H. exocellata* successfully built nets around these artificial structures, which were easy to photograph. We placed the individuals in the trays (8 trays; 10 individuals per tray; total N = 80 individuals) containing 3 L of river water from the stream with the lowest conductivity (B10) and we let them acclimate for 72 h (i.e. no treatment applied). We kept the trays in an environmental chamber at a constant temperature of 14 °C and dark conditions. We used dark conditions because *Hydropsyche* tends to be more active in the dark (Gerhardt, 1996; Macedo-Sousa et al., 2008) and it is usually found in shadow areas under the river cobbles.

2.3. Effluent reconstitution

We collected effluents from meat, mining and paper industries and analysed their Cl^- and SO_4^{2-} concentrations (Table 1) following standard methods (Eaton et al., 2005). The salt mining effluent was collected from a stream that receives salt-enriched groundwater contaminated by mine tailing leachate (Otero and Soler, 2002). The meat and paper industry effluents where directly provided by the industry, prior to being discharged to the public sewer. We prepared salt-saturated solutions (i.e. salinity = 250 g L^{-1}) to mimic the Cl^-:SO_4^{2-} ratio of each of the effluents (Table 1) by mixing distilled water with NaCl and Na_2SO_4 salts. Thus, we had a total of 3 reconstituted waters with different Cl^-:SO_4^{2-} ratios. We used reconstituted waters instead of real effluents to avoid the possible confounding effects of other stressors (e.g. low oxygen caused by high organic matter content, other pollutants, etc.).

2.4. Experimental design

A scheme of the experimental design is provided in Fig. 1. The reconstituted waters were added to the trays containing river water until reaching a conductivity of 2.5 mS cm^{-1}. This conductivity was chosen because it is above those generally (not always) reported in polluted rivers in Spain (López-Doval et al., 2012; Prat et al., 2014) and, at the same time, it is within the tolerance range of *H. exocellata* (Bonada et al., 2004a). Also, *H. exocellata* were likely to be stressed but tolerate these exposures because *Hydropsyche* larvae exhibited minor effects in a previous study using artificial streams (Cañedo-Argüelles et al., 2012). Thus, we expected that the treatment would not cause mortality but it could trigger sub-lethal effects. We had 4 different treatments that were named and arranged according to their Cl^-:SO_4^{2-} ratios:

- Control = river water (conductivity of 1 mS cm^{-1}).
- Low = river water + 0.55 Cl^-:SO_4^{2-} reconstituted water until reaching a conductivity of 2.5 mS cm^{-1}.
- Moderate = river water + 5.38 Cl^-:SO_4^{2-} reconstituted water until reaching a conductivity of 2.5 mS cm^{-1}.
- High = river water + 171.94 Cl^-:SO_4^{2-} reconstituted water until reaching a conductivity of 2.5 mS cm^{-1}.

Because the study involved two different populations (B10 and L10) that needed to be separated, we had a total of 8 trays (2 population × 4 treatments). The experiment lasted 72 h.

Table 1
Concentration of the dominant anions (Cl^- and SO_4^{2-}) and their ratio in the characterized effluents (coming from the mining, paper and meat industries) and the river water from the two sites from which *H. exocellata* were collected (B10 and L102).

	Cl^- (mg/l)	SO_4^{2-} (mg/l)	Cl^-:SO_4^{2-}
Mining	213,000	1246	171.94
Paper	1065	197.8	5.38
B10	98.2	99.0	0.99
L102	250.5	149.5	1.67
Meat	74.5	135.9	0.55

2.5. Net symmetry

Each 24 h we counted, photographed and removed the nets built by *H. exocellata*. We used a Nikon 7100 camera, a Sigma 17 70 f1:2.8 lens and a Sigma EM-140 DG flash for taking the photographs and Matlab (The MathWorks Inc.) to analyse them. First of all, we converted each image to grayscale because the colour information was not distinctive of the net structure. Furthermore, we wanted to extract the contours of the net so the background did not affect the final result. We applied a Prewitt Filter (Prewitt, 1970) giving a result as the one seen in Fig. 2b. After all the pre-processing steps, we computed the 2D Discrete Cosine Transform (DCT) (Makhoul, 1980) for each image (Fig. 2c). The DCT is a representation of a discrete signal in terms of a sum of cosine functions with different oscillating frequencies. Therefore, a sinusoidal signal with a certain pulsation ω would be represented as a δ function centred on ω at the DCT domain. On the other hand, a noise signal would be hard to decompose in a linear combination of cosines and, consequently, would have several components at its DCT transform. Hence, if a unitary energy distribution along the coefficients is assumed (defining energy as sum of the squares of the elements), those signals with a higher periodicity will hold most of their energy in just a few coefficients. This idea is one of the basis of Entropy Encoding, used in the popular image compression standard JPEG (Watson, 1994).

In our particular scenario, we used the DCT to measure each net's level of disorder. Assuming a unitary distribution of the energy in the DCT domain, i.e. the total energy equals one regardless the original image, we could use the percentage of coefficients holding a particular portion of the total energy as an indicator of how entropic is the distribution. The images with more regular patterns needed a smaller number of coefficients to reach the energy value than those looking more entropic (since the DCT was flatter). We sorted the DCT values according to the magnitude of the coefficients and squared summed them until a value of 0.99 was reached. We divided the obtained value by the total number of elements in the DCT, giving us the percentage of coefficients holding that energy value. We used this output to compare the symmetry of the nets. We assumed low values to belong to symmetric nets, whereas high values were assumed to indicate highly disorganized ones.

2.6. Assessment of movement

At the end of the study we collected 5 individuals from each tray to analyse their movement. The larvae of *Hydropsyche* have a very distinctive movement, contracting their abdomen rhythmically from side to side. We placed the individuals on a white tray with river water and recorded them for a minute to register these movements. Then we played

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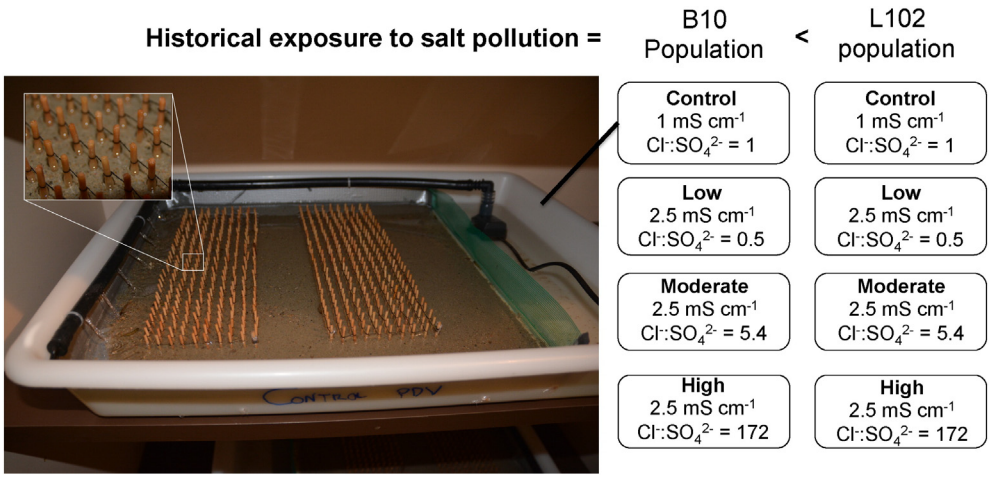

Fig. 1. Scheme of the experimental design. Control = river water (conductivity of 1 mS cm^{-1}). Low = river water + 0.55 Cl^-:SO_4^{2-} reconstituted water until reaching a conductivity of 2.5 mS cm^{-1}. Moderate = river water + 5.38 Cl^-:SO_4^{2-} reconstituted water until reaching a conductivity of 2.5 mS cm^{-1}. High = river water + 171.94 Cl^-:SO_4^{2-} reconstituted water until reaching a conductivity of 2.5 mS cm^{-1}. Since we collected two different populations coming from stream with different background conductivities (B10 and L10), we used a total of 8 trays (2 per treatment, corresponding to each population). The experiment lasted 72 h. On the left we show a picture of the experimental trays. We created artificial habitats by adhering sand to polystyrene blocks. These were separated from the pump by a net to prevent the individuals from being drawn into the pump. We provided the individuals a structure where they build their nets by systematically inserting several rows of toothpicks into polystyrene and then looping sewing thread down each row like a fence (zoomed in; upper left corner of the picture).

the video and recorded the number of abdominal contractions during 10 s. We only recorded each individual once (5 individuals per treatment; total N = 40).

2.7. Biomarkers

At the end of the study we collected 5 individuals from each tray to analyse general stress biomarkers that had been correlated with salinity stress in a previous study (Damásio et al., 2011a, 2011b). We analysed seven biomarkers in the whole animal tissue of *H. exocellata* (Table 2), including: the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT); the endogenous antioxidant molecule, reduced glutathione (GSH); the phase II metabolic conjugate enzyme, glutathione *S* transferase (GST); the metabolic enzymatic activity of lactate dehydrogenase (LDH); the cellular membrane oxidative damage marker lipid peroxidation (LPO); and the enzyme activity acetylcholinesterase (AChE), which is an indicator of neurotoxicity. We performed measurements of all biomarkers according to Damásio et al. (2011a, 2011b).

Whole body of *H. exocellta* was homogenized individually in ice cold 0.1 M phosphate buffer with 150 mM KCl and 0.1 m methylenediaminetetraacetic acid, disodium, salt and dihydrate (EDTA) in a 1:20 (weight/volume) proportion. Then, it was centrifuged at 10,000×*g*, 4 °C for

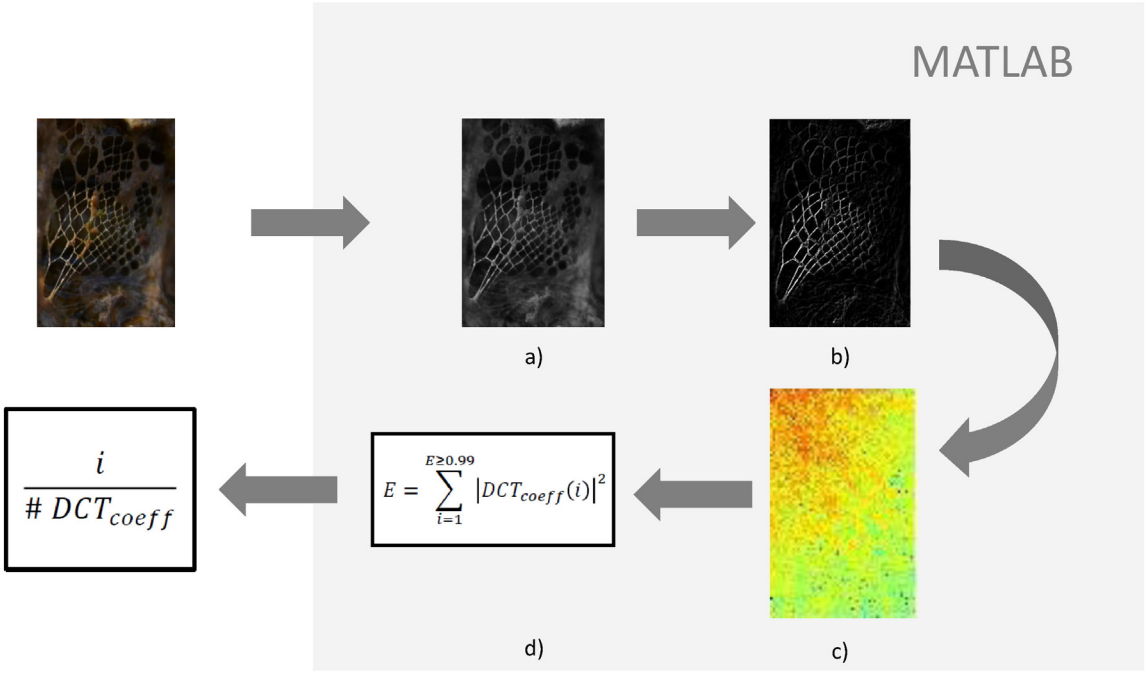

Fig. 2. Scheme of the processing of the images of *H. exocellata* nets, used to calculate their symmetry. In step b) the white lines are the contours detected on the image (i.e. in most of the cases the net). DCT = Discrete Cosine Transform. In step c red dots indicate a high magnitude of the DCT coefficients (i.e. recurrent frequencies), while green dots indicate low magnitude of DCT coefficients (i.e. rarely found frequencies). Step d shows the algorithm used for energy calculation. The output will be the number of coefficients needed to reach the required Energy Value (i.e. 0.99) divided by the total number of coefficients of the DCT. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Table 2
Differences in the activity or levels of the different analysed biomarkers between treatments, populations and the interaction of both factors (Population × Treatment). For those biomarkers registering significant differences we have ranked treatment groups according to Tukey's post-hoc test. The treatments consisted of adding 3 different reconstituted waters to river water until reaching a conductivity of 2.5 mS cm^{-1}. The reconstituted waters reproduced the Cl^-:SO_4^{2-} ratios found in the 3 industrial effluents characterized in Table 1 by mixing distilled water with NaCl and Na_2SO_4 salts. C: control (river water); L: low (river water + 0.55 Cl^-:SO_4^{2-} reconstituted water); M: moderate (river water + 5.38 Cl^-:SO_4^{2-} reconstituted water); H: high (river water + 171.94 Cl^-:SO_4^{2-} reconstituted water). Populations: B10 = historically exposed to lower salt concentrations; L102 = historically exposed to higher salt concentrations. Significant results are shown in bold.

	Treatment			Population			Population × Treatment	
	F	p		F	p		F	p
Acetylcholinesterase	0.09	0.554	–	3.30	0.080	–	1.03	0.394
Catalase	2.24	0.104	–	0.69	0.412	–	**5.40**	**0.005**
Glutathione *S* transferase	0.76	0.524	–	**9.80**	**0.004**	B10 > L102	0.26	0.852
Lactate dehydrogenase	**5.64**	**0.004**	M > C,H,L	0.23	0.634	–	2.50	0.082
Lipid peroxidation	**0.96**	**0.450**	–	**19.93**	$\mathbf{9.3 \times 10^{-5}}$	B10 > L102	**4.01**	**0.016**
Reduced glutathione	0.87	0.464	–	4.88	**0.034**	L102 > B10	1.46	0.242
Superoxide dismutase	2.08	0.124	–	19.30	$\mathbf{1.3 \times 10^{-4}}$	B10 > L102	**4.67**	**0.009**

20 min. The supernatant was collected, aliquoted and stored at −80 °C prior to biomarker determination.

Catalase (CAT) activity was measured by a decrease in absorbance at 240 nm due to H_2O_2 (52.5 mM) consumption according to Aebi (1974). Results are shown as μmol/min/mg of protein.

Superoxide dismutase (SOD) activity was determined according to McCord and Fridovich (1969) by measuring the degree of inhibition caused by SOD on the reduction of cytocrome c (0.01 mM) by free oxygen radicals ($O^{\bullet}_2^-$) released by the xanthine oxidase (0.0017 U/mL)/xanthine (0.023 mM) reaction. Final results were normalized by tissue total protein content and expressed as U/mg of total protein.

Glutathion *S* transferase (GST) activity towards 1-Cl, 2.4 dinitro benceno (CDNB) (1 mM) in the presence of glutathion-GSH (1 mM) was measured as described by Habig et al. (1974). Results were normalized by tissue total protein content and expressed as nmol/min/mg of protein. Reduced glutathione (GSH) quantification was measured following the fluorometric assay of Kamencic et al. (2000), which measures the conjugate complex formed between GSH present in the sample with a fluorescent probe monochlorobimane (mCB) (0.1 mM) by GST (1 U/mL). Results were expressed as nmol/g *ww* (tissue wet weight).

Lactate dehydrogenase (LDH) activity was determined by monitoring the absorbance decrease at 340 nm due to NADH (0.18 mM) oxidation in the presence of Pyruvate (1.18 mM) following Diamantino et al. (2001). Results were expressed as nmol/min/mg of protein. Lipid peroxidation (LPO) was determined by quantifying the levels of malondialdehyde (MDA) according to Esterbauer et al. (1990) and results were expressed as nmol/g of *ww* (tissue wet weight).

Acetylcholinesterase activity (AChE) was determined following the method described by Ellman et al. (1961) and modified by Escartín and Porte (1997). We measured the product formed by the combination of 5,59-dithiobis- 2-dinitrobenzoic acid (DTNB) (0.33 mM) with thiocholine that resulted from the hydrolysis of acetylthiocholine (2 mM) by AChE activity. Final results were expressed in nmol/min/mg protein. Proteins were determined following Bradford (1976), using Bovine serum albumin (BSA) as a standard. Except for catalase activity, which was measured using a cuvette assay (Life Science UV/Vis Spectrophotometer DU® 730, Beckman Coulter – Fullerton, CA, USA), all the bioassays were performed in microplate (Synergy 2 Multi-Mode Microplate Reader, BioTek® Instuments – Vermont, USA).

2.8. Statistical analyses

All statistical analyses were performed in R software (R Core Team, 2015). Data were square root transformed to stabilize variance. We performed two-way ANOVAs for analysing differences in biomarkers and movement (which were tested just once, at the end of the study) between treatments and populations. In the case of the nets' symmetry (which was analysed every 24 h), we performed repeated-measures ANOVA to assess overall differences between populations and treatments at each time (24, 48 and 72 h after treatment) by using the "ezANOVA" function in R package ez (Lawrence, 2015). The Tukey's 'Honest Significant Difference' method was used (function "TukeyHSD") to compare differences in the mean between the different treatments, obtaining a p-value after adjustment for the multiple comparisons. Normality was checked performing the Shapiro-Wilk test of normality using the "shapiro.test" function. In the cases where normality was not met, alpha was set to 0.01 to reduce the risk of finding false positives. Heteroscedasticity was checked by performing the Breusch-Pagan test using the "bptest" function the R package "lmtest" (Zeileis and Hothorn, 2002). In the case of heteroscedasticity, a recently suggested robust statistical test ("glht" function in R package "multcomp") was used as a post hoc test with adjustment of p-values for multiple pairwise comparisons between treatment levels (Herberich et al., 2010).

3. Results

Conductivities were significantly different (ANOVA tests: F = 14.58, p = 0.002) between both sampling sites for the period 2007–2014 (Fig. 3). There were no significant differences between treatments in the number of nets (N = 208) built by *H. exocellata* or their symmetry, but there were significant differences between populations. The B10 population (i.e. individuals collected from the site with lower historical conductivities) built a significantly lower number of nets (Fig. 4) and

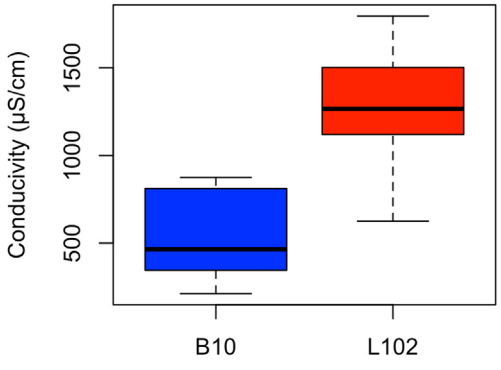

Fig. 3. Conductivities from 2007 to 2014 (spring and summer samples) at the two streams (B10 and L102) from which *H. exocellata* were collected. The data belong to water monitoring campaigns conducted by the Freshwater Ecology and Management group of the University of Barcelona, and they are mostly available in their website (FEM, 2016). Codes correspond to those assigned by the Catalonian Water Agency water quality monitoring program (Prat and Rieradevall, 2006). B10 = La Garriga; L102 = Pont de Vilomara.

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they were significantly less symmetric, but only 48 h after treatment (Fig. 5). The movement of *H. exocellata* (i.e. number of abdominal contractions per second) was significantly different between treatments (treatment effect: $F = 7.48$; p-value $= 6.9 \times 10^{-4}$), and these differences varied significantly between populations (treatment*population effect: $F = 10.78$; p-value $= 5.6 \times 10^{-5}$). Tukey's test revealed no significant differences between treatments in the B10 population, whereas the low treatment resulted in significantly higher abdominal contractions in the L102 population (i.e. individuals collected from the site with higher historical conductivities) (Fig. 6).

Treatment only had an overall effect (i.e. across populations) on lactate dehydrogenase (LDH) activity, but there was a population-dependent effect for lipid peroxidation (LPO) and catalase (CAT) and superoxide dismutase (SOD) activities (Table 2, Fig. 7). We found significant differences between populations for LDH, LPO, SOD, GST and GSH, with all of them exhibiting higher concentrations or activities in the B10 population, except for GSH (Table 2).

4. Discussion

Overall our results do not unequivocally support our hypotheses. Although most of the measured endpoints (e.g. several biomarkers, movement) varied significantly among treatments with different SO_4^{2-}:Cl^- ratios, we did not find clear evidences of any of the treatments being more toxic to *H. exocellata* than the rest. We did find significant differences in the response of the studied populations to treatment, with the population historically exposed to lower conductivities (B10) showing higher levels of stress than the population historically exposed to higher conductivities (L102). However, these differences were only found for some specific endpoints and treatments. Thus, we can't claim that historical exposure to salt determined the sensitivity of *H. exocellata* populations to SO_4^{2-} and Cl^- in this study.

Net building was not significantly affected by treatment, but we found significant differences between populations. The B10 population constructed a significantly lower number of nets and these were less symmetric (i.e. had a higher entropy). Although Gerhardt (1996) found no significant changes in net building of *H. angustipennis* exposed to polluted water, several studies have reported otherwise. For example, Petersen and Petersen (1984) recorded a significant increase in net anomalies in of *H. angustipennis* exposed to kraft mill pulp effluent; Becker (1987) found diminished net-spinning activity of *H. pellucidula* under low oxygen conditions; Wendt-Rasch et al. (1998) reported increased mesh-opening and a decreased symmetry of the nets of *H. siltalai* exposed to fenvalerate; and Tessier et al. (2000) found a distortion of the midline meshes and a significant decrease in net symmetry of *H. slossonae* exposed to malathion. Thus, net-symmetry of *Hydropsyche* larvae seems to be a reliable endpoint that could be used as an early signal of river pollution. Although in this study net symmetry

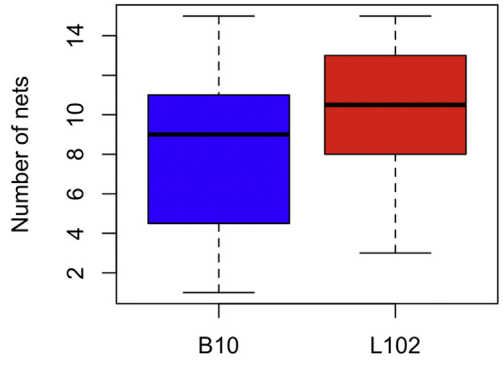

Fig. 4. Number of nets built by each population of *H. exocellta*. ANOVA test: $F = 4.599$; $p = 0.0167$. Sensitive = individuals collected from site B10; Tolerant = individuals collected from site L102. Differences among the different effluent treatments are not shown because of lack of statistical significance.

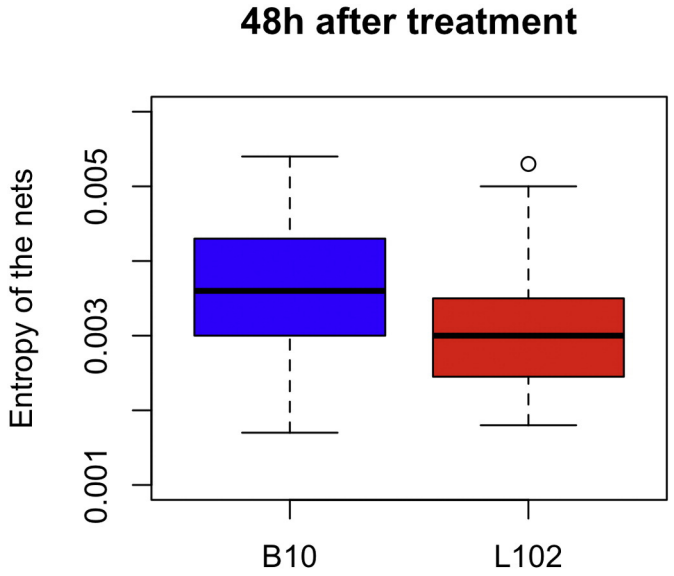

Fig. 5. Entropy (i.e. disorder) of the nets built by each population of *H. exocellata*. ANOVA test: $F = 5.313$; $p = 0.0238$. Sensitive = individuals collected from site B10; Tolerant = individuals collected from sie L102. Differences among the different effluent treatments and among populations for other times (i.e. 24 and 72 h) are not shown because of lack of statistical significance.

was not significantly different between treatments, the recorded differences between populations suggest that salt could have affected net building. The above-mentioned studies analysed net symmetry by carefully removing, cleaning and mounting the nets and then looking at them under the microscope. This is a difficult and time-consuming process that precludes this endpoint from being routinely used for monitoring water quality. Here, we present an easier and faster way of analysing net symmetry by using a camera and computer software. We believe that this method deserves to be further tested (i.e. other pollutants and concentrations) to evaluate its suitability for water quality monitoring in rivers. Moreover, image processing represents a promising tool to be considered in biomonitoring. For example, the stress level of ecosystem engineers could affect the structures that they build in streams (Moore, 2006), and this could be analysed using image processing.

Abdominal contractions (i.e. undulatory movements of their abdomen) of *Hydropsyche* are related to ventilation. Under salinity stress metabolism can be enhanced due to the activation of ionoregulatory mechanisms (Guerriero et al., 2002; Martínez-Álvarez et al., 2002). This can lead to an increase in undulatory movements to facilitate ventilation and oxygen uptake (Van der Geest, 2007). In our study, the number of abdominal contractions per second of *H. exocellata* varied significantly across treatments and populations. L102 individuals exposed to the low treatment (i.e. river water + 0.55 Cl^-:SO_4^{2-} reconstituted water) registered significantly higher number of contractions than those exposed to the rest of treatments, whereas we found no significant differences between treatments in the B10 population. Thus, our results suggest that the L102 individuals subjected to the low treatment were under significantly higher stress than the rest of individuals. This would mean that the L102 population was more sensitive to SO_4^{2-} toxicity than the B10 population. This is counter to what was expected, since the L102 population should be less stressed than the B10 population and the low treatment should be the least harmful according to its lowest Cl^- concentrations (Mount et al., 1997). Moreover, the biomarker results (e.g. lipid peroxidation) and the net building analyses seem to contradict these results. Thus, it is not clear yet if locomotion can be a viable endpoint for assessing the effects of pollution on *Hydropsyche* larvae. Accordingly, Macedo-Sousa et al. (2008) found that *H. pellucidula* movement was unaffected by drops in pH caused by acid mine drainage. At the same time, they reported a higher movement of individuals

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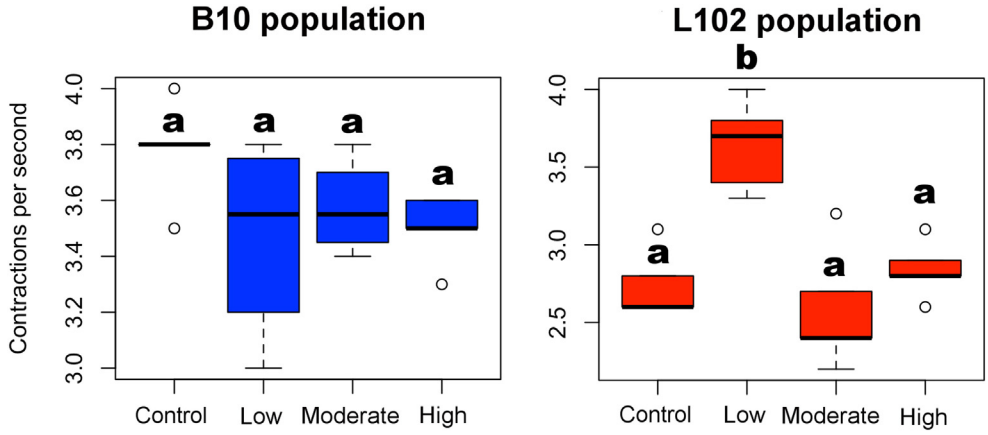

Fig. 6. Number of contractions per second of *H. exocellata* for each treatment and population. Treatments are arranged by their Cl^-:SO_4^{2-} ratio. Meat = meat industry effluent; Control = river water; Paper = paper industry effluent; Mine = mining industry leachate. Sensitive = individuals collected from site B10; Tolerant = individuals collected from site L102. Letters refer to Tukey's post-hoc test; different letters mean significant differences among groups. N = 40 individuals (5 per treatment per population).

placed in treatment than in control chambers that could not be explained by treatment (i.e. pH drop), suggesting that movement could vary according to intrinsic factors. However, Gerhardt (1996) reported increased locomotion of *H. angustipennis* exposed to polluted surface water from an industrial effluent and Van der Geest (2007) showed that exposure to hypoxia increased movement of the same species. We suggest that movement is a sub-lethal endpoint that also deserves further study using different methodologies to record it, different sources of stress and different *Hydropsyche* species and populations.

The only biomarker showing an overall significant response to treatment was LDH, although CAT, LPO and SOD responded to at least one treatment in the B10 population. The activity of LDH, which is linked to anaerobic metabolism, has been related to toxic stress in aquatic invertebrates (Diamantino et al., 2001) and specifically to salt pollution in *H. exocellata* (Damásio et al., 2011b) and the leech *Dina lineata* (Cañedo-Argüelles et al., 2015). Individuals exposed to the moderate treatment showed significantly higher LDH activity than the rest, indicating a greater toxicity of this treatment (with a moderate Cl^-: SO_4^{2-} ratio). This suggests that toxicity could be unimodally related to the Cl^-: SO_4^{2-} ratio. Concordantly, the SOD activity of the B10 population was significantly higher in the moderate than in the high treatment. LPO and SOD were significantly higher in the B10 than in the L102 population. SOD provides the first line of antioxidant defense against reactive oxygen species to prevent oxidative tissue damage (lipid peroxidation). Thus, oxidative stress was highest in the B10 population. GST was significantly higher in the B10 than in the L102 population, whereas GSH showed the opposite pattern. Glutathion has a dual role in cells/organisms: it is conjugated by GST and other enzymes to detoxify reactive oxygen species and other nocive metabolites and, at the same time, it helps to maintain the cell oxidative status (Halliwell and Gutteridge, 2015). Observed population differences in GST and GSH indicate that B10 and L102 populations may have used high constitutive activities of GST and GSH to detoxify excess salt ions and/or reactive oxygen species, respectively. The increase in GST activity in the B10 population matches the results obtained by Damásio et al. (2011a, 2011b) when analyzing biomarker responses in *H. exocellata* along a salt-pollution gradient.

According to biomarkers and net building, the population historically exposed to lower conductivities (B10) showed higher levels of stress than the population historically exposed to higher conductivities (L102). However, the differences between populations were not strong. For example, net symmetry was lower in the B10 than in the L102 only 48 h after treatment was applied, and biomarkers showed a variety of responses, with no discernable pattern.

Also, treatment effects were rather weak, i.e. only some endpoints, and in most cases only in the B10 population, showed a significant response to treatment. In this study, both populations were collected from streams with relatively high conductivities (mean conductivity for the period 2007–2014: B10 = 0.55 mS cm^{-1}, L102 = 1.26 mS cm^{-1}). Thus, the lack of consistent differences between populations and treatments could be related to the high salt tolerance of *H. exocellata* (Bonada et al., 2004a; Damásio et al., 2011b; Gallardo-Mayenco, 1994). In that case, future studies testing higher conductivities (i.e. above 2.5 mS cm^{-1}) could reveal population-dependent differences in the response of *H. exocellata* to salt pollution. Another possibility is that aquatic insects, in general, show little intraspecific variation in their sensitivity to salt pollution. Concordantly, Dunlop et al. (2008) and Kefford et al. (2003) found little variation in the LC50 of populations of aquatic insects coming from streams with different background conductivities. Also, Kefford et al. (2012) compared the salt sensitivity of freshwater invertebrates from Eastern Australia, France, Israel and South Africa and found that the greatest source of variation in species sensitivity was between taxonomic groups (Order and Class) and not between the regions. On the contrary, Clements and Kotalik (2016) reported significant differences in the response to salt pollution of invertebrate communities from two streams with different background conductivities. However, such differences could be related to differences in species composition rather than intraspecific variation in salt sensitivity. Moreover, background conductivities at both streams were very low (34–133 and 200–250 μS cm^{-1}) when compared to the ones reported in our study. Finally, the lack of intraspecific differences could be related to factors other than salinity affecting salinity tolerance (e.g. genetics of the populations, migration, ionic composition, feeding history, other stressors in their environments). The influence of such factors could swamp the effect of salinity exposure history.

The response to treatment varied widely across endpoints, preventing us from determining which of the tested ions (i.e. Cl^- and SO_4^{2-}) was more toxic to *H. exocellata*. However, since treatments with the same conductivity but different Cl^-: SO_4^{2-} ratios had different effects on the studied populations, our results suggest that salt toxicity was somehow dependent on the ionic composition of the treatments. Although some important work has already been done in identifying the toxicity of different ions to freshwater biodiversity (e.g. Mount et al., 1997), further investigations on the toxicity of salts representative of different pollution sources (e.g. the ones tested in this study) are required (Cañedo-Argüelles et al., 2016). Additionally, water treatment technologies that specifically remove those ions and/or salts that are more harmful to freshwater ecosystems should be explored. One of the most appropriate technologies for that purpose is nanofiltration. Nanofiltration has been successfully used in several applications because it can selectivity separate different ions. Depending on the application, a membrane with suitable selectivity should be selected in order

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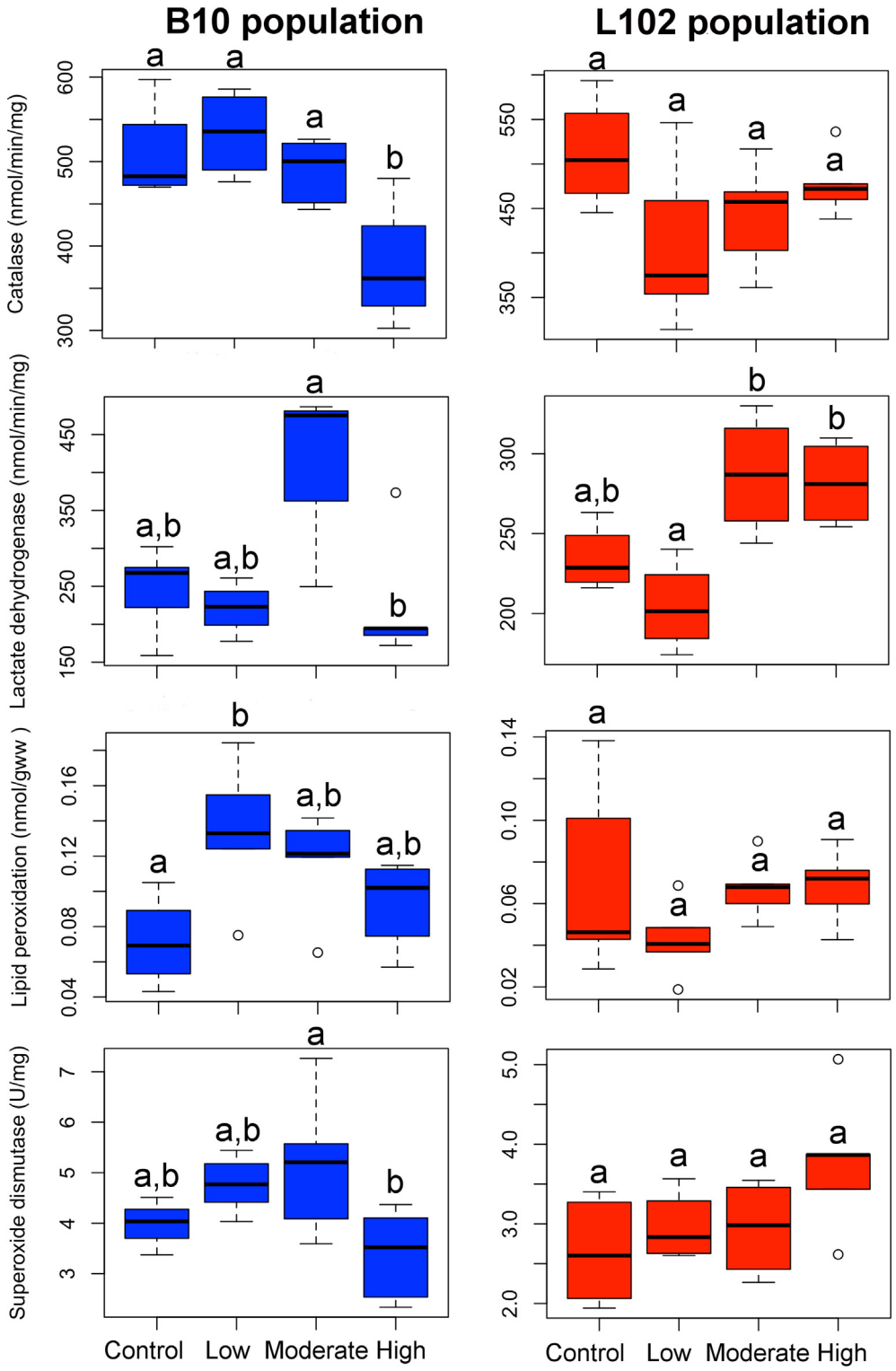

Fig. 7. Biomarker responses for each treatment and population. Only those biomarkers registering any significant response to treatment are shown (Table 2). Treatments are arranged by their Cl^-:SO_4^{2-} ratio (from lowest, i.e. meat, to highest, i.e. mine). Control = river water; Meat = meat industry effluent; Paper = paper industry effluent; Mine = mining industry leachate. Sensitive = individuals collected from site B10; Tolerant = individuals collected from site L102. Letters refer to Tukey's post-hoc test; different letters mean significant differences among groups. N = 40 individuals (5 per treatment per population).

to provide the most optimum separation (Mohammad et al., 2015). Electrodialysis is another membrane technology that could be suitable for this purpose. There are commercial ion-exchange membranes that are monovalent anion or monovalent cation selective, and with this type of membranes an almost complete separation between ions of different charge can be achieved (Sata, 2004). However, these technologies are expensive. Thus, a better understanding of the ecological impacts of different effluents with different ion signatures is needed to assess costs and benefits of targeting specific ions in wastewater treatments and to guide water management decisions in the face of global salinization of freshwaters (Cañedo-Argüelles et al., 2016). In this regard, the sub-lethal effects tested in this study could offer an

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interesting and promising tool to monitor freshwater ecosystems by combining physiological and behavioural bioindicators.

Author contributions

MC and MS designed and performed the experiment; NB, NP and SP helped with the experimental design; CB, MF and MS analysed biomarkers; NS and MS analysed net symmetry; MC and MS performed statistical analyses; MC wrote the bulk of the manuscript; ALL authors contributed to writing the manuscript.

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