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Haemolymph [Na^+] and [Cl^-] loss in *Gammarus fossarum* exposed *in situ* to a wide range of acidic streams

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ABSTRACT: The acid-sensitive amphipod *Gammarus fossarum* was exposed *in situ* for 24, 96 and 168 h to 18 streams (9 draining from granite and 9 draining from sandstone bedrock), selected in order to provide a wide range of acidification. After 24 h, exposure to slightly acidic ($6.00 \leq pH \leq 5.50$) and strongly acidic water ($pH < 5.50$) led to a severe and significant depletion in haemolymph [Na^+] and [Cl^-] compared to organisms exposed in circumneutral water. Highly significant linear correlations between stream mean pH value and haemolymph [Na^+] and [Cl^-] were observed for each exposure time on each bedrock. Organisms exposed to slightly acid streams draining granite bedrock (pH = 5.71, pH = 5.81) showed a physiological adaptation after 96 h of exposure, while animals in acidic sandstone streams did not. Results of this study indicate that haemolymph [Na^+] and [Cl^-] in *G. fossarum* could be an effective ecophysiological marker for monitoring freshwater ecosystem acidification.

KEY WORDS: *Gammarus fossarum* · Haemolymph · Ion loss · Acid stress · *In situ* markers

INTRODUCTION

Acidification of freshwater ecosystems related to anthropogenic emissions of SO_2 and NO_X has been one of the most striking ecological problems throughout the northern hemisphere during the 20th century. National and international legislation in the 1980s and 1990s aimed at reducing the emissions of acidifying pollutants (e.g. Clean Air Act in the USA and the Convention on Long-Range Transboundary Air Pollution in Europe) have led to the decline in acidic depositions across wide areas of Europe and North America (Stoddard et al. 1999, Lawrence et al. 2000, Likens et al. 2001). Consequently, it has been assumed that the 'acidification problem' was solved. Recent studies have shown that recovery of alkalinity has occurred in several areas of Europe (such as Great Britain, Nordic countries and North/Central Europe), but in only one region of North America (Stoddard et al. 1999, Skjelkvale et al. 2001). However, acidification per se still occurs in many areas (Guerold et al. 2000, Driscoll et al. 2001, Evans et al. 2001). Concomitantly, a decline of base cations (mainly Ca^{2+} and Mg^{2+}) in soils and surface waters has been reported in most areas where high rates of sulphur depositions occurred previously (Likens et al. 1996, 1998, Bouchard 1997, Lawrence et al. 1999, Castro & Morgan 2000, Driscoll et al. 2001, Tessier et al. 2002). If such cation depletion continues, it will represent another serious threat to aquatic ecosystems. In addition, acidification of aquatic ecosystems is now reported across other large areas of the world, such as China (Thorjørn et al. 1999, Tang et al. 2001) and India (Aggarwal et al. 2001). Therefore, it appears that acidification of soils and water is an important problem presently and will remain so in the future.

In order to evaluate the degree of acidification and the long-term trends and variation in aquatic chemistry and aquatic biota, monitoring programmes have been in place for several decades in Europe and North

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America. For example, the International Cooperative Program on Assessment and Monitoring of Acidification of Rivers and Lakes (ICP Waters) was established in 1985 under the UN/ECE (United Nations Economic Commission for Europe). Presently, 23 countries in Europe and North America participate in this programme. Although chemical trends have been well documented at numerous sites, the effects of acidification (or recovery) on aquatic biota have been largely neglected, except for reporting the presence/absence of benthic invertebrates (index 1: NIVA 1994).

The use of acid-sensitive indicators to monitor acidification has been a useful tool (Raddum 1999), but they provide limited information about how organisms respond to and recover from environmental stressors. A more powerful and relevant approach is a combination of bioindicators at the population/community level, together with rapidly responding exposure biomarkers, such as alterations in haemolymph chemistry. By using an integrated approach we can, not only better assess the link between environmental change and observed biological effects (Adams & Greeley 2000), but also determine streamwater quality (combination of several chemical parameters), compare acidification levels of several brooks and then monitor the acidification state (during the year or with the passing of years) and evolution (degradation or recovery). In the same way, we could evaluate if the water quality allows *Gammarus fossarum* and other sensitive species to settle.

One of the most striking consequences of freshwater acidification is the erosion of biodiversity (Sutcliffe & Carrick 1973, Leivestad & Muniz 1976, Dillon et al. 1984, Brown & Sadler 1989, Muniz 1991). Numerous studies have shown clear evidence of a failure to regulate blood or haemolymph Na^+ and Cl^- levels in acid-stressed fish, clams (Unionidae) and decapods (Massabuau 1985, McMahon & Stuart 1989, Pynnönen 1991, Gonzales et al. 1997). However, most of the studies have focused on large species, and relatively little is known about physiological responses in smaller acid-sensitive species of macroinvertebrates.

Crustaceans are among the most acid-sensitive macroinvertebrate species (Sutcliffe & Carrick 1973, Guerold et al. 2000). In a previous laboratory study (Felten & Guerold 2001), we showed that *Gammarus fossarum* (Crustacea: Amphipoda), a common acid-sensitive species in west Palearctica, also sustained a severe depletion of haemolymph Na^+ and Cl^- ions when exposed to realistic acidic conditions.

The purpose of the present study was to investigate whether haemolymph ion concentrations in the acid-sensitive species *Gammarus fossarum* were effective biomarkers for monitoring the acidification of running waters. Moreover, this experiment was conducted to determine whether there was a relationship between a describing level of acidification and haemolymph ion losses. In this context we assessed *in situ* the short-term response of haemolymph [Na^+] and [Cl^-] in *G. fossarum* transferred to 18 headwater streams providing a wide range of acidification levels (defined by: pH, acid-neutralising capacity (ANC), [Mg^{2+}], [Ca^{2+}] and [Al_{tot}]).

MATERIALS AND METHODS

Study organisms. Experiments were performed on *Gammarus fossarum* (Crustacea: Amphipoda) because this species presents several interesting characteristics for ecotoxicological investigations: it is (1) an acid-sensitive species (Guerold et al. 2000, Felten & Guerold 2001), (2) widespread and common in Paleartica (Barnard & Barnard 1983), (3) often occurs in high density, (4) easy to identify to species level, (5) characterised by sexual dimorphism and (6) plays a major role in the leaf breakdown process and consequently in the entire food web (Pöckl 1995).

Experimental design. The study was conducted in the Vosges Mountains (northeastern France). Since substantial chemical differences between streams draining sandstone and granite were shown in a previous study (Guerold et al. 1997), 2 separate experiments were carried out during late spring in order to distinguish potentially different streamwater toxicity levels. Each experiment was conducted in 9 headwater streams providing exposure to a range of acidification levels (from near neutral to strongly acidified). The first experiment included a set of 9 streams draining granite bedrock: Meurthe (ME), Tihay (TI), Haut Rupt (HR), Moyen Rupt (MR), Grand Clos (GC), Rouge Rupt (RR), Bas Rupt (BR), Longfoigneux (LO) and Morbieux (MO). The second experiment included a set of 9 streams draining sandstone bedrock: La Maix (LM), Plaine (PL), Truite (TR), Madeleine (MA), Repafosse (RE), Ravine (RA), Chevrosgoutte (CH), Grand Bras (GB) and Gentil Sapin (GS).

For each type of substratum, 1620 males of *Gammarus fossarum* with 8 to 10 mm body lengths were collected from neutral streams, the Meurthe stream for granite and the La Maix stream for sandstone.

The experimental design we used is shown in Fig. 1. Briefly, 180 organisms were placed in 2 Plexiglas flow-through enclosures each consisting of 18 compartments each with 5 organisms. The enclosures were then transferred to each of the 18 streams (9 on granite and 9 on sandstone) including the neutral streams (ME and LM). For each stream, one enclosure was used for haemolymph analyses and the other for the assessment of survival. In order to evaluate the initial concentrations of haemolymph Cl^- and Na^+ in *Gammarus fos-*

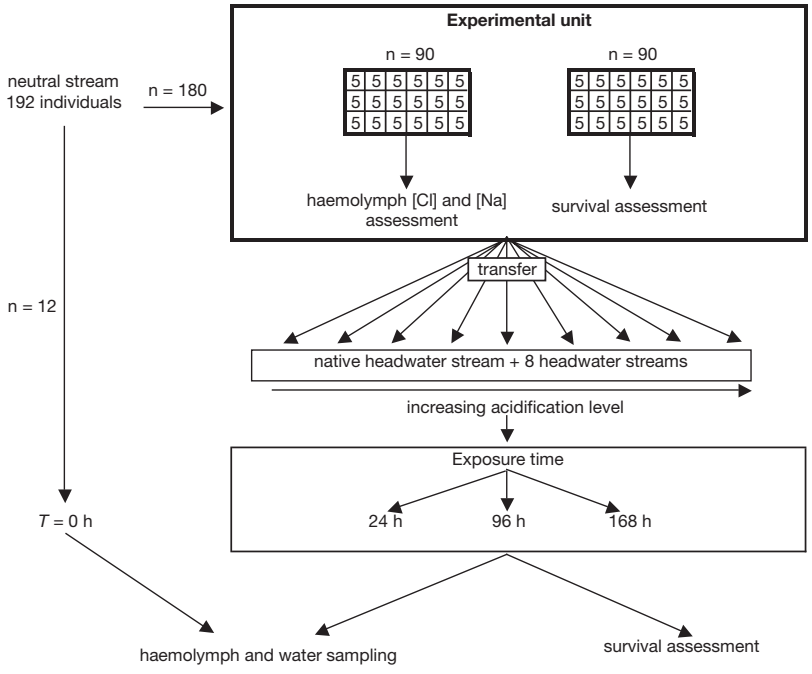

Fig. 1. Experimental design used for each experiment (i.e. each type of bedrock)

sarum, 12 organisms were sampled in each neutral stream just before the onset of the experiments (control). At 24, 96 and 168 h after transfer, samples of haemolymph from 10 organisms were randomly collected in each stream for analysis.

Survival, haemolymph sampling and analysis. The survival was assessed in each stream for each exposure time (18 replicates of 5 organisms). Samples of haemolymph (0.8 to 1.2 µl) were taken from the telson of each individual (n = 10) using a microsyringe, transferred to a gauged 5 µl microcapillary tube and centrifuged for 10 min at 6596 × *g*. After centrifugation the liquid phase was diluted in 2 ml of Nanopur water to determine chloride and sodium concentrations in haemolymph by ionic chromatography (Dionex 4500i with Ion Pac AS4A column) and atomic absorption spectrophotometry (AAS) (Perkin Elmer Analyst 100), respectively.

Water analysis. Water was collected at the initiation of the experiment (T_0) and at each exposure time (24, 96 and 168 h). Cations were analysed by flame AAS and anions by ionic chromatography as described previously. Total aluminium was determined by graphite furnace AAS (Varian Spectraa 300) after acidification with 0.25% HNO_3. ANC was measured by Gran's titration and pH (glass electrode), and conductivity with multi-parametric equipment (WTW). Chemical characteristics of water from each stream are given in Table 1 for granite bedrock stream and in Table 2 for streams draining sandstone bedrock.

Statistical analysis. All data are reported as mean ± SD. We used a 2-way analysis of variance (ANOVA) to detect the effect of Stream, Exposure Time and the interaction of the 2 factors (Stream × Exposure Time) on haemolymph parameters and survival. For each exposure time, parametric tests (Student's *t*-test) were used to test for differences in mean chloride and sodium haemolymph concentrations between control (T_0) and transferred organisms. In order to determine the parameters most relevant in describing the effect of acidic stress (survival, ion loss), correlation analysis between water parameters, haemolymph concentrations, survival rates (Pearson correlation coefficient) and multiple regressions (not presented here) were carried out. Linear regressions were performed in order to establish a

Table 1. Mean (±SD) values (n = 4) of various chemicals of each exposure stream draining granite bedrock (see 'Materials and methods' for site abbreviations)

	ME	TI	HR	MR	GC	RR	BR	LO	MO
pH	6.23 (0.02)	6.51 (0.04)	6.26 (0.12)	5.81 (0.20)	5.71 (0.16)	5.16 (0.10)	5.15 (0.35)	5.03 (0.10)	4.71 (0.06)
Temperature (°C)	6.3 (0.4)	11.8 (1.7)	7.6 (0.6)	7.7 (0.8)	10.1 (1.1)	9.0 (9.0)	7.9 (0.9)	8.8 (0.8)	10.1 (1.3)
Conductivity (µS cm^{-1})	56.3 (2.6)	26.6 (1.9)	18.0 (0.7)	13.9 (0.3)	13.8 (0.5)	10.9 (0.3)	12.5 (0.4)	12.0 (0.4)	13.1 (0.5)
ANC (µmol l^{-1})	102.8 (5.9)	132.7 (25.0)	87.8 (8.9)	30.1 (3.4)	23.4 (4.3)	−0.6 (2.8)	3.6 (3.9)	−2.5 (4.2)	−9.9 (2.1)
Ca (µmol l^{-1})	84.8 (1.5)	50.1 (2.7)	39.3 (2.3)	28.3 (1.2)	26.8 (1.8)	14.7 (1.0)	19.5 (3.1)	12.8 (0.4)	12.4 (1.5)
Mg (µmol l^{-1})	49.1 (0.8)	38.6 (4.5)	30.7 (1.2)	19.6 (0.2)	14.4 (0.3)	6.5 (0.2)	13.7 (0.2)	7.1 (0.4)	4.9 (0.3)
Na (µmol l^{-1})	277.7 (10.3)	97.1 (9.1)	57.2 (2.4)	45.7 (0.9)	44.7 (0.9)	43.0 (0.9)	38.7 (1.2)	45.8 (1.2)	43.6 (1.1)
K (µmol l^{-1})	8.3 (0.8)	8.8 (0.8)	10.0 (0.4)	6.0 (0.3)	7.4 (0.8)	5.5 (4.2)	3.8 (0.2)	2.6 (0.4)	3.2 (0.3)
SO_4 (µmol l^{-1})	36.2 (0.2)	30.1 (1.1)	23.9 (1.5)	29.5 (1.6)	33.0 (0.6)	25.4 (1.4)	30.8 (1.3)	30.5 (0.4)	26.4 (1.4)
NO_3 (µmol l^{-1})	25.7 (0.2)	16.9 (1.9)	6.2 (0.5)	9.0 (0.9)	11.1 (0.7)	5.6 (0.7)	8.7 (0.5)	11.2 (2.3)	11.0 (1.6)
Cl (µmol l^{-1})	27.5 (7.0)	54.2 (3.7)	26.5 (1.3)	17.3 (0.8)	17.3 (0.6)	16.2 (0.8)	17.0 (0.3)	18.1 (0.8)	16.6 (0.7)
Total Al (µmol l^{-1})	0.3 (0.0)	1.5 (0.7)	4.0 (1.0)	6.3 (1.5)	2.5 (0.5)	8.3 (0.7)	9.2 (1.8)	9.8 (1.2)	13.5 (0.1)

Table 2. Mean (±SD) values (n = 4) of various chemicals of each exposure stream draining sandstone bedrock (see 'Materials and methods' for site abbreviations)

	LM	PL	TR	MA	RE	RA	CH	GB	GS
pH	7.22 (0.11)	6.90 (0.14)	6.90 (0.09)	6.00 (0.18)	5.50 (0.14)	5.50 (0.09)	4.91 (0.10)	4.71 (0.13)	4.53 (0.03)
Temperature(°C)	10.2 (0.4)	13.6 (0.9)	12.0 (0.6)	12.6 (0.7)	13.0 (0.8)	11.6 (0.5)	11.3 (0.3)	11.4 (0.7)	12.9 (0.6)
Conductivity (µS cm^{-1})	75.6 (0.8)	67.3 (0.5)	45.9 (1.7)	24.7 (0.5)	31.0 (0.4)	31.8 (0.3)	44.9 (0.6)	25.9 (0.9)	28.1 (0.6)
ANC (µmol l^{-1})	586.2 (4.4)	286.2 (4.8)	282.6 (3.9)	51.0 (8.2)	−0.3 (5.8)	3.1 (2.6)	−3.8 (5.4)	−16.1 (8.5)	−14.3 (7.0)
Ca (µmol l^{-1})	260.8 (0.6)	160.6 (3.2)	129.1 (2.3)	52.3 (0.6)	52.6 (0.9)	56.5 (3.3)	82.5 (0.6)	40.9 (0.7)	36.9 (0.2)
Mg (µmol l^{-1})	172.8 (1.1)	99.8 (1.4)	87.4 (2.0)	30.1 (0.4)	38.1 (0.8)	36.0 (0.5)	55.7 (0.5)	24.9 (0.4)	25.7 (0.5)
Na (µmol l^{-1})	45.9 (0.8)	176.7 (1.8)	40.4 (0.0)	34.8 (0.4)	52.2 (0.4)	51.2 (1.1)	66.2 (0.7)	33.0 (0.0)	34.6 (2.5)
K (µmol l^{-1})	42.4 (0.2)	39.1 (0.4)	41.4 (0.7)	34.6 (0.7)	39.0 (0.4)	42.3 (1.0)	50.3 (0.6)	28.8 (0.8)	26.4 (0.2)
SO_4 (µmol l^{-1})	65.9 (0.4)	63.2 (1.1)	46.6 (0.5)	38.3 (0.2)	83.9 (0.5)	78.6 (0.4)	126.4 (0.7)	55.9 (0.5)	54.2 (0.4)
NO_3 (µmol l^{-1})	53.5 (3.5)	67.6 (2.1)	40.4 (2.1)	50.8 (3.4)	33.6 (0.4)	51.7 (0.7)	70.3 (1.8)	61.3 (5.6)	74.2 (1.2)
Cl (µmol l^{-1})	38.0 (0.2)	158.4 (0.9)	34.6 (0.7)	34.2 (0.2)	44.5 (1.1)	44.4 (1.2)	56.1 (1.1)	30.6 (0.7)	32.2 (0.3)
Total Al (µmol l^{-1})	1.0 (0.0)	1.2 (0.2)	1.7 (0.1)	5.6 (0.9)	4.1 (0.4)	3.1 (0.2)	9.3 (0.5)	13.5 (0.7)	16.7 (0.7)

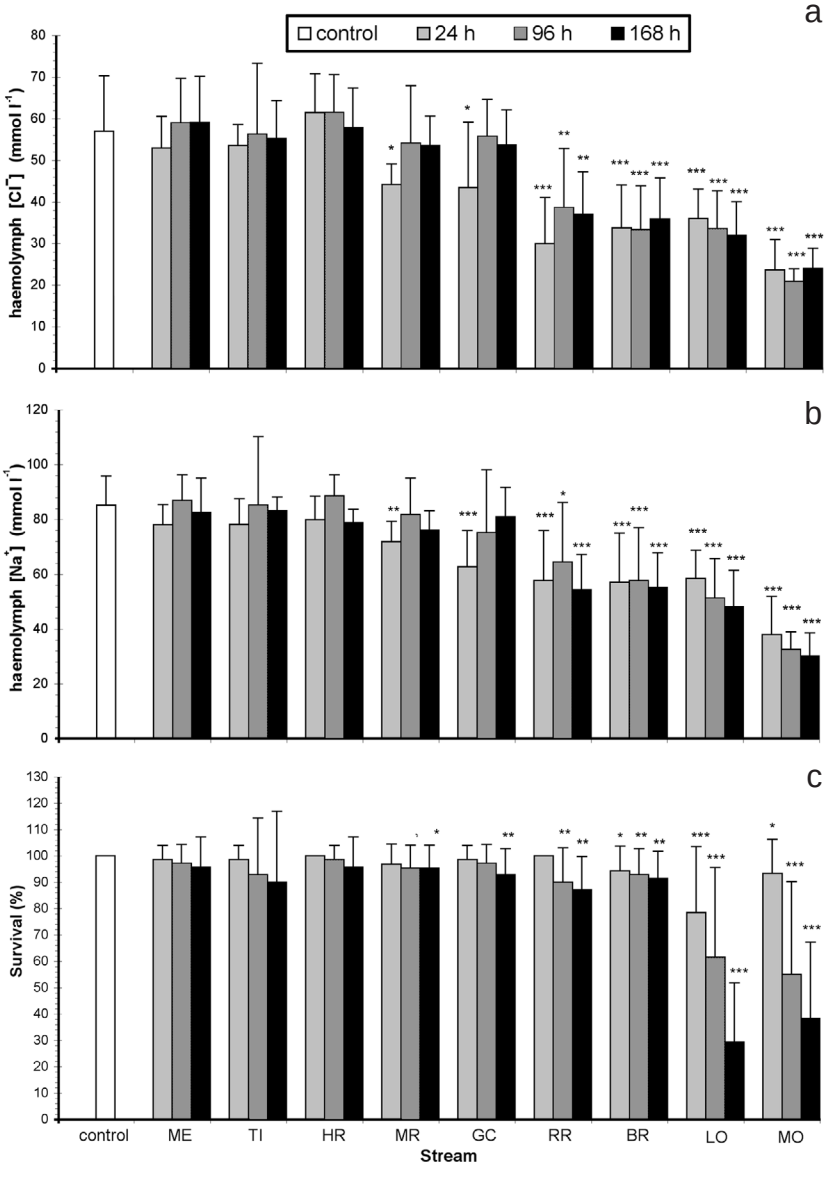

Fig. 2. *Gammarus fossarum*. Mean (±SD) (a) haemolymph [Cl^-], (b) haemolymph [Na^+] and (c) survival when exposed to granite bedrock stream. Significant differences against T_0 are indicated by asterisks (Student's *t*-test, $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$)

relationship between ion loss (haemolymph [Na^+] and [Cl^-]) and the most relevant physico-chemical parameter describing acid level. Critical values of the Pearson correlation coefficient were used to evaluate the significance of the linear regressions. The analyses were carried out using STATISTICA (Microsoft), with a probability limit of $p \leq 0.05$ considered as significant.

RESULTS

Streamwater chemistry

Acidified streams were characterized by low pH (i.e. high [H^+]), low ANC, low [Mg^{2+}] and low [Ca^{2+}] and high [Al_{tot}] (Tables 1 & 2). Streams draining granite ranged from pH 4.71 to 6.51, for [Ca^{2+}] from 12.4 to 84.8 µmol l^{-1} and for [Al_{tot}] from 0.3 to 13.5 µmol l^{-1}. Streams draining sandstone ranged from pH 4.53 to 7.22, for [Ca^{2+}] from 36.9 to 260.8 µmol l^{-1} and for [Al_{tot}] from 1.0 to 16.7 µmol l^{-1}. Note that for the same level of acidification, streams draining granite showed lower conductivity (mainly lower [Ca^{2+}], [Mg^{2+}] and [Al_{tot}]) than streams draining sandstone.

General results

To study the effect of acidification on haemolymph [Na^+] and [Cl^-], specimens of *Gammarus fossarum* were transferred from their natal non-acidified streams to streams showing different levels of acidification. The baseline levels of haemolymph [Cl^-] and [Na^+] were 57 ± 13.3 and 85.2 ± 10.7 mmol l^{-1} (mean ± SD), respectively, in control organisms collected in ME (granite bedrock, Fig. 2a,b) and 61.4 ± 6.3 and 77.5 ± 5.7 mmol l^{-1} in organisms sampled in LM (sandstone bedrock, Fig. 3a,b). For each type of bedrock, the 2-way analysis of variance (ANOVA) indicated that Stream (acidification level), Exposure Time and the interaction between them (Stream × Exposure Time) exerted a significant effect on haemolymph [Na^+] or [Cl^-] and survival (Table 3). The pH was chosen as the indicator of acidification level because it had the best correlation with haemolymph [Na^+] or [Cl^-] (for each exposure time $r \leq -0.90$) and because multiple regression analysis showed that the use of pH, alone, represented the best variance explanation (> 90%).

Table 3. Summary of 2-way analysis of variance showing the effects of stream water, exposure time and the interaction between them on mortality and haemolymph parameters. Degrees of freedom were 8 for Stream, 3 for Exposure time, and 24 for the interaction

	Stream		Exposure time		Interaction		df
	F	p	*F*	p	*F*	p	error
Sandstone							
Survival	84.1	$<10^{-3}$	199.0	$<10^{-3}$	21.2	$<10^{-3}$	495
Haemolymph [Cl^-]	39.8	$<10^{-3}$	95.7	$<10^{-3}$	6.0	$<10^{-3}$	293
Haemolymph [Na^+]	66.7	$<10^{-3}$	102.2	$<10^{-3}$	11.7	$<10^{-3}$	314
Granite							
Survival	45.6	$<10^{-3}$	64.2	$<10^{-3}$	11.6	$<10^{-3}$	492
Haemolymph [Cl^-]	31.8	$<10^{-3}$	33.1	$<10^{-3}$	4.4	$<10^{-3}$	320
Haemolymph [Na^+]	40.0	$<10^{-3}$	52.9	$<10^{-3}$	5.7	$<10^{-3}$	320

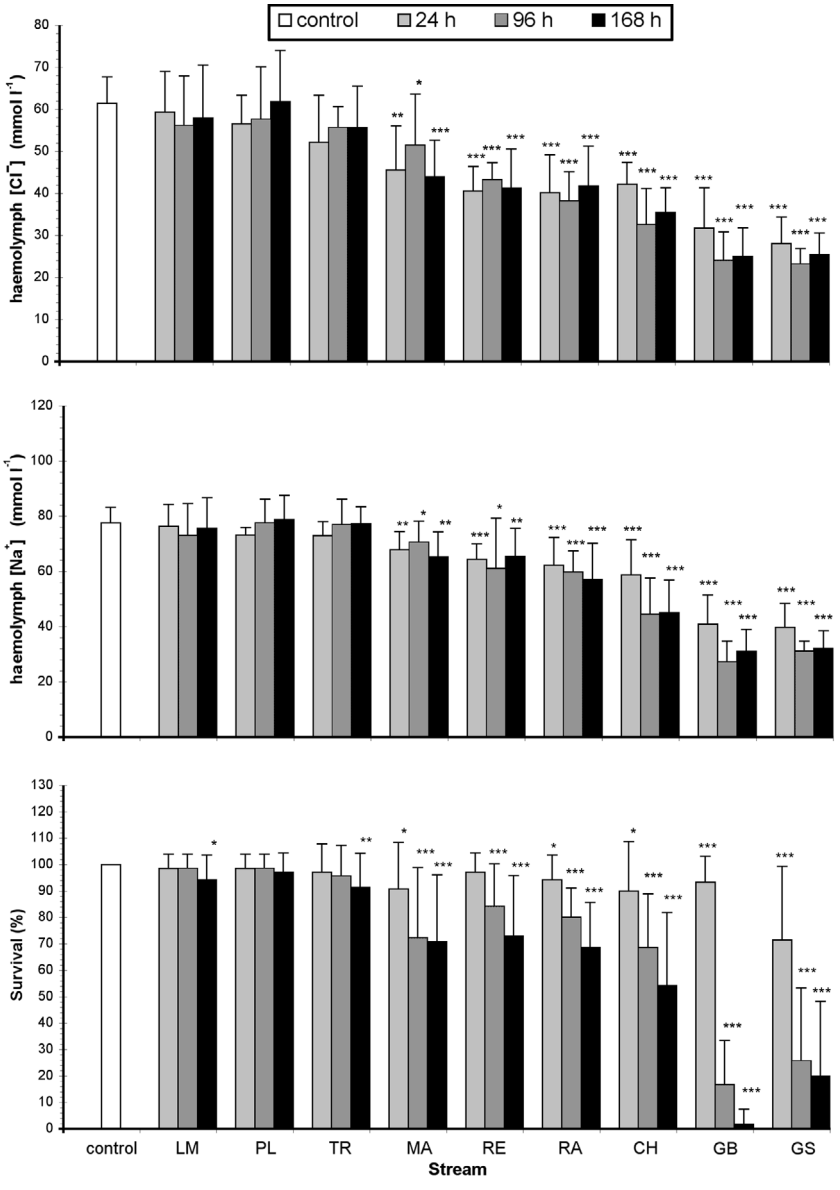

Fig. 3. *Gammarus fossarum*. Mean (±SD) (a) haemolymph [Cl^-], (b) haemolymph [Na^+] and (c) survival when exposed to sandstone bedrock stream. Significant differences against T_0 are indicated by asterisks (Student's *t*-test, $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$)

Granite bedrock

Haemolymph [Na^+] and [Cl^-] in *Gammarus fossarum* exposed to circumneutral streams (ME, TI, HR; pH ≥ 6.23) remained constant over a 168 h exposure period, but significantly decreased in organisms exposed to slightly acidic and acidic streams (pH ≤ 5.81) during the first 24 h (Fig. 2a, b). Indeed, after 24 h of exposure, the loss of haemolymph Cl^- ranged from 22.5% in a slightly acidic stream (MR, pH = 5.81, mean haemo-

lymph [Cl^-] = 44.2 ± 4.9 mmol l^{-1}) to 58.4% in the most acidic one (MO, pH = 4.71, mean haemolymph [Cl^-] = 23.7 ± 7.2 mmol l^{-1}) in comparison to the control (ME, pH = 6.23, mean haemolymph [Cl^-] = 57 ± 13.3 mmol l^{-1}) (Fig. 2a). The same trend was observed for haemolymph [Na^+]. After 24 h of exposure, the loss of haemolymph Na^+ ranged from 15.7% in MR (mean haemolymph [Na^+] = 71.9 ± 7.4 mmol l^{-1}) to 55.5% in MO (mean haemolymph [Na^+] = 37.9 ± 13.9 mmol l^{-1}) in comparison to the control (ME, mean haemolymph [Na^+] = 85.2 ± 10.7 mmol l^{-1}) (Fig. 2b).

After 96 h of exposure, the haemolymph [Na^+] and [Cl^-] of the organisms exposed to slightly acidic streams (MR pH 5.81 and GC pH 5.71) returned to near-control values (T_0) and remained constant until the end of the experiment. For example, individuals transferred for 96 h to MR had a haemolymph [Cl^-] of 54.1 ± 13.8 mmol l^{-1} and [Na^+] of 81.8 ± 13.2 mmol l^{-1}, representing a chloride loss of only 5% and a sodium loss of 4% ($p > 0.05$; not significant). Thus, recovery of haemolymph [Na^+] and [Cl^-] took place between 24 and 96 h of exposure in organisms transferred to slightly acidic streams. On the contrary, the loss of haemolymph Na^+ and Cl^- remained constant or increased in organisms exposed to strongly acidic streams characterised by a pH ≤ 5.16.

For each exposure time, the survival rate was >92.9% for *Gammarus fossarum* exposed to streams with a mean pH ≥ 5.71 (GC, MR, HR, TI, ME). Concomitantly to the haemolymph ion loss in organisms exposed to strongly acidic waters (pH ≤ 5.16), the survival rate significantly decreased, reaching 38.3% after 168 h of exposure in the most acidic stream (MO).

The loss of haemolymph Na^+ and Cl^- was highly correlated with the mean pH for each exposure time (Table 4: $r^2 \geq 0.872$, $p < 0.001$).

Sandstone bedrock

The haemolymph [Na^+] and [Cl^-] in *Gammarus fossarum* exposed in circumneutral streams (LM, PL, TR: pH ≥ 6.90) remained constant over a 168 h exposure period, but significantly decreased in organisms exposed to slightly acidic and acidic waters (pH ≤ 6.00) during the first 24 h (Fig. 3a,b). After 24 h exposure time, the loss of haemolymph Cl^- ranged from 25.7% in the slightly acidic stream (MA, pH = 6.00, mean haemolymph [Cl^-] = 45.6 ± 10.5 mmol l^{-1}) to 54.3% in the most acidic one (GS, pH = 4.53, mean haemolymph [Cl^-] = 28 ± 6.3 mmol l^{-1}) in comparison to the control (LM, pH = 7.22, mean haemolymph [Cl^-] = 61.4 ± 6.3 mmol l^{-1}) (Fig. 3a). The same tendency was observed for haemolymph [Na^+]. After 24 h of exposure, the loss of haemolymph Na^+ ranged from 12.5% in MA (mean haemolymph [Na^+] = 67.8 ± 6.6 mmol l^{-1}) to 48.8% in GS (mean haemolymph [Na^+] = 39.7 ± 8.6 mmol l^{-1}) in comparison to the control (LM, mean haemolymph [Na^+] = 77.5 ± 5.7 mmol l^{-1}) (Fig. 3b).

At each exposure time, mean haemolymph Cl^- and Na^+ concentrations in individuals exposed to strongly acidic or slightly acidic water (pH ≤ 6.00) were significantly different from mean control values. With exposure time, loss of haemolymph Na^+ and Cl^- increased or remained constant for organisms exposed to strongly acidic water (pH ≤ 6.00).

At each exposure time, a survival rate >91.4% was observed for the organisms exposed to streams characterised by pH ≥ 6.23 (LM, PL, TR). The haemolymph ion loss in organisms exposed to acidic waters (pH ≤ 6.00), correlated with significant decreases in survival rate, reaching 20% in the most acidic stream (GS) after 168 h of exposure.

Table 4. *Gammarus fossarum*. Linear regression equation between acid level (pH) and haemolymph [Cl^-], haemolymph [Na^+] in streams draining granite and sandstone bedrock. Significance of the regression is indicated by asterisk (Pearson test, ns: not significant; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$)

	Granite bedrock		Sandstone bedrock	
	Haemolymph [Cl^-]	Haemolymph [Na^+]	Haemolymph [Cl^-]	Haemolymph [Na^+]
T_{24}				
Slope	18.414	20.402	9.9972	12.051
Ordinate	−61.325	−49.934	−13.934	−8.0984
r^2	0.8989***	0.8931***	0.9173***	0.8304***
T_{96}				
Slope	21.322	28.45	12.956	17.535
Ordinate	−73.862	−90.489	−32.635	−43.675
r^2	0.8787***	0.8854***	0.9239***	0.848***
T_{168}				
Slope	19.546	27.963	12.828	17.286
Ordinate	−64.415	−91.585	−31.231	−41.607
r^2	0.8989***	0.8716***	0.9451***	0.8844***

As observed in the granite bedrock experiment, the loss of haemolymph Na^+ and Cl^- was highly correlated with the mean pH for each exposure time (Table 4: $r^2 \geq 0.830$, $p < 0.001$).

DISCUSSION

Several studies have clearly shown that crustaceans exposed to water-borne pollutants, environmental stressors and pathological agents usually exhibit disruption of ionic regulation (Lignot et al. 2000). Different causes include alterations in the structure and ultrastructure of the branchial and excretory organs, and changes in Na^+, K^+-ATPase activity, ionic fluxes and surface permeability (Lignot et al. 2000). Ion-regulation failure leading to a severe deficiency of extracellular ions (i.e. Na^+ and Cl^-) has been recognised to be the major response of fish to acid stress (McDonald et al. 1989, Potts & McWilliams 1989, Wood 1989, Gonzales et al. 1997). Similar results have been reported in crayfish (Appelberg 1985, Fjeld et al. 1988, McMahon & Stuart 1989, Jensen & Malte 1990), gammarids (Rupprecht 1992) and molluscs (Pynnönen 1991). Surprisingly, and despite the numerous papers reporting detrimental effects of acidification on invertebrate communities, few studies have been performed on the ecophysiology of smaller acid-sensitive macroinvertebrate species.

Although some studies have clearly shown a depletion of ion concentrations in invertebrate exposed to acidic waters, most of these studies were performed on moderately acid-sensitive species (*Corixia dentipes*, *C. punctata*: Vangenechten et al. 1989; *Cenocorixia blaisdelli*: Needham 1990; *Libellula julia*: Rockwood & Coler 1991) and/or on organisms exposed to non-environmentally relevant concentrations of aluminium (Rockwood & Cooler 1991). Similar results have also been obtained with pooled samples of haemolymph (Needham 1990) or from whole body analyses (Hermann 1987, Havens 1992).

In the present study, we demonstrated that the exposure of *Gammarus fossarum* to strongly acidic or slightly acidic water caused significant losses of haemolymph [Na^+] and [Cl^-] after as little as 24 h of exposure. These losses were significantly correlated to the pH. Moreover, failure in ionic regulation is accompanied by significant mortality. A significant decrease of haemolymph [Na^+] and [Cl^-] after 24 h of exposure was seen in slightly acidic streams draining granite bedrock (GC and MR); however, they did not differ significantly from control values after 96 and 168 h of exposure. Moreover, the associated survival was very high for each exposure time, showing that *G. fossarum* is able to compensate ion loss.

The process of acidification on sandstone bedrock seems to have a greater impact on the mortality of *Gammarus fossarum* and on ion loss. Moreover, no physiological adaptation was observed on sandstone bedrock: a significant ion loss at each exposure time was observed for organisms transferred to streamwater in which the pH ≤ 6.00. Two hypotheses may explain these differences: (1) the greater toxicity of sandstone draining streamwater (due to the greater [Al_{tot}]), (2) the difference in sensitivity of the 2 gammarid populations (the pH in granite control streamwater, ME, was 6.23, whereas the sandstone value was 7.22). Although the second hypothesis seems to be the most relevant, it would be necessary to perform another experiment in order to compare the physiological responses of 2 or more gammarid populations (sampling in different streamwater qualities, i.e. pH) in order to confirm these trends.

This study confirms previous results obtained in the laboratory (Felten & Guerold 2001). Since the effect of acid stress on the ecophysiology of *Gammarus fossarum* was detectable at sublethal levels, it may be possible to use haemolymph [Na^+] and [Cl^-] to monitor acidification pollution (Handy & Depledge 1999, Lignot et al. 2000). Moreover, the sensitivity and the rapid response (24 h) of these physiological markers are interesting criteria to monitor the evolution of freshwater acidification and base cation depletion in response to the reduction of atmospheric emissions. Thus, the use of these markers could permit us to better assess streamwater toxicity (combination of several chemical parameters: pH, ANC, [Mg^{2+}], [Ca^{2+}] and [Al_{tot}]), compare acidification levels of several brooks and to evaluate if water quality is sufficient to allow *G. fossarum* and even other less sensitive species to settle (within the framework of a possible reintroduction). Consequently, such physiological markers should be useful diagnostic tools for programmes aiming to assess both acidification and recovery from acidification. A multiparametric approach, including biotic indices and chemical variables, would enhance the power of monitoring programmes. These biomarkers would be of particular interest in studying ecosystems chemically recovering from acidification, since recolonisation by macroinvertebrates is a very slow process (Soulsby et al. 1997, Bradley & Ormerod 2002), meaning that indices examined on a community level fail to reveal the biogenic capacity of such aquatic ecosystems.

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