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► To cite this version:

V. Felten, F. Guerold. Short-term physiological responses to a severe acid stress in three macroinvertebrate species: A comparative study. *Chemosphere*, 2006, 63, p. 1427 - p. 1435. 10.1016/j.chemosphere.2005.10.003 . hal-00452244

HAL Id: hal-00452244

<https://hal.science/hal-00452244>

Submitted on 1 Feb 2010

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Author-produced version of the article published in Chemosphere
2006, 63, 1427-1435.
The original publication is available at <http://www.sciencedirect.com/>
doi : 10.1016/j.chemosphere.2005.10.003

1 **Short-term physiological responses to a severe acid stress in three**
2 **macroinvertebrate species: a comparative study.**

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4

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11

12 **Abstract**

13 The present study focuses on the sensitivity among freshwater invertebrate species to acidic
14 stress. Three common macroinvertebrate species in the Vosges Mountains (North-Eastern
15 France), *Gammarus fossarum* (Amphipoda), *Hydropsyche pellucidula* (Trichoptera) and
16 *Dinocras cephalotes* (Plecoptera) were exposed for 24, 72 and 120 hours to natural acidified
17 water (pH= 4.73 ± 0.08 , $[Ca^{2+}] = 39.1 \pm 0.6 \mu\text{mol.L}^{-1}$, $[Al_{\text{tot}}] = 28.4 \pm 1 \mu\text{mol.L}^{-1}$). Short-term
18 exposure to acid stress caused significant decreases both in survival rate and haemolymph
19 ions ($[Cl^-]$ and $[Na^+]$). The relative sensitivity to a natural acidic stress slightly differed among
20 the species and was in the following order: *G. fossarum*, as the most sensitive, then *H.*
21 *pellucidula* and *D. cephalotes*. Results of this study confirm the interest of *in situ* tests to
22 assess the toxicity of short-term acid exposure. Finally, our results reinforce the hypothesis
23 that transient acidification can offset the recovery of sensitive species of macroinvertebrates
24 in streams chemically recovering from acidification either through liming or declining
25 deposition.

26 **Keywords:** Acidification, Macroinvertebrate, Ion loss, Survival, Acid-sensitivity.

27

28 INTRODUCTION

29 Acidification of freshwater ecosystems related to anthropogenic emissions of SO₂ and NO_x
30 has been one of the most striking ecological problems throughout the northern hemisphere
31 during the 20th century. National and international legislation in the 1980s and 1990s aimed
32 at reducing the emissions of acidifying pollutants (e.g. Clean Air Act in the USA and the
33 Convention on Long-Range Transboundary Air Pollution in Europe) have led to the decline in
34 acidic depositions across wide areas of Europe and North America (Stoddard et al., 1999,
35 Lawrence et al., 2000, Evans et al., 2001, Likens et al., 2001, Folster and Wilander, 2002).
36 Consequently, it has been assumed that the 'acidification problem' was solved. However, even
37 if recent studies have shown that recovery of alkalinity has occurred in several areas of
38 Europe and North America (Stoddard et al., 1999, Skjelkvale et al., 2001), acidification still
39 occurs in many areas. (Driscoll et al., 2001, Evans et al., 2001, Wright et al., 2005).

40 Concomitantly, a decline of base cations (mainly Ca²⁺ and Mg²⁺) in soils and surface waters
41 has been reported in most areas where high rates of sulphur depositions occurred previously
42 (Likens et al., 1996, 1998, Bouchard, 1997, Lawrence et al., 1999, Castro & Morgan, 2000,
43 Driscoll et al., 2001, Tessier et al., 2002). If such cation depletion continues, it will represent
44 another serious threat to aquatic ecosystems. In addition, acidification of aquatic ecosystems
45 is now reported across other large areas of the world, such as China (Thorjörn et al., 1999,
46 Tang et al., 2001) and India (Aggarwal et al., 2001). Therefore, it appears that acidification of
47 soils and water remains an important environmental problem.

48 One of the most striking consequences of freshwater acidification is the loss of biodiversity
49 (Muniz, 1991). An ion-regulation failure leading to a severe deficiency of extracellular ions
50 (i.e. Na⁺ and Cl⁻) has been recognised to be the major response in fish to acid stress (Neville,

51 1985, Booth *et al.*, 1988, Wood *et al.*, 1988, McDonald *et al.*, 1989, Potts & McWilliams,
52 1989, Wood, 1989, Masson *et al.*, 2002). Similar results have been also reported in crayfish
53 (Morgan & McMahon, 1982, Wood & Ronago, 1986, McMahon & Stuart, 1989, Jensen &
54 Malte, 1990), and molluscs (Malley *et al.*, 1988, Pynnönen, 1990, 1991).
55 Surprisingly, and despite the numerous papers reporting detrimental effects of acidification on
56 macroinvertebrate communities, few realistic studies have been performed to assess the
57 ecophysiology of smaller acid-sensitive species and finally, relatively little is known about
58 their physiological responses to short-term acid stress. In previous studies (Felten & Guérol,
59 2001, 2004), we showed that *Gammarus fossarum* (Crustacea: Amphipoda), a common acid-
60 sensitive species in west Palearctica, also sustained a severe depletion of haemolymph Na^+
61 and Cl^- ions when exposed to acidic conditions. Conjointly, we determined relationship
62 between acidification level and haemolymph ion losses (Felten & Guérol, 2004).
63 In order to better understand the ecology of ecosystems recovering from acidification and
64 because episodic acid-stresses generated by heavy rainfall and snowmelt continue to affect
65 streams, the purpose of the present study was to investigate and to compare *in situ* the
66 physiological responses and survival rates of three common acid-sensitive species to realistic
67 episodic acid stress.

68

69 MATERIALS AND METHODS

70 **Study sites.** The study was performed in the Vosges Mountains (North-Eastern France). Two
71 headwater streams located in the same area were selected because they presented very similar
72 morphological characteristics (**Table 1**) but contrasted buffer capacities: a circumneutral
73 stream (La Maix) and a strongly acidified stream (Gentil Sapin).

74

75 **Study organisms.** Three macroinvertebrate species were selected because they are known to
76 be acid-sensitive, widespread in European inland waters and easy to collect: *Hydropsyche*
77 *pellucidula* (Trichoptera, Hydropsychidae), *Dinocras cephalotes* (Plecoptera, Perlidae) and
78 *Gammarus fossarum* (Amphipoda, Gammaridae). Organisms were collected by using a net
79 from a circumneutral stream, La Maix.

80

81 **Experimental design.** A total of 1728 males of *Gammarus fossarum* with 8 to 10mm body
82 lengths were collected. 288 organisms were placed in 2 Plexiglas flow-through enclosures
83 each consisting of 18 compartments each with 8 organisms.

84 Conjointly, we used 216 *Dinocras Cephalotes* and 216 *Hydropsyche pellucidula* with body
85 lengths of 20mm and 15 mm respectively. For both species, 36 organisms were placed in 2
86 Plexiglas flow-through enclosures each consisting of 18 compartments (10-cm long and 5-cm
87 i.d.) each filled with 1 organism to avoid potential cannibalism.

88 The enclosures were then transferred to the 2 streams (circumneutral and strongly acid). For
89 each stream, one enclosure was used for haemolymph analyses and the other for the
90 assessment of survival. For each stream and species, there were 3 pairs of enclosure each
91 corresponding to one exposure time (24, 72 and 120 h).

92 In order to evaluate the initial concentrations of haemolymph Cl^- and Na^+ in each species,
93 organisms (12 individuals for *G. fossarum*, 8 individuals for *D. cephalotes* and 8 individuals
94 for *H. pellucidula*) were sampled in the neutral “native” stream just before the onset of the
95 experiments (T_0 , control). At 24, 72 and 120 h after exposure transfer, survival was assessed
96 and samples of haemolymph from 8 (*G. fossarum*) or 6 (*D. cephalotes*, *H. pellucidula*)
97 organisms were randomly collected in each stream for analysis (in enclosure corresponding to
98 exposure time). The organisms were not fed during the experiment. However, the
99 experimental design allowed drifting material (organic particles, algae and small invertebrates

100 < 1 mm) to enter the compartments. For more details concerning enclosure technique see
101 Felten & Guérold (2004).

102

103 **Survival, haemolymph sampling and analysis.** The survival was assessed in each stream for
104 each species and exposure time (*G. fossarum*: 3 replicates of 48 organisms; *D. cephalotes* and
105 *H. pellucidula*: 3 replicates of 6 organisms).

106 For *D. cephalotes* and *H. pellucidula* samples of haemolymph were taken from the base of the
107 cephalic capsule with a 5- μ l microcapillary tube (n = 6). For *G. fossarum*, samples were taken
108 from the telson (n = 8) using a microsyringe and then transferred to a gauged 5- μ l
109 microcapillary. After centrifugation of the microcapillary tubes (10 min at 6596 g), the liquid
110 phase was diluted in 2 ml of Nanopur water to determine chloride and sodium concentrations
111 in haemolymph by ionic chromatography (Dionex 4500i with Ion Pac AS4A column) and
112 atomic absorption spectrophotometry (AAS) (Perkin Elmer Analyst 100), respectively.

113

114 **Water analysis.** Water was collected at the initiation of the experiment (T_0) and at each
115 exposure time (24, 72 and 120 h). Cations were analysed by flame AAS and anions by ionic
116 chromatography as described previously. Total aluminium was determined by graphite
117 furnace AAS (Varian Spectraa 300) after acidification with 0.25% HNO_3 . Acid neutralising
118 capacity (ANC) was measured by Gran's titration and pH (glass electrode), and conductivity
119 with a multi-parametric equipment (WTW). Chemical characteristics of water of each stream
120 are given in **Table 2**.

121

122 **Statistical analysis.** All data are reported as mean \pm SD. Statistical comparisons of
123 experimental data were performed by two-way analysis of variance (ANOVA) and Fisher's
124 Least Significant Difference test (LSD). The analyses were carried out using STATISTICA

125 (Microsoft), with a probability limit of $p \leq 0.05$ considered as significant.

126

127 **RESULTS**

128 To study and compare the effect of acidification on haemolymph $[\text{Na}^+]$ and $[\text{Cl}^-]$ and survival
129 of the 3 species, specimens were transferred from the native circumneutral stream to a
130 strongly acidified stream. Acidified stream was characterized by low pH (i.e. high $[\text{H}^+]$), low
131 ANC, low $[\text{Mg}^{2+}]$ and low $[\text{Ca}^{2+}]$ and high $[\text{Al}_{\text{tot}}]$. Note that, small-scale differences in
132 mineral composition of the underlying bedrock explain the marked differences in buffering
133 capacity, ion content and acidification (**Table2**). The 2-way analysis of variance (ANOVA)
134 indicated that the factors “exposure time” and “stream” (acidification level) exerted a
135 significant effect on haemolymph parameters ($[\text{Na}^+]$, $[\text{Cl}^-]$) and survival (**Table 3**).

136

137 ***Gammarus fossarum***

138 *G. fossarum* baseline levels of haemolymph $[\text{Cl}^-]$ and $[\text{Na}^+]$ on the onset of the exposure were
139 61.4 ± 9.2 and 77.5 ± 15.5 mmol.L^{-1} (mean \pm SD), respectively, in control organisms
140 collected in La Maix (**Fig. 1a&b**).

141 Haemolymph $[\text{Na}^+]$ and $[\text{Cl}^-]$ in organisms exposed in the circumneutral stream (mean pH =
142 7.42) remained constant over the 120-h exposure period, but significantly decreased in
143 organisms exposed in the strongly acidic one (mean pH = 4.73) (**Fig. 1a&b**). Indeed, in the
144 acidic stream, the loss of haemolymph Cl^- (**Fig. 1a**) ranged from about 44.4% after 72h (mean
145 haemolymph $[\text{Cl}^-] = 33.6 \pm 4.8$ mmol.L^{-1}) to 58.7% after 120 h of exposure (mean
146 haemolymph $\text{Cl}^- = 23.2 \pm 3.7$ mmol.L^{-1}) compared with the control (mean haemolymph $\text{Cl}^- =$
147 61.4 ± 9.2 mmol.L^{-1}).

148 The same trend was observed for haemolymph $[\text{Na}^+]$ (**Fig. 1b**). In the acidic stream, the
149 significant loss of haemolymph Na^+ ranged from 48.5% after 72 h of exposure (mean

150 haemolymph $\text{Na}^+ = 38.5 \pm 6.1 \text{ mmol.L}^{-1}$) to 57.4% after 120 h of exposure (mean
151 haemolymph $\text{Na}^+ = 31.1 \pm 3.5 \text{ mmol.L}^{-1}$) compared with the control (mean haemolymph Na^+
152 $= 77.5 \pm 15.5 \text{ mmol.L}^{-1}$).

153 For each exposure time, the survival rate in *G. fossarum* transferred to the circumneutral
154 stream (La Maix) remained very high and above 96%. On the contrary, the survival rate in
155 organisms exposed to acidic water significantly decreased from $79.9 \pm 10.8\%$ after 24h of
156 exposure to $20.5 \pm 6.2\%$ after 120h of exposure (**Fig. 1c**).

157

158 *Hydropsyche pellucidula*

159 As observed for *G. fossarum*, the factors “exposure time” and “stream” (acidification level)
160 exerted a significant effect on haemolymph parameters ($[\text{Na}^+]$, $[\text{Cl}^-]$) and survival (2-way
161 ANOVA, **Table 3**). *H. pellucidula* baseline levels of haemolymph $[\text{Cl}^-]$ and $[\text{Na}^+]$ on the
162 onset of the exposure were 37.2 ± 5.7 and $132.2 \pm 8.8 \text{ mmol.L}^{-1}$ (mean \pm SD) respectively, in
163 control organisms collected in the neutral stream (**Fig. 2a&b**).

164 Haemolymph $[\text{Na}^+]$ and $[\text{Cl}^-]$ in organisms exposed into the circumneutral stream remained
165 constant over the 120-h exposure period, but significantly decreased in organisms exposed to
166 the strongly acidic stream. Indeed, in the acidic stream, the loss of haemolymph Cl^- ranged
167 from 33.6% after 24 h of exposure (mean haemolymph $[\text{Cl}^-] = 23.5 \pm 6.3 \text{ mmol.L}^{-1}$) to 51%
168 after 120 h of exposure (mean haemolymph $\text{Cl}^- = 16.7 \pm 5.6 \text{ mmol.L}^{-1}$) compared with the
169 control (mean haemolymph $\text{Cl}^- = 37.2 \pm 5.7 \text{ mmol.L}^{-1}$) (**Fig. 2a**).

170 A similar trend was observed for haemolymph $[\text{Na}^+]$. In the acidic stream, the significant loss
171 of haemolymph Na^+ ranged from 12.1% after 24 h of exposure (mean haemolymph $\text{Na}^+ =$
172 $120.7 \pm 5.3 \text{ mmol.L}^{-1}$) to 20.8% after 120 h of exposure (mean haemolymph $\text{Na}^+ = 100.5 \pm$
173 9.2 mmol.L^{-1}) compared with the control (mean haemolymph $\text{Na}^+ = 132.2 \pm 8.8 \text{ mmol.L}^{-1}$)
174 (**Fig. 2b**).

175 Throughout the exposure period the survival rate in *H. pellucidula* transferred to the
176 circumneutral stream remained very high and above 94%. In contrast, survival decreased
177 significantly in organisms exposed to the acidic stream, where the survival rate ranged from
178 $72.2 \pm 9.6\%$ after 24h of exposure to $58.3 \pm 14.4 \%$ after 120h (**Fig. 2c**).

179

180 ***Dinocras cephalotes***

181 The exposure time had a significant effect on haemolymph $[\text{Cl}^-]$ and $[\text{Na}^+]$ and survival, but a
182 significant effect of stream was only observed on $[\text{Cl}^-]$ and survival (2-way ANOVA, **Table**
183 **3**). *D. cephalotes* baseline levels of haemolymph $[\text{Cl}^-]$ and $[\text{Na}^+]$ on the onset of the exposure
184 were 126.6 ± 12.6 and $133.0 \pm 6.8 \text{ mmol.L}^{-1}$ (mean \pm SD), respectively, in control organisms
185 collected in the neutral stream (**Fig. 3a&b**).

186 Haemolymph $[\text{Na}^+]$ and $[\text{Cl}^-]$ in *D. cephalotes* exposed into the circumneutral stream
187 remained constant over the 120-h exposure period. In organisms exposed into the acidic
188 stream, a significant loss was observed only after 120h of exposure. The loss of haemolymph
189 Cl^- and Na^+ reached respectively 20.6% (mean haemolymph $[\text{Cl}^-] = 96.7 \pm 17.3 \text{ mmol.L}^{-1}$)
190 and 13.1% (mean haemolymph $\text{Na}^+ = 109.3 \pm 22.2 \text{ mmol.L}^{-1}$) compared with the control
191 (mean haemolymph $\text{Cl}^- = 126.6 \pm 12.6 \text{ mmol.L}^{-1}$; mean haemolymph $\text{Na}^+ = 133.0 \pm 6.8$
192 mmol.L^{-1}).

193 No mortality was observed in *D. cephalotes* transferred to the circumneutral stream (La Maix)
194 during the experiment. Survival rate in organisms exposed to the acidic stream remained also
195 high after 24h, but decreased significantly after 72h and 120h exposure (reaching $83.3 \pm$
196 8.3% , **Fig. 3c**).

197

198 **Species sensitivity comparison**

199 The baseline level of haemolymph $[\text{Cl}^-]$ in *D. cephalotes* was respectively 2 and 3.5 times

200 higher than in *G. fossarum* and *H. pellucidula*. Haemolymph $[Na^+]$ baseline levels in *D.*
201 *cephalotes* and *H. pellucidula* were similar and 1.8 times higher than in *G. fossarum*.
202 Haemolymph $[Na^+]$ was always higher than $[Cl^-]$ representing a difference of 5%, 21.7% and
203 74.4% in *D. cephalotes*, *G. fossarum* and *H. pellucidula*, respectively.
204 Under the experimental conditions of this study, *D. cephalotes* was the least acid-sensitive
205 species, since it presented the lowest ion losses and mortality rate. *G. fossarum* was the most
206 acid-sensitive species, showing both the highest losses of ions and mortality.

207

208 **DISCUSSION**

209 Over recent years, the loss of biodiversity has become a major concern (see Gaston, 2000,
210 Loreau et al., 2001, Naeem et al., 1999). Acidification of freshwater due to anthropogenic
211 activities has been recognized as an important cause of biodiversity loss in ecosystems located
212 in remote areas. In the Vosges Mountains, acidification of headwater streams has been
213 identified as being the major threat to aquatic biota. Acidified streams are strongly
214 impoverished. For example, macroinvertebrate communities have lost between 50 and 70% of
215 the original species (Guérol et al., 2000).

216 Although all major taxonomic groups are affected by acidification, species sensitivity appears
217 different among macroinvertebrates groups. Studies carried out in Europe and in North
218 America have highlighted the high sensitivity of molluscs, Ephemeroptera and crustacean
219 (Hall et al., 1980, Harriman & Morison, 1982, Sutcliffe, 1983, Engblom & Lingbell, 1984,
220 Peterson et al., 1985, Guérol et al., 2000). The response of Trichoptera to acidification is
221 more variable: several species disappear (e.g. *Hydropsyche* sp.) whereas some others exhibit
222 higher densities (e.g. *Plectrocnemia conspersa*). Thus, many works have reported the acid-
223 sensitivity of Hydropsychidae and Philopotamidae as well as the acid-tolerance of
224 *Plectrocnemia*, *Rhyacophyla* and Limnephilidae (Sutcliffe & Carrick, 1973, Ziemann, 1975,

225 Harriman & Morrison, 1982, Havas & Hutchinson, 1982, Townsend et al., 1983, Raddum &
226 Fjellheim, 1984, Simpson et al., 1985). Although Perlidae (e.g., *Dinocras cephalotes*) and, to
227 a lesser extent, Perlodidae are recognized as being acid-sensitive, the majority of Plecoptera
228 (especially Nemouridae and Leuctridae) are acid-tolerant and can be very abundant in acid
229 streams (Sutcliffe & Carrick, 1973, Raddum & Fjellheim, 1984, Tixier & Guérol, 2005).

230 The toxicity of acid water seems to be the principal hypothesis (often referred to as the
231 ecotoxicological hypothesis) explaining the loss of species. In the present study we showed
232 that a short-term exposure of *G. fossarum*, *H. pellucidula* and *D. cephalotes* to strongly
233 natural acidic water caused significant losses of haemolymph $[Na^+]$ and $[Cl^-]$ accompanied by
234 significant mortality. However, the intensity and rapidity of ion loss depends on the species
235 considered: in *G. fossarum* and *H. pellucidula* significant ion losses occurred as early as 24h
236 of exposure in an acidic stream whereas in *D. cephalotes* a significant decrease of
237 haemolymph $[Cl^-]$ and $[Na^+]$ was observed only after 120h of exposure.

238 The magnitude of ion loss also differs among species: after 120h of exposure in acidic stream,
239 haemolymph $[Cl^-]$ and $[Na^+]$ losses reached respectively 58.7% and 57.4% in *G. fossarum*,
240 51% and 20.8% in *H. pellucidula* and only 20.6% and 13.1% in *D. cephalotes*. Similarly, the
241 lowest survival rate was observed for *G. fossarum* (20.5%), the highest for *D. cephalotes*
242 (83.3%) whereas it was intermediate for *H. pellucidula* (58.3%).

243 Although some studies have clearly identified a depletion of ion concentrations in invertebrate
244 exposed to acidic waters, most of these studies were performed on moderately acid-sensitive
245 species (*Corixia dentipes*, *C. punctata*: Vangenechten et al., 1989; *Cenocorixia blaisdelli*:
246 Needham, 1990; *Libellula julia*: Rockwood & Coler, 1991; *Pteronarcys proteus*: Leichleitner
247 et al., 1985) and/or on organisms experimentally exposed to conditions that were not
248 environmentally meaningful; e.g. pH 2.8-3.0 (Leichleitner et al., 1985; pH 3.0, Rupprecht,
249 1992) or aluminium concentrations of 30 mg.L⁻¹ (Rockwood & Coler, 1991). Similar results

250 have also been obtained from pooled samples of haemolymph (Needham, 1990) or from
251 whole body analyses (Hermann, 1987, Rowe *et al.* 1988, 1989, Havens, 1992).

252 Because of the protocol and the realistic *in situ* conditions of exposure, our results study
253 provides more rigorous results and consolidates the experimental studies previously
254 mentioned (see above). There is no doubt that significant ion loss and mortality in acid
255 sensitive species can be induced by short exposures to acidic stresses. This is particularly
256 pertinent to running water ecosystems experiencing acute acid stresses that are, for most of
257 the time, within the pH range that could sustain such taxa. For example, in the Vosges
258 Mountains, several circumneutral streams draining sandstone are subjected to rare acid
259 stresses. These streams exhibit communities of invertebrates that are rather typical of
260 moderately acidified streams despite their average chemical characteristics.

261 Depending on their duration, magnitude and frequency, such acid stresses can lead to the loss
262 of sensitive species or simply impair their abundances. This has been recently evidenced by
263 Lepori and Ormerod (2005) who have shown that the distribution and the density of the
264 bivoltine species, *Baetis alpinus* (Ephemeroptera) in areas subjected to acidification in the
265 Alps, depend on the toxicity of acid run-off during snowmelt. However, concerning species
266 which are monovoltine or semi-voltine, repeated acid stresses are more likely to eradicate
267 them from sensitive ecosystems.

268 Finally, our results sustain the hypothesis proposed by Bradley and Ormerod (2002) that short
269 episodes of low pH can offset the recovery of sensitive species of macroinvertebrates in
270 streams that have chemically recovered from acidification following catchment liming.

271 Understanding both the biological effect and ecological consequences of episodic acidity has
272 real management implications in defining strategies. Given the general context of recovery
273 from acidification, in response to reduced acidic depositions as well as following liming, the
274 use of physiological variables such as haemolymph $[Cl^+]$ and $[Na^-]$, would be helpful to

275 predict whether the ecosystem meets again the chemical quality that can allow the re-
276 colonization of acid-sensitive species. Likewise, the development and the use of methods
277 based on macroinvertebrate communities would provide important information of ecological
278 relevance (for example on the settlement of sensitive species populations and the
279 sustainability of these populations). Finally, in the context of ecological toxicology (*sensu*
280 Chapman, 2002) a combination of both approaches would greatly enhance the pertinence of
281 biological monitoring with minimal uncertainty, especially in national or international survey
282 programs aiming to assess the consequences of the reduction of atmospheric emissions of
283 acidic pollutants as well as the effectiveness of liming.

284

285 *Acknowledgements.* We are grateful to Marie-Danielle Murard and two anonymous referees
286 for helpful comments and linguistic corrections to improve the manuscript.

287

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290

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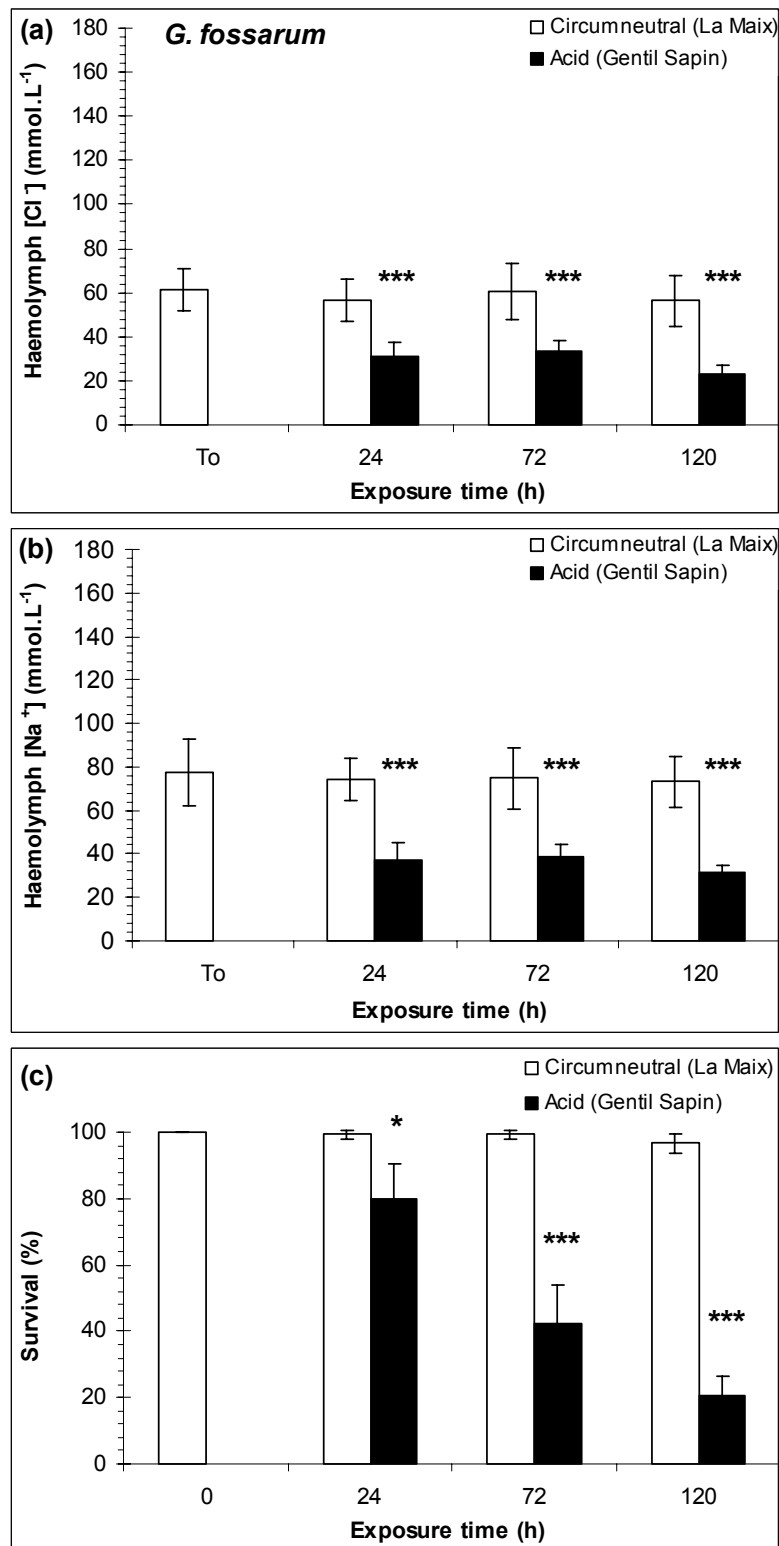
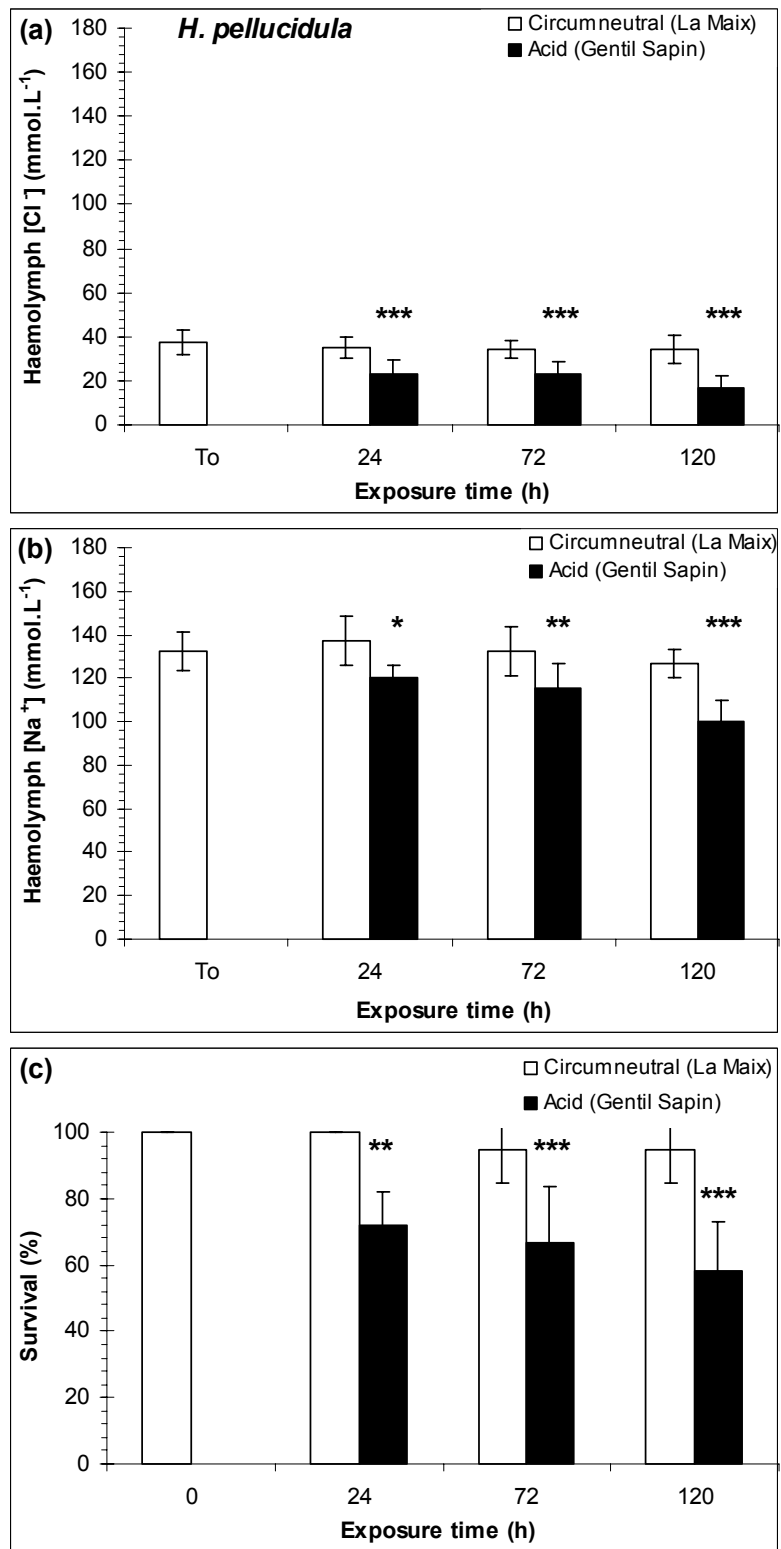


Figure 1. Mean (\pm SD) [Cl⁻] haemolymph (a), [Na⁺] haemolymph (b), and survival (c) of *Gammarus fossarum* exposed to circumneutral and acidic water. Significant differences against T₀ are indicated by asterisks (Fischer's Least Significant Difference test; * : p < 0.05; ** : p < 0.01; *** : p < 0.001).

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517 **Figure 2.** Mean (\pm SD) [Cl⁻] haemolymph (a), [Na⁺] haemolymph (b), and survival (c) of
518 *Hydropsyche pellucidula* exposed to circumneutral and acidic water. Significant differences
519 against T₀ are indicated by asterisks (Fischer's Least Significant Difference test; * : p < 0.05;
520 ** : p < 0.01; *** : p < 0.001).

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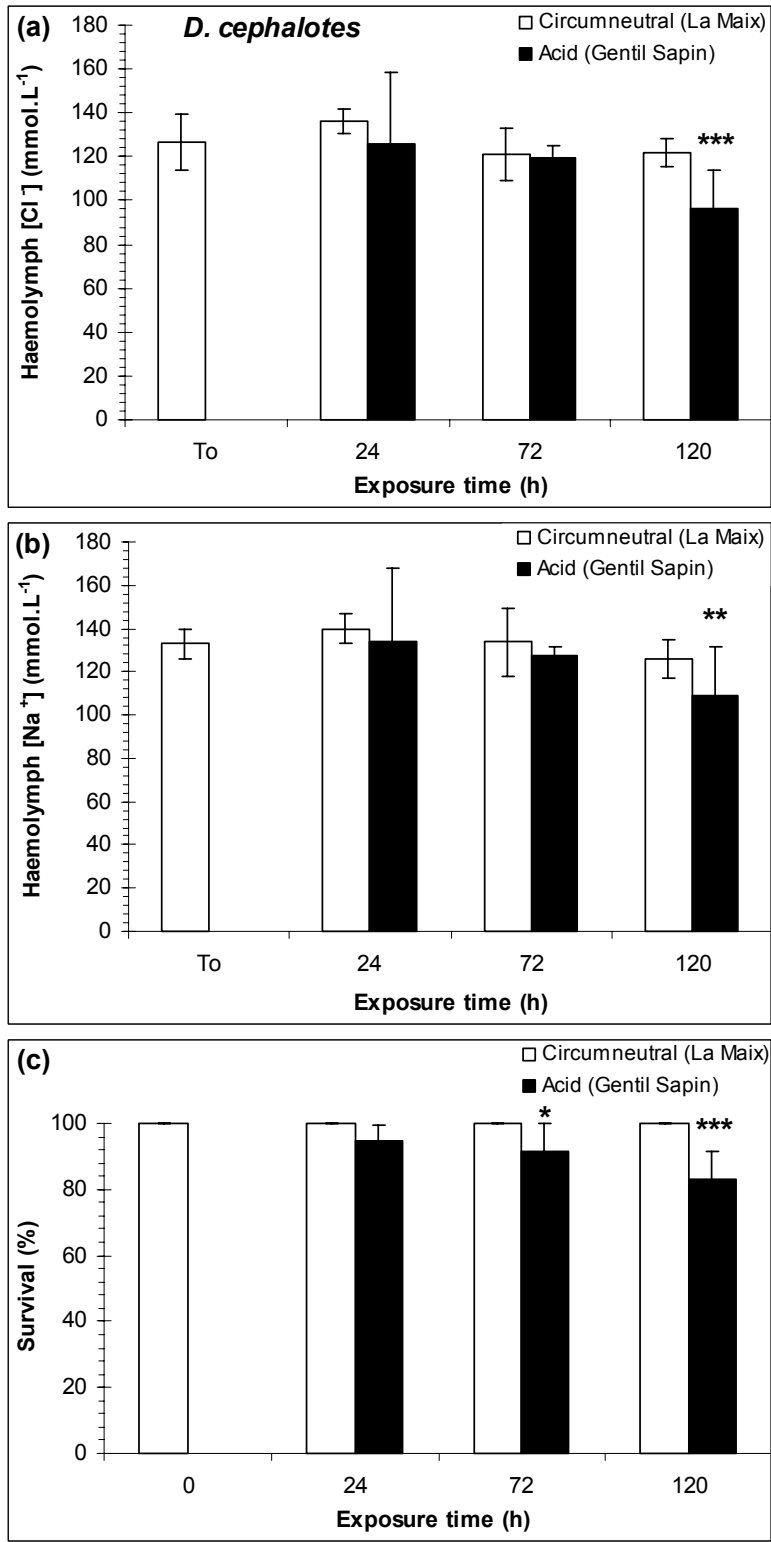


Figure 3. Mean (\pm SD) [Cl⁻] haemolymph (a), [Na⁺] haemolymph (b), and survival (c) of *Dinocras cephalotes* exposed to circumneutral and acidic waters. Significant differences against T₀ are indicated by asterisks (Fischer's Least Significant Difference test; * : p < 0.05; ** : p < 0.01; *** : p < 0.001).

546 **Table 1.** Description of the two study sites.

	stream name	La Maix	Gentil Sapin
550	stream order	2	2
	elevation (m)	439	501
552	distance from spring (m)	3870	3280
554	latitude N	48°29'02.1"	48°26'56.8"
	longitude E	007°04'08.5"	007°03'33.5"
556	width (m)	1.8	2.3
558	depth (m)	0.35	0.30

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576 **Table 2.** Mean (\pm SD) values of various chemicals of each exposure stream (n=4). ANC: Acid
 577 Neutralizing Capacity.

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	La Maix		Gentil Sapin	
	Mean	SD	Mean	SD
pH	7,42	0,13	4,73	0,08
Temperature ($^{\circ}$ C)	11,9	0,7	14,0	1,5
conductivity (μ S.cm $^{-1}$)	74,3	4,3	31,5	2,4
ANC (μ eq.L $^{-1}$)	618,3	13,5	-15,5	7,0
Ca $^{2+}$ (μ mol.L $^{-1}$)	222,9	3,1	39,1	0,6
Mg $^{2+}$ (μ mol.L $^{-1}$)	188,3	0,8	28,6	0,2
Na $^{+}$ (μ mol.L $^{-1}$)	64,1	0,4	49,4	1,4
K $^{+}$ (μ mol.L $^{-1}$)	56,4	0,3	35,4	1,3
SO $_4^{2-}$ (μ mol.L $^{-1}$)	75,7	0,6	62,5	0,8
NO $_3^{-}$ (μ mol.L $^{-1}$)	60,0	0,9	88,5	1,1
Cl $^{-}$ (μ mol.L $^{-1}$)	54,0	0,6	44,9	0,7
Total Al (μ mol.L $^{-1}$)	2,1	0,4	28,4	1,0

600 **Table 3.** Summary of the 2-way analysis of variance. F : F ratio ; p : probability; d.f. : degrees
 601 of freedom.

	[Cl ⁻] haemolymph			[Na ⁺] haemolymph			Survival		
	d.f.	F	p	d.f.	F	p	d.f.	F	p
a) <i>Gammarus fossarum</i>									
Factor a (Stream)	1	79,4	< 10 ⁻³	1	99,3	< 10 ⁻³	1	228,3	< 10 ⁻³
Factor b (Exposure time)	3	18,1	< 10 ⁻³	3	20,1	< 10 ⁻³	3	54,2	< 10 ⁻³
Interaction	3	10,7	< 10 ⁻³	3	14,2	< 10 ⁻³	3	47,2	< 10 ⁻³
b) <i>Hydropsyche pellucidula</i>									
Factor a (Stream)	1	32,3	< 10 ⁻³	1	28,9	< 10 ⁻³	1	33,0	< 10 ⁻³
Factor b (Exposure time)	3	12,9	< 10 ⁻³	3	9,3	< 10 ⁻³	3	6,7	< 5*10 ⁻⁵
Interaction	3	6,8	< 10 ⁻³	3	5,9	< 5*10 ⁻⁵	3	3,9	< 10 ⁻²
c) <i>Dinocras cephalotes</i>									
Factor a (Stream)	1	5,4	< 10 ⁻²	1	3,0	n.s.	1	16,8	< 10 ⁻³
Factor b (Exposure time)	3	4,9	< 5*10 ⁻⁵	3	3,7	< 10 ⁻²	3	3,5	< 10 ⁻²
Interaction	3	2,0	n.s.	3	0,8	n.s.	3	3,5	< 10 ⁻²

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