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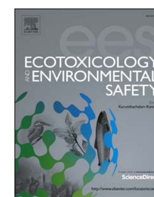

Combined effect of temperature and ammonia on molecular response and survival of the freshwater crustacean *Gammarus pulex*

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ABSTRACT

Freshwater ecosystems are experiencing mounting pressures from agriculture, urbanization, and climate change, which could drastically impair aquatic biodiversity. As nutrient inputs increase and temperatures rise, ammonia (NH_3) concentration is likely to be associated with stressful temperatures. To investigate the interaction between NH_3 and temperature on aquatic invertebrate survival, we performed a factorial experiment on the survival and molecular response of *Gammarus pulex*, with temperature (10, 15, 20, and 25 °C) and NH_3 (0, 0.5, 1, 2, 3, and 4 mg NH_3/L) treatments. We observed an unexpected antagonistic interaction between temperature and NH_3 concentration, meaning survival in the 4 mg NH_3/L treatment was higher at 25 °C than at the control temperature of 10 °C. A toxicokinetic-toxicodynamic (TK-TD) model was built to describe this antagonistic interaction. While the No Effect Concentration showed no significant variation across temperatures, the 50% lethal concentration at the end of the experiment increased from 2.7 (2.1–3.6) at 10 °C to 5.5 (3.5- 23.4) mg NH_3/L at 25 °C. Based on qPCR data, we associated these survival patterns to variations in the expression of the *hsp70* gene, a generic biomarker of stress. However, though there was a 14-fold increase in *hsp70* mRNA expression for gammarids exposed to 25 °C compared to controls, NH_3 concentration had no effect on *hsp70* mRNA synthesis across temperatures. Our results demonstrate that the effects of combined environmental stressors, like temperature and NH_3, may strongly differ from simple additive effects, and that stress response to temperature can actually increase resilience to nutrient pollution in some circumstances.

1. Introduction

Consequences of global change are often considered independently as isolated drivers of biodiversity loss (Chapin et al., 2010; Loreau et al., 2001; Steudel et al., 2012). In natural ecosystems, multiple environmental forces interact, leading to multi-stress situations (Dehedin et al., 2013a; Travis, 2003). Despite the importance of considering these combined effects (Dehedin et al., 2013a, 2013b; Didham et al., 2007; Dukes and Mooney, 1999; Heino et al., 2009; Walther et al., 2002), synergisms and interactions between multiple stressors are difficult to conceptualize and quantify, and are often overlooked in ecological studies. Individual treatment of multi-dimensional stressors introduces uncertainty in predictive models for species distribution patterns (Chapin et al., 2000; Seneviratne et al., 2006).

Ammonia is a common anthropogenic pollutant in stream ecosystems (Alonso and Camargo, 2004; Piscart et al., 2009; Prenter et al., 2004). The most common sources of ammonia inputs include urban and agricultural runoff, industrial activity, and mismanaged waste water (Jeppesen et al., 2009; Maltby, 1995; Piscart et al., 2009; Wagner and Benndorf, 2007). While background concentration of ammonia is usually low in the environment, it may rise locally and periodically (Alonso and Camargo, 2015; Maltby, 1995) due to precipitation events or waste water runoff (Seager and Maltby, 1989). High water temperature can aggravate ammonia pollution because the decreased dissolved oxygen concentration associated with warmer water can impede nitrification and promote reduction of nitrate to ammonia by microorganisms, increasing ammonia concentration, particularly when nitrate concentration is high (Jensen et al., 1994; Navel et al., 2013).

Ammonium (NH_4^+), is typically inert in aquatic environments, whereas the un-ionized form, the ammonia (NH_3), is highly toxic (Alonso and Camargo, 2004). Ammonia induces severe stress on cells by disrupting respiratory metabolism and membrane Na^+/K^+-ATPase activity, impairing organism survival, activity and growth (Dehedin et al., 2013a; Li et al., 2014; Mummert et al., 2003; Naqvi et al., 2007; Prenter et al., 2004). Environmental factors such as water temperature and pH determine the equilibrium between ammonium and ammonia, with warm and alkaline water strongly favoring NH_3 (*e.g.* at neutral pH, an increase from 10 °C to 20 °C will approximately double the

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concentration of NH_3) (Emerson et al., 1975). While multiple consequences of ammonia on biodiversity have been described (Piscart et al., 2009; Williams et al., 1985), its physiological effects in combination with other environmental stressors have rarely been assessed (Maltby, 1995). In a context of global warming, exogenous and endogenous ammonia inputs as well as NH_4^+/NH_3 equilibrium shifts may become much more common, potentially enhancing deleterious environmental effects from ammonia in the future (Dehedin et al., 2013b; Handy, 1994).

The present experimental study was conducted on the species *Gammarus pulex* (L. 1758, Amphipoda: Gammaridae). This species plays a central role in leaf litter decomposition in streams (Piscart et al., 2009, 2011a, 2011b), and population crashes could trigger cascades through the whole trophic network. Gammarids are sensitive to both NH_3 concentration (Piscart et al., 2009) and temperature (Cottin et al., 2015; Foucreau et al., 2014) and are therefore good biological models to assess interactions between these parameters. Our study quantified gammarids survival under continuous exposure to temperature and ammonia, alone and in combination. Additionally, we measured expression of the *hsp70* gene to assess the molecular response to temperature and ammonia stressors. Several studies have demonstrated up-regulation of *hsp70* in response to a wide array of stressors, including thermal and NH_3 stress, in a variety of arthropod taxa (Feder and Hofmann, 1999; Sung et al., 2014). Therefore, we expected some response of *hsp70* transcript expression to isolated stressors and their combination.

The combination of multiple stressors can result in various interactions. Folt et al. (1999) and more recently Côté et al. (2016) defined these patterns in relation to the neutral additive interaction in which the effect of multiple stress is equal to the sum of each isolated stress. Therefore, any effect stronger than the one predicted using the additive hypothesis is as a synergism, while any lesser response is an antagonism. We predicted (i) an additive or a synergistic effect from high temperature and NH_3 concentrations on the survival of *G. pulex* and (ii) an up-regulation of the *hsp70* gene in response to both temperature and NH_3 stress, as well as a synergistic interaction between these stressors.

2. Material and methods

2.1. Organism sampling and rearing

Adult gammarids were manually harvested in a stream (47°32′27″N, 2°3′25″W, Sévérac, France) between February and March 2015. Stream water temperature at the end of the sampling campaign was 9 °C, pH was 6.8, and dissolved O_2 concentration 11.8 mg/L (was 100% saturation). The stream's surrounding was wooded and did not have intensive agricultural activity. Adult gammarids were stored 24 h in a climate chamber (Percival, CLF PlantClimatics, Germany) set at 15 °C, with a 12 h:12 h day/night cycle and with continuously oxygenated water collected from the stream. They were then transferred into plastic boxes containing synthetic freshwater (96 mg/L $NaHCO_3$, 60 mg/L $CaSO_4$, 60 mg/L $MgSO_4$, and 4 mg/L KCl in deionized water) with pH buffered at 7 according to the US EPA method (Anon, 1991). Gammarids were left to acclimate for 5 days in this water at 15 °C with a 12 h:12 h day/night cycle and with *ad libitum* industrial food for shrimp (Novo Prawn, JBL, Neuhofen, Germany). We performed this acclimation process to standardize the abiotic factors before exposing gammarids to stressful conditions.

2.2. Combined exposure to NH_3 and temperature, and measures of survival

The experiment was performed in open glass petri dishes (Ø 15 cm) filled with 350 mL of synthetic water. Four temperatures were selected to be comparable with previous studies on gammarids: 10, 15, 20 and 25 °C (Cottin et al., 2012, 2015; Foucreau et al., 2014). This range includes optimal (10 °, 15 °C), mildly stressful (20 °C), and strongly stressful (25 °C) temperatures. These thermal conditions where crossed with six nominal NH_3 concentrations (0, 0.5, 1, 2, 3 and 4 mg NH_3/L) in a full factorial experimental design, leading to a total of 24 experimental conditions. The NH_3 concentrations were selected after several pretests, allowing us to adjust the treatments used by Dehedin et al. (2013b), to get a range of mortality going from 0 to at least 90% for the strongest dose at the end of the experiment. NH_3 concentration was increased by adding ammonium chloride (NH_4Cl), taking into account the influence of temperature and pH on the chemical equilibrium NH_3/NH_4^+ (Emerson et al., 1975). Each treatment was applied to 10 randomly selected adult gammarids, replicated three times and maintained 196 h under experimental conditions. Mortality was checked at least twice a day, dead individuals were counted and then removed. Water was renewed on a daily basis in order to limit any marginal decrease in NH_3 concentration due to oxidation or volatilization, therefore ensuring stable and continuous experimental conditions.

2.3. Combined exposure to NH_3 and temperature, and hsp70 expression measurement

For measurements of *hsp70* mRNA expression, gammarids were acclimated as previously described (15 °C, 5 d) and then exposed to four temperatures (10, 15, 20 and 25 °C) crossed with three NH_3 concentrations (0, 1 and 4 mg NH_3/L), resulting in a total of 12 experimental conditions. Gammarids were exposed to each experimental condition for 6 or 24 h. We looked at *hsp70* mRNA expression after 6 and 24 h to get an estimate of *hsp70* expression after short term exposure (mimicking pollutant spikes) and after longer exposure. We did not perform longer exposures to avoid sampling after the onset of mortality, which could bias the sampling in favor of tolerant individuals. Three replicates of three pooled gammarids from each experimental condition and each exposure duration were flash-frozen in liquid nitrogen and stored at −80 °C for subsequent rt-qPCR analyses.

2.4. RNA extraction and cDNA preparation

For each treatment combination, pools of three gammarids were ground in liquid nitrogen using a pestle. The RNA was extracted in 600 µL of extraction buffer (Nucleospin® kit, Macherey-Nagel, Düren, Germany) with 1% β-mercaptoethanol (Sigma-Aldrich, Saint Louis, MO, USA) and then isolated on mini-spin columns (Macherey-Nagel) following the manufacturer instructions. We thus extracted three RNA replicates for each treatment combination. The quality of RNA was checked with NanoDrop® 1000 (Thermo Fisher Scientific, Wilmington, DE, USA) and by running 1 µL on 1% agarose gel. Samples were diluted in RNase-free water in order to standardize concentrations of purified RNA. Five hundred nanograms of poly(A)$^+$ total RNA were used in the reverse transcription to complementary DNA (cDNA) using Superscript III First-Strand Synthesis System for qRT-PCR (Invitrogen™, Carlsbad, CA, USA), according to manufacturer instructions. The cDNA was diluted 10 times in DEPC-treated water and stored at −20 °C until use.

2.5. Quantitative real-time PCR

We quantified *hsp70* transcripts with rt-qPCR for all the 24 combined treatments (12 conditions x 2 exposure durations). We investigated mRNA expression of an inducible gene, *hsp70* (form 1), as well as a housekeeping reference gene *Gapdh* for *G. pulex*, as described by Cottin et al. (2015). Primer sequences used for *hsp70* gene were CCGAAGCTTACCTTGGAGGCACTG for the forward strand and GTTCGCCCCCAGTTTTCTTGTCC for the reverse strand. Primer sequences used for *Gapdh* gene were CCGAAGCTTACCTTGGAG GCACTG for the forward strand and GTTCGCCCCCAGTTTTCTTGTCC for the reverse strand. Reactions were performed in a LightCycler® 480 system (Roche™, Boulogne-Billancourt, France) with a SybrGreen I mix (Roche™) according to Colinet et al. (2010). Two technical replicates

Fig. 1. The relationship between temperature, NH_3 concentration and survivorship. Rows show the treatment NH_3 concentrations and columns the treatment temperatures. Graphs show observed numbers of survivors (dots) as a function of time superimposed to predicted numbers of survivors as represented by the median tendency (plain broken line) and the 95% credible band (shaded zones). The blue color refers to controls, the red colored gradient to increasing NH_3 concentrations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

were performed for each sample of synthesized cDNA. A post-amplification melting curve was used as described by Colinet et al. (2010) to verify the specificity of the amplification. qPCR data were analyzed using the LightCycler® 480 software ver. 1.5.1. Cycle threshold values (CT) indicate the minimum number of cycles necessary to detect the fluorescence signal. CT values can be used to compute the relative quantity of mRNA *hsp70* (denoted *Ratio* and standing for "relative expression Ratio") with Pfaffl's formula (Eq. (1)) (Pfaffl, 2001), once the CT_{target} associated with the *hsp70* gene is normalized to $CT_{reference}$ which is associated with the reference gene *Gapdh*. Expression of *hsp70* transcripts is given relative to the expression observed in control condition (10 °C and 0 mg NH_3/L NH_3 during 6 h). "*E*" corresponds to the amplification efficiency for each cycle, and was assumed to equal two.

$$Ratio = \frac{(E_{target})^{\Delta CT_{target}(control-sample)}}{(E_{reférence})^{\Delta CT_{reférence}(control-sample)}} \quad (1)$$

Relative expression ratios were analyzed in R (R development core team, 2014). Normality and *homoscedasticity* were checked with Shapiro-Wilk and Bartlett tests. A three-way ANOVA with temperature, NH_3 concentration and exposure duration as main factors was used to test for difference among *hsp70* expression rate and pairwise comparisons were computed using posthoc Tukey tests.

2.6. Toxicokinetic-toxicodynamic modelling of survival

The time-dependency of survival was described as a function of NH_3 concentration, using a toxicokinetic-toxicodynamic (TK-TD) model inspired from the general unified threshold model developed by Jager et al. (2011). NH_3 kinetics are fast, and we assumed that the concentration inside the organisms was equal to the concentration in water C_w. To model survival, we assumed there was a concentration threshold effect (denoted *NEC* and standing for "No Effect Concentration"), before which no effect on survival was detected, other than background mortality, even after prolonged exposure. We considered that all individuals had the same tolerance of NH_3. The hazard rate of an individual was assumed to increase linearly when the external concentration C_w exceeded the *NEC*, and that mortality in control treatments was constant over time, a reasonable assumption for short-term exposure.

In the end, the survival probability $S(C_w,t)$ of an organism in the presence of an external contaminant C_w at time t, and at concentrations over threshold *NEC* is given by the following equations:

$$\begin{cases} S(C_w,\ t)=e^{-h_0 t} \text{if} C_w < NEC \\ S(C_w,\ t)=e^{-(h_0+k_s(C_w-NEC))t} \text{if} C_w \geq NEC \end{cases} \quad (2)$$

In addition, the number of survivors at time *t* and concentration C_w was assumed to follow a conditional binomial distribution of parameters $N_{(C_w,t-1)}$ and $p_{(C_w,t)}=\frac{S(C_w,t)}{S(C_w,t-1)}$ (see Forfait-Dubuc et al. (2012) for details); where $N_{(C_w,t-1)}$ is the number of alive individuals at the previous time step $t-1$ and concentration C_w, and $p_{(C_w,t)}$ is the probability of an individual to be alive at time *t* knowing that it was alive at time $t-1$.

The three parameters of the survival model (Eq. (2)), namely *NEC*, the killing rate k_s and the background hazard rate h_0, were estimated in a Bayesian framework with JAGS software and the R package *rjags* (Plummer, 2003). Priors were defined as recommended by Forfait-Dubuc et al. (2012). For each model, three independent MCMC chains were run in parallel. After an initial burn-in period of 5,000 iterations, the Bayesian algorithm needed 15,000 iterations to converge, for each temperature. Convergence was checked with the Gelman and Rubin statistics (Gelman and Rubin, 1992).

Bayesian inference provides posterior probability distributions for all model parameters, from which any posterior probability distribution of a combination of these parameters can be extracted. In particular, LC_{50} estimates at any time *t*, denoted $LC_{50,t}$, can be obtained as follows:

$$LC_{50,t}=NEC+\frac{\ln\ (2)}{k_s t} \quad (3)$$

This formula implies that $LC_{50,t}$ declines gradually with time and converges at the NEC; such a property still verifies for any *x* of $LC_{x,t}$ (Jager et al., 2006; Jager, 2014).

3. Results

3.1. Toxicokinetic-toxicodynamic modelling for survival

There was generally good agreement between modeled and observed survival data for all tested NH_3 concentrations at 10, 15 and 20 °C (Fig. 1; see Fig. S1 for complementary information on the goodness-of-fit). Ninety percent of experimental data (Fig. 1, dots) were within the 95% credible bands (shaded zones) of the models. As expected, for each temperature, our model converged towards stable posterior probability distributions (Fig. S2). Effect parameters, namely NEC and k_s, were cross-correlated but not with the mortality in the control treatments h_O (Fig. S3). At all temperatures except 25 °C, increasing NH_3 concentrations had a negative effect on the number of survivors (Fig. 1, per column), indicating a typical dose-dependent effect of NH_3 as a stressor. At 25 °C, the fit was poor, and no marked effect of NH_3 concentration on mortality was detected (Fig. 1). Contrary to our prediction, the overall effect of NH_3 concentrations decreased when temperature rose, suggesting an antagonistic interaction (Fig. 2). Median values of the parameter NEC were rather stable across temperatures and NEC estimates were not significantly different (overlapping 95% credible bands; Fig. 2(a)). However, the LC_{50} at the end of the experiment increased at higher temperatures (Fig. 2(b)). Between the two extreme values of 10 and 25 °C, the estimated NH_3 LC_{50} values more than doubled from 2.7 (2.1–3.6) to 5.5 (3.5–23.4) mg NH_3/L.

3.2. Hsp70 expression

The three-way ANOVA revealed a significant effect of temperature on the expression of *hsp70* ($F_{(3,48)}=11.94$, $p < 0.001$). Posthoc Tukey tests showed that *hsp70* expression was highest in gammarids exposed to 25 °C compared to all the other conditions including the control and regardless of NH_3 concentration ($p < 0.001$). A maximum 14-fold change (± 1.3 SE) in the transcript abundance was detected in individuals coming from the 25 °C - 0 mg NH_3/L condition (Fig. 3). A significant time-temperature interaction ($F_{(3,48)}=4.37$, $p < 0.01$) indicated that high temperature did not affect *hsp70* expression equally over time, with weakened observed expression after 24 h of exposure. The ANOVA did not highlight any NH_3 effect ($F_{(2,48)}=0.93$, $p=0.40$) nor interactions including NH_3, like NH_3-temperature interaction ($F_{(6,48)}=0.84$, $p=0.54$) or NH_3-duration of exposure interaction ($F_{(2,48)}=2.96$, $p=0.06$) on the transcripts abundance.

4. Discussion

This study characterized the effect of continuous exposure of temperature and NH_3 on survival and molecular stress response of gammarids. Our full factorial design allowed quantification of interaction effects as well as individual effects. First, we observed strong individual effects of temperature and NH_3 on gammarid survival. These results are consistent with previous findings (Dehedin et al., 2013b; Maltby, 1995; McCahon et al., 1991; Prenter et al., 2004; Williams et al., 1984). However, the *G. pulex* population used in the present study was more tolerant than expected, with estimated 96 h LC_{50} equal to 3.1 (2.6–4.0) mg NH_3/L, in comparison with reported 96 h LC_{50} in the literature ranging from 1.2 to 2 mg NH_3/L (Dehedin et al., 2013b; Prenter el al., 2004; Williams et al., 1984). This difference may be due to the regional context were populations of aquatic invertebrates are

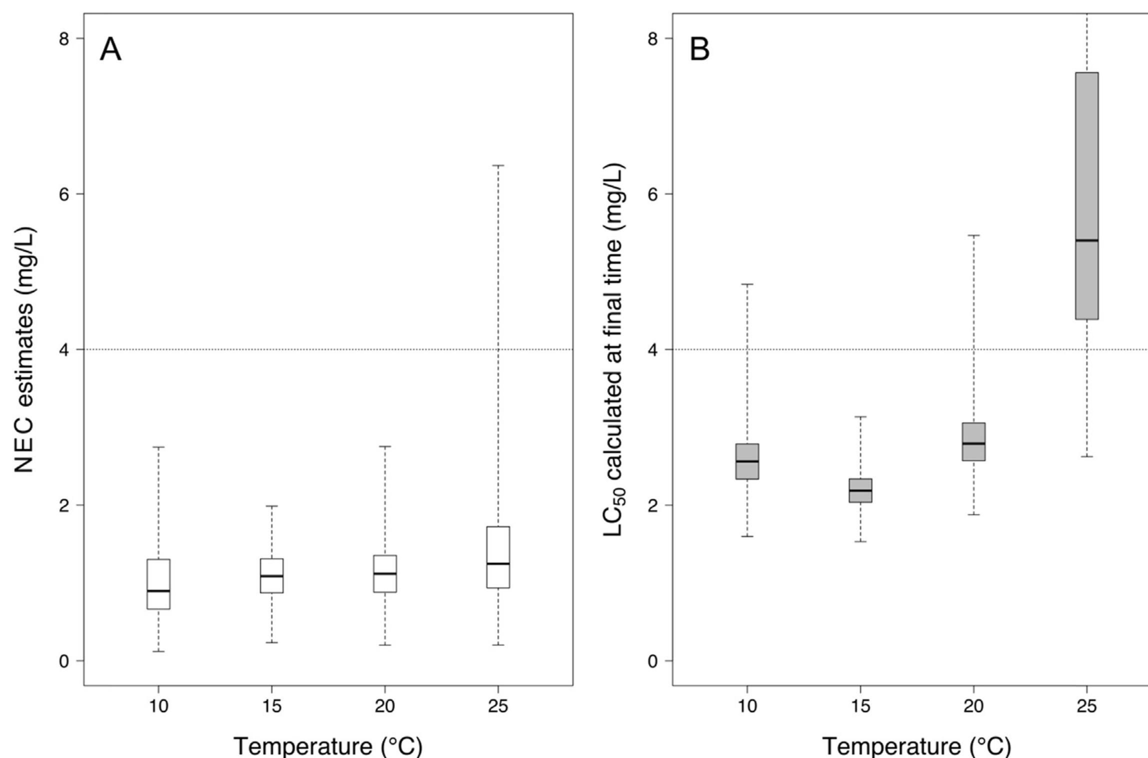

Fig. 2. Box plots of posterior probability distributions of (A) NEC estimated at each tested temperature; (B) LC_{50} calculated at final time for each tested temperature: the extreme of the lower (resp. upper) whisker stand for the minimum (resp. maximum) of the distribution, the box is delimited by the first and the third quantiles, and the black segment corresponds to the median. The dotted horizontal line corresponds to the highest NH_3 tested concentration.

exposed to high nutrient concentrations due to intensive farming (Piscart et al., 2009). Indeed, even if the stream where *G. pulex* population has been collected did not show high nutrient concentrations, aquatic invertebrates in this stream could be selected at a regional scale (Cottin et al., 2012). In addition, the organisms were collected in winter, and the seasonal effects may have a great importance regarding the NH_3 tolerance, as suggested by Dehedin et al. (2013b). Temperature effects were especially harmful over 20 °C, with higher estimated Lethal Temperature for 50% of individuals at final time. This result is consistent with previous studies showing that mortality of *G. pulex* increases between 21 °C and 24 °C after 10 days (Foucreau et al., 2014), and between 20 °C and 25 °C after 15 days (Maazouzi et al., 2011).

Surprisingly, the combination of NH_3 and temperature generated a lower mortality than expected. Whereas the predicted NEC was rather homogeneous, the LC_{50} increased with temperature. In other words, more NH_3 was necessary to kill 50% of the population at higher temperatures. It is therefore possible to qualify this response as antagonistic, which is the opposite conclusion of several similar studies, like in Di Lorenzo et al. (2015) using the copepod *Eucyclops serrulatus*. A reduction in NH_3-induced stress at high temperature has been reported in some fish species (Jeney et al., 1992; Thurston and Russo, 1983). However, these authors did not propose any mechanistic explanations for these observations. Multiple functional processes can be involved in antagonistic responses, and various hypotheses can be developed. Erickson (1985) explained the temperature-NH_3 antagonism with the joint toxicity hypothesis, which assumes that both NH_3 and NH_4^+ are toxic (instead of only NH_3). In these conditions, a temperature increase would reduce the amount of NH_4^+, hence decreasing the strength of the stress. However, no convincing evidence has been generated in support of this hypothesis to explain the temperature-dependence of the NH_3 effect. Alternatively, it was suggested that enhanced metabolism at high temperatures allowed organisms to more quickly eliminate toxic compounds (Donker et al., 1998). Elevated oxygen consumption and locomotion at increasing temperatures support the idea of a positive relation between temperature and metabolic rate in amphipod species (Foucreau et al., 2014; Issartel et al., 2005). However, even if enhanced metabolism can boost the elimination of the toxins, it can also increase the intake rate. Without any measurement of toxicokinetics, the characterization of the NH_3 clearance/intake balance is not possible, but this mechanism should be investigated in further studies. Other explanations for the NH_3-temperature antagonism can be proposed, including cross-tolerance mechanisms. Heat stress is well known to induce up-regulation of numerous stress-related genes among which *hsp70* is one of the most ubiquitous (Kültz, 1995). The presence of HSP70 protein at high temperatures may mitigate or protect organisms from the deleterious effects of other stressors. Indeed, HSPs have already been associated with tolerance to NH_3 (Sung et al., 2012, 2014). Therefore, high *hsp70* gene up-regulation at stressing temperature may allow a better tolerance to subsequent heat exposure, but also grant a cross-protection against NH_3 stress. Moreover, this idea of NH_3-temperature cross-tolerance has been experimentally validated on a shrimp species *Penaeus monodon* by Peaydee et al. (2014), who found a cross-tolerance mechanism mediated by an aquaporin gene.

In aquatic ecology, the HSP70 protein has been widely used as a biomarker to assess thermal stress in invertebrate species (Bedulina et al., 2013; Cottin et al., 2015; Shatilina et al., 2010). However, only few studies have investigated the relationship between *hsp70* expression and NH_3 exposure. Up-regulation of *hsp70* has been reported for *G. pulex* at temperatures above 24 °C (Cottin et al., 2015). We also found such a pattern in our experiments: from weak to no variation in the regulation of *hsp70* gene at temperatures below 20 °C, and a strong up-regulation at 25 °C. This suggests that 25 °C was rather stressful. We also note a global decrease in *hsp70* expression after 24 h which was consistent with previous observations (Bahrndorff et al., 2009; Koštál and Tollarová-Borovanská, 2009). Generally, transcript expression shows a rapid increase before returning to the basal expression level within a few hours. The main cause of such a dynamic probably lies in

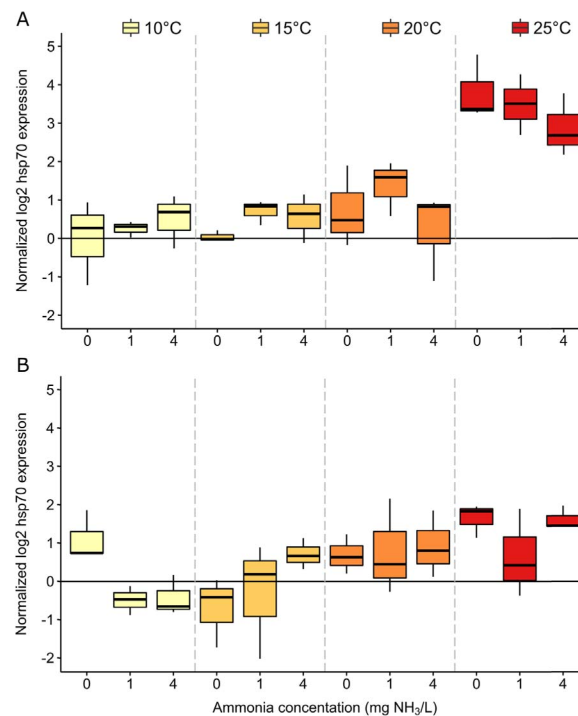

Fig. 3. Boxplots of the relative expression of *hsp70* transcripts, based on log2 transformation of qRT-PCR ratios. The *hsp70* expression values are expressed relative to the reference gene expression (*Gapdh*) and the control value (10 °C, 0 mg NH_3/L NH_3, 6 h) was subtracted from all the measures. Measures of the *hsp70* transcripts expression were realized after 6 h (A) and 24 h (B) of exposure to different combinations of temperatures and NH_3 concentrations. For each treatment, black lines and boxes represent the median, first and third quartiles of the distribution of three biological replicates. Shades of colors correspond (from yellow to red) to the 10, 15, 20 and 25 °C temperature conditions. For each shade, first, second and third bars respectively represent 0, 1 and 4 mg NH_3/L. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the high energetic cost of maintaining a substantial protein production (Krebs and Loeschcke, 1994). Contrary to our second prediction, we observed no significant effect of either NH_3 or NH_3-temperature interaction on the *hsp70* mRNA expression for *G. pulex*. While some studies found a link between *hsp* and NH_3 (Sung et al., 2014), our data indicated that *hsp70* was not a robust biomarker of NH_3 stress in *G. pulex*. This result is unexpected because HSP70 response to stress is highly general, and reports in literature of HSP induction exists for most known stressors (Feder and Hoffman, 1999). In addition, previous studies noticed significant fold changes in *hsp70* (Sung et al., 2014; Wang et al., 2012) and *hsp90* regulation (Wang et al., 2012) in fish and bivalve species, following acute exposure to NH_3.

Piscart et al. (2009) measured total amphipod biomass in pristine and disturbed sites bordered by agricultural activity and large farm effluents. They reported a drastic loss of amphipod abundance, representing 15–85% of total invertebrate biomass. Such a loss was particularly alarming, since the disturbed sites were primarily impacted by high NH_3 concentration. However, in a context of global warming, the increasing concentration of the toxic NH_3 over NH_4^+ caused by the warmer temperature might not be as toxic as expected for all species. Here we show an antagonistic interaction in multi-stress situations can partly counterbalance the effects of an increasing stress applied on organisms, though the mechanisms underlying this antagonism are not yet known. This study highlights the need for a more integrative approach towards stress-related issues. Indeed, responses of organisms to multiple stressors should not be considered as the simple sum of responses to multiple individual stressors. Complex and unexpected patterns in multi-stress experiments have been observed in an increasing number of studies, and should be carefully considered to avoid unreliable predictive models. Therefore, we should prioritize research for such effects in hydrosystems that are already facing mounting and multiple pressures.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2016.11.011.

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