

# Temperature effect on the sensitivity of the copepod *Eucyclops serrulatus* (Crustacea, Copepoda, Cyclopoida) to agricultural pollutants in the hyporheic zone

Tiziana DI LORENZO<sup>1</sup>, Walter D. DI MARZIO<sup>2</sup>, Marco CIFONI<sup>3</sup>, Barbara FIASCA<sup>3</sup>, M. BARATTI<sup>1</sup>, Maria E. SÁENZ<sup>2</sup>, Diana M.P. GALASSI<sup>3\*</sup>

<sup>1</sup> Institute of Ecosystem Study - CNR - National Research Council of Italy, Via Madonna del Piano 10, 50019 Sesto Fiorentino, Florence, Italy

<sup>2</sup> Programa de Investigación en Ecotoxicología, Departamento de Ciencias Básicas, Universidad Nacional de Luján - Comisión Nacional de Investigaciones Científicas y Técnicas CONICET, Argentina

<sup>3</sup> Department of Life, Health and Environmental Sciences, University of L'Aquila, Via Vetoio 1, Coppito 67100, L'Aquila, Italy

**Abstract** The sensitivity of freshwater invertebrates to agricultural pollutants is supposed to increase with rising temperature due to global warming. The aim of this study was to measure the effect of temperature on the lethal toxicity of ammonia-N, the herbicide Imazamox and the mixture of the two chemicals, in the adults and the juveniles of a population of the copepod *Eucyclops serrulatus*. This is a widely distributed species found in surface waters, in transitional habitats between surface water and groundwater, and in genuine groundwater environments. We tested the sensitivity by short-term bioassays (96 h) at 15°C and 18°C, respectively. Our results highlighted the following: (1) increasing temperature affected the sensitivity of the adults to ammonia-N and of the juveniles to the mixture, all of which were more sensitive to its detrimental effects at 18°C; (2) the juvenile stages were more sensitive than the adults to all toxicants, and (3) for all combinations of chemicals and temperatures, the effects were synergistic and approximately one order of magnitude greater than those expected according to a concentration addition model when comparing the LC50 for each chemical in the mixture with the LC50s of chemicals individually assayed. Overall, in a context of global change, ammonia-N and mixtures of agricultural pollutants may affect the survival rate of species that spend a part or the whole life-cycle in the hyporheic habitat, with detrimental effects to biodiversity and ecosystem services provided by the hyporheic biota [*Current Zoology* 61 (4): 629–640, 2015].

**Keywords** Hyporheic, Global warming, Copepods, Ammonia, Herbicide, Mixtures

The projected impact of global warming will cause several shifts in the biotic and abiotic conditions of water bodies (Stern, 2006; IPCC, 2007; Kernan et al., 2010). A global increasing of 3°C is expected to lead to falling crop yields, reduced water availability, and increased flood risk in many areas (Kernan et al., 2010). Despite the increasing knowledge on biodiversity threats under global warming for terrestrial communities and single species, predictions of the potential effects of climate change on freshwater biodiversity are still poor (Sala et al., 2000; Thomas et al., 2004; Stern, 2006; Heino et al., 2009; Sandin et al., 2014), especially for hyporheic habitats.

The hyporheic zone (Orghidan, 1959), which is a transitional zone between surface water and groundwater in streams and rivers, plays a crucial role in the functioning of river ecosystems (Findlay et al., 1993;

Boulton et al., 1998; Hinkle et al., 2001; Vervier et al., 2009). Both epigeal and true groundwater invertebrates spend a part or their whole life-cycle in this habitat (Boulton, 2000). Invertebrates have physical effects on hyporheic sediment permeability, promote microbial activity, are involved in organic matter and energy transfer between surface benthic and groundwater habitats (Boulton 2000; Boulton et al., 2008) and are indicators of water exchanges (Lafont and Vivier, 2006; Di Lorenzo et al., 2013; Iepure et al., 2014), habitat heterogeneity and ecosystem status and trends (Boulton, 2000; Hancock et al., 2005; Boulton et al., 2008). In the hyporheic zone, thermal fluctuations, due to surface-groundwater exchanges, are lagged with respect to stream waters and are attenuated with increasing depth and distance from infiltration sites (Ward and Stanford, 1982; Brunke and Gonser, 1997; Dole-Olivier, 2011).

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\* Corresponding author. E-mail: dianamariapaola.galassi@univaq.it

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An increase in temperature is likely to alter the physiology of the invertebrate species living occasionally or permanently in the hyporheic habitat (e.g. by doubling locomotion activity, altering phenology, increasing oxygen consumption rates, and even inducing death when a critical threshold is reached), especially in those species with a narrow thermal tolerance (Issartel et al., 2005; Colson-Proch et al., 2010; Dehedin et al., 2013a; Stoks et al., 2014).

The sensitivity of hyporheic organisms to toxicants might increase with temperature similar to what has been observed for surface benthic invertebrates (Bat et al., 2000; Dehedin et al., 2013b). However, this issue has been not fully addressed by in-field (Iepure and Selescu, 2009; Moldovan et al., 2011; Dehedin et al., 2013a; Iepure et al., 2013) or laboratory experimentation (Marmonier et al., 2012 and references herein). In Europe, data from the last decades showed that many freshwater bodies suffer from diffuse pollution of ammonia-N ( $\text{NH}_3 + \text{NH}_4^+$ ; Camargo and Alonso, 2006) and pesticides from agriculture (EEA, 2013). The high concentrations of nitrogen (N) compounds detected in groundwater (Di Lorenzo et al., 2012; Di Lorenzo and Galassi, 2013), and in those environments dependent on groundwater, such as the hyporheic zone, are often due to intensive agriculture, through the use of fertilizers in the forms of ammonium nitrate, urea, calcium nitrate, ammonium bicarbonate, and mixtures of nitrogen, potassium and phosphorus (Erisman et al., 2007). Pesticides form an integral part of modern agriculture and horticulture and, although subject to strict regulations, they still have an impact on non-target organisms and the natural environment through spray drift, leaching or run-off (European Union, 2012; EEA, 2013). Ammonia-N causes mortality and other toxic effects on aquatic invertebrates (Alonso and Camargo, 2004; Prenter et al., 2004; Dehedin et al., 2013b). Their toxic effect occurs by interfering with respiratory metabolism and by changing the hemolymph pH (Cheng et al., 2013) and  $\text{Na}^+/\text{K}^+$ -ATPase membrane activities (Naqvi et al., 2007; Romano and Zeng, 2013). The effect of ammonia-N on copepods, which are among the most abundant invertebrate groups in the hyporheic zone (Galassi et al., 2009a), has been documented only recently (Di Marzio et al., 2009, 2013; Di Lorenzo et al., 2014, 2015a).

Herbicides and their degradation products are common artificial chemicals detected in freshwater ecosystems (EEA, 2013). Herbicides are known to affect non-target species, such as fishes and crustaceans, inducing alteration of metabolic, hematological and oxidative

parameters (Neškovich et al., 1996; Di Lorenzo et al., 2014).

Although the great majority of ecotoxicological studies dealing with agricultural pollutants and aquatic organisms have been focused on exposure to individual chemicals (Camargo and Alonso, 2006; Di Lorenzo et al., 2014), freshwater bodies in rural areas are typically contaminated by complex and changing mixtures of fertilizers and pesticides (EEA, 2013). When chemicals collectively exist, the toxic response may be highly different from that occurring under exposure to a single chemical. Accordingly, understanding the joint action of chemical mixtures is crucial for the risk assessment of chemicals in aquatic environment. However, despite the substantial information available regarding the effects of single substances on some temporary or permanent hyporheic copepods (Brown et al., 2003; Turesson et al., 2007), there is a lack of awareness concerning the toxicity of mixtures of fertilizers and herbicides.

The aim of this study was to experimentally measure the combined effect of temperature, ammonia-N, the herbicide Imazamox and the mixture of the two chemicals, on the survival rate of the cyclopoid copepod *Eucyclops serrulatus* by means of assessing lethal responses (i.e. mortality). We selected *E. serrulatus* since it is a widely distributed and well-studied freshwater species (Di Lorenzo et al., 2014; Baratti et al., 2015; Di Lorenzo et al., 2015b and references therein). The species is primarily hyperbenthic, swimming in proximity of the benthic sediment layers of lakes, streams, rivers and springs. It is commonly found in epikarst, saturated karst, alluvial aquifers and hyporheic zones where it can live from a few centimeters below the streambed up to 1.50 m deep (Galassi et al., 2009a, b; Galassi et al., 2014), as member of the permanent hyporheos (Gibert et al., 1994). It is frequently the most abundant epigeic cyclopoid in meiofaunal communities (Galassi et al., 2009a; Fiasca et al., 2014). We selected the herbicide Imazamox for its widespread diffusion in Italian freshwater bodies (ISPRA, 2014), where it is frequently found at concentrations exceeding  $1\mu\text{g/L}$  (ARPAP, 2014), which is ten times the threshold value ( $0.1\mu\text{g/L}$ ) assessed by European Directives 2006/118/CE and 2000/60/CE for pristine freshwater bodies. We tested the sensitivity of *E. serrulatus* at two temperatures: at  $15^\circ\text{C}$  (control temperature), corresponding to the maximum temperature in the sites where the species has been collected, and at  $18^\circ\text{C}$ , respectively, since an increase of  $3^\circ\text{C}$  corresponds to the maximum temperature increase predicted for European inland waters in the next 40

years (Dokulil, 2013). The methods for risk assessment of mixture toxicity are usually based on the two concepts of concentration addition (CA) and independent action. CA has been widely recognized as a valid method to estimate joint toxicity of mixtures sharing the same or similar action mechanisms (Backhaus et al., 2004; Huang et al., 2011) and also dissimilar mode of action, as reported in Syberg et al. (2008) for binary and ternary mixtures. Hence, a further aim of this study was to determine if the effect that has been detected in the mixture bioassays can be either explained by a concentration addition (CA) model or if the two chemicals have synergistic or antagonistic effects on the assayed species.

## 1 Materials and Methods

### 1.1 Rearing

Individuals of *E. serrulatus* were collected in the hyporheic zone of the upper reach of the River Aterno, Italy (coordinates: N 42.481166, E 13.244952), in a pristine area in July 2014. Ten samples were collected from the same reach with a Bou-Rouch pump (Bou and Rouch, 1967) and mobile pipes hammered at each sampling site to a depth of 30 cm below the riverbed. Ten liters of interstitial water and sediments were withdrawn at each site and filtered through a 60- $\mu\text{m}$  mesh net. Specimens were transported to the lab with the same hyporheic water in a cooling box within 2 hours after collection. Copepods were sorted in the laboratory under a stereomicroscope and individuals of *E. serrulatus* were separated from the remaining fauna. Up to 100 individuals were maintained in a 1 L plastic baker with the hyporheic water withdrawn from the River Aterno (pH 7.8, electrical conductivity 479  $\mu\text{S cm}^{-1}$ , total hardness 241 mg/L as  $\text{CaCO}_3$ , N-  $\text{NH}_4^+$  < 0.02, N- $\text{NO}_3^-$  0.9,  $\text{SO}_4^{2-}$  19, all expressed in mg/L), in permanent darkness and in a laboratory thermostatic cabinet (Pol-Eko-Aparatura Mod. ST 3) at 15°C, corresponding to the maximum annual temperature in the field. Neither sediment nor additional food were offered in order to allow specimens to feed on the microbial biofilm of their native habitat. After a week, 30 ovigerous females (each carrying 2 egg-sacs with  $18 \pm 2$  eggs each) were separated from the original stock culture and maintained in a 500-ml baker, under the same rearing condition of the stock culture. After 17 days (the average time needed to allow the embryos to develop to C3-C4 copepodid stages at 15°C; Maier, 1990), the juveniles (copepodids C3-C4) required for the tests were picked-up from the culture of the ovigerous females. Similarly, after 24

days (the average time needed for embryos to reach the adult stage at 15°C; Maier, 1990), the adults required for the tests were picked-up. Before testing, both adults and juveniles were acclimated in two different glass containers (500 ml) for 3 days in a standard water (pH 7.4, electrical conductivity 415  $\mu\text{S cm}^{-1}$ ,  $\text{HCO}_3^-$  301,  $\text{Ca}^{2+}$  48.6,  $\text{Mg}^{2+}$  28.2,  $\text{SiO}_2$  15.2,  $\text{NO}_3^-$  8.5,  $\text{Na}^+$  5.8,  $\text{SO}_4^{2-}$  4.1,  $\text{K}^+$  1,  $\text{F}^-$  < 0.15,  $\text{NH}_4^+$  absent; pesticides absent; all expressed in mg/L) at the temperatures required for the tests (15°C or 18°C). During acclimation, the individuals were deprived of food in order to allow the gut to empty completely. The digestive tract of *E. serrulatus* is clearly visible under stereomicroscope at 80 x magnification, and gut emptying was confirmed visually. After this procedure, feces were removed, and only actively swimming copepods were selected for each bioassay.

### 1.2 Ecotoxicological testing

Experiments were carried out with ammonia-N, Imazamox and a mixture of the two toxicants, at 15°C and 18°C for a total of 12 trials (6 trials for adults and 6 trials for juveniles). For each trial and for each stage (adults and juveniles), 4 or 5 nominal concentrations (Table 1) of each toxicant and the toxicant mixture were prepared by diluting the appropriate volumes of stock solutions. Ammonia-N was dosed as  $\text{NH}_4^+$ , starting from ammonium nitrate (pure crystalline solid, Chemical Abstracts Service [CAS]: 6484-52-2), the herbicide Imazamox was sourced from BEYOND commercial mixture (BASF Italy with 3.7% Imazamox, CAS: 114311-32-9). The appropriate weight of ammonium nitrate and the herbicide Imazamox were dissolved in the standard water (500 ml) used for acclimation to prepare the stock solutions. The experimental concentrations (Table 1) were chosen based on preliminary range-finding experiments according to the species tolerance (> 90% survivorship in control treatment and 100% mortality at the maximal concentration; U.S. EPA, 2002). Two control (no toxicant) treatments, for adults and juveniles respectively, were prepared, for each temperature. A minimum of 20 organisms were exposed to each concentration (U.S. EPA, 2002), for a total of about 1200 utilized organisms. According to the U.S. EPA (2002), to enhance the reliability of our trials, 4 test chambers (5 cm-diameter Petri dishes) were provided for each concentration and the control, to the end of performing 4 subtrials for each trial. Every 24 h, each replicate was checked for the presence of dead individuals (no movement after gentle stimulation by a sorting needle). At the end of the trials, the mortality responses in each subtrial were used to determine the LC50 values.

Afterwards, a mean LC50 and the relative standard deviation was computed for each trial. The mixture effects were measured using the so-called fixed-ratio design: the mixture of interest was analyzed at a constant concentration ratio (Table 1) while the total concentration of the mixture was varied. A concentration-response curve of the mixture was recorded and then analyzed just as the concentration-response curve of a single chemical.

### 1.3 Statistical analyses

Lethal concentration values 50% (LC50) at 96 h were calculated using Probit analysis whenever the data structure was suitable (U.S. EPA, 1994a). If insufficient numbers of partial kills were present in a trial, the data were alternatively analyzed by the Trimmed Spearman-Kärber method (U.S. EPA, 1994b). To test if temperature variation altered the sensitivity, one-sample *t*-values were performed for each toxicant and for each stage (6 tests in total). One-sample *t*-values (Student's or Welch's test) were applied after checking for normal distribution (Shapiro's test) and variance homogeneity (Levene's or Bartlett's tests). Univariate analyses were performed using R software v. 2.15.0 (R Development Core Team, 2008).

The data from the single chemicals and the mixture assays were fitted according to the non-linear equation:

$$\%Survival = \frac{a}{1 + (x/EC\%)^b}$$

where *a* is the maximum survival percentage, *x* is the chemical concentration and *b* is the Hill slope. The toxic responses in the mixtures were evaluated to assess

whether they followed the expectations set by the concentration addition model (CA) or if they had antagonistic or synergistic effects. For a mixture of *n* components, the relationship can be mathematically expressed as:

$$\sum_{i=1}^n (ci/ECxi) = 1 \quad (\text{equation 1})$$

where *ci* gives the concentration of the *i*th component in a *n*-compound mixture that elicits *x*% total effect and *ECxi* denotes the concentration of that substance that provokes *x*% effect if applied singly (Jonker et al., 2011). The ratio *ci/ECxi* is known as toxic unit (TU) and gives the concentration of a compound in the mixture scaled for its relative potency. CA is held when the sum of TU of the mixture components equals 1 at a mixture concentration provoking *x*% effect. If instead antagonistic or synergistic effects of exposure to the mixture are present, this value will be higher or lower than 1.

## 2 Results

None of the control animals died, thus demonstrating that holding facilities and handling techniques were acceptable to run the trials, as required in the standard U.S. EPA/COE protocol (U.S. EPA, 2002). The LC50 mean values at 96 h and the standard deviation (SD) for NH<sub>4</sub><sup>+</sup>, Imazamox and the mixture for each trial are provided in Table 2. The LC50 values at 15°C were significantly different from those at 18°C for the adults stressed with NH<sub>4</sub><sup>+</sup> (*t*-value = 4.2042, *P*-value = 0.02362; Fig. 1A) and for the juveniles stressed with the mixture (*t*-value = -3.4598, *P*-value = 0.01659; Fig. 1F). Conversely, the LC50 values at 15°C were not significantly

**Table 1** NH<sub>4</sub><sup>+</sup>, Imazamox and mixture concentrations tested at 15°C and 18°C

Stage	Concentration	NH <sub>4</sub> <sup>+</sup> (mg/L)		Imazamox (mg/L)		Mix (mg/L)	
		15°C	18°C	15°C	18°C	15°C	18°C
adults	C0	0	0	0	0	0	0
	C1	4.9	6.9	84	84	8.9	8.9
	C2	27.7	13.8	120	120	17.7	17.7
	C3	55.4	20.0	171	171	26.6	26.6
	C4	110.7	27.7	245	245	35.5	35.5
	C5	221.5		350	350		
juveniles	C0	0	0	0	0	0	0
	C1	4.9	6.9	84	84	8.9	8.9
	C2	27.7	13.8	120	120	17.7	17.7
	C3	55.4	20.7	171	171	26.6	26.6
	C4	110.7	27.7	245	245	35.5	35.5
	C5			350	350		

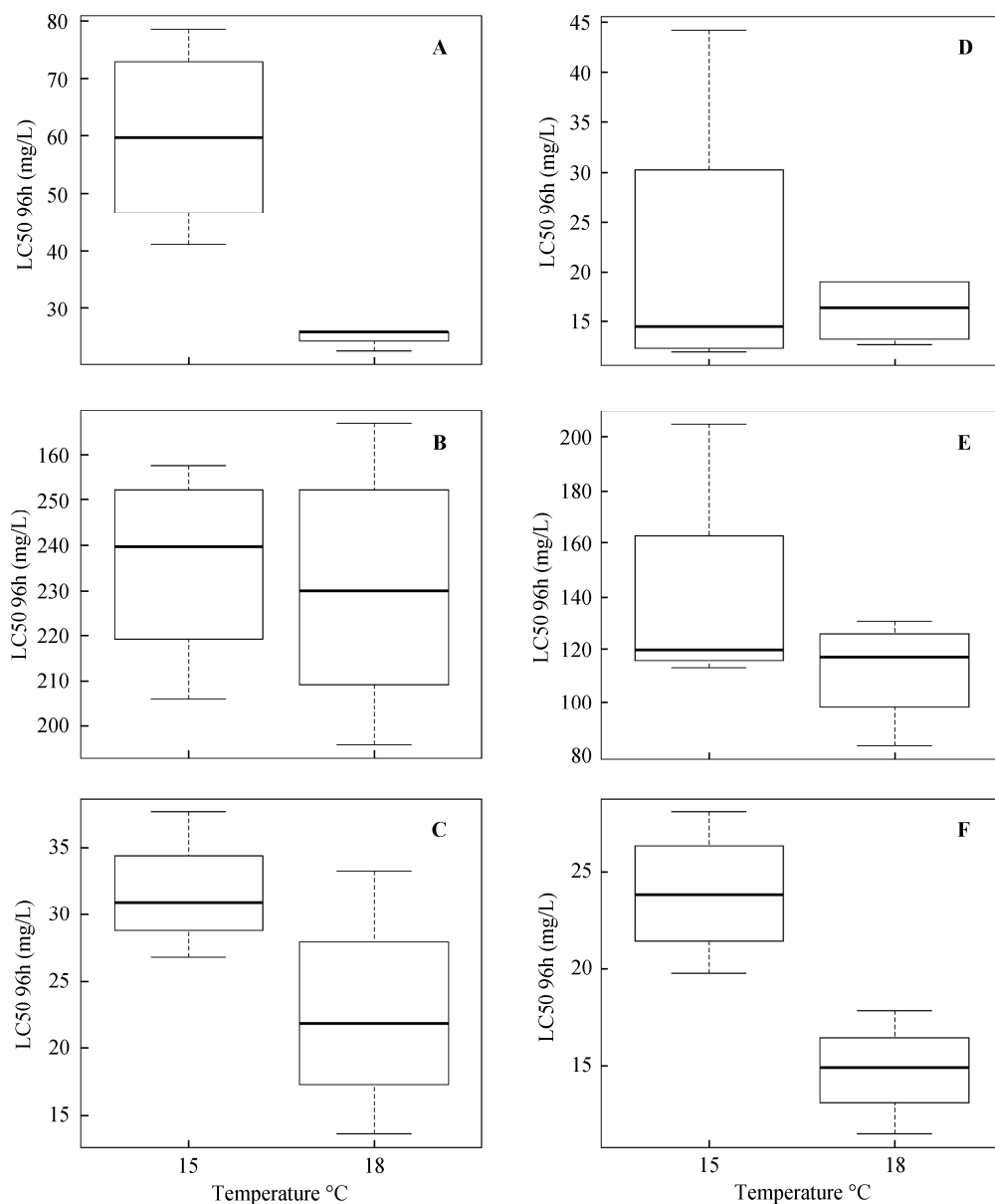
In the mixture, the ratio of NH<sub>4</sub><sup>+</sup> to Imazamox is 4:1.

different from those at 18°C for both adults stressed with either Imazamox ( $t$ -value = 0.2712,  $P$ -value = 0.796; Fig. 1B) or the mixture ( $t$ -value = 1.9315,  $P$ -value = 0.1151; Fig. 1C), and for juveniles stressed with either  $\text{NH}_4^+$  ( $t$ -value = -0.4545,  $P$ -value = 0.6797; Fig. 1D) or Imazamox ( $t$ -value = 0.2873,  $P$ -value = 0.788; Fig. 1E). Imazamox was truly toxic only at very high concentrations (> 100 mg/L) at both temperatures for both stages. The mixture was more toxic than either  $\text{NH}_4^+$  or Imazamox alone for both adults and juveniles (Table 3). The data analysis of the mixture assays showed that, for all combinations of chemicals and temperatures, the effects were higher than those expected according to the con-

centration addition model (Table 3), which suggested the occurrence of synergistic effects (Figs. 2, 3; Table 3). These synergistic effects reached approximately an in-

**Table 2** LC50 mean values (mg/L) at 96 h and standard deviation (SD) for *Eucyclops serrulatus* (a: adults; j: juveniles) exposed to  $\text{NH}_4^+$ , Imazamox (IMA) and the mixture (MIX) of Imazamox and ammonia-N at 15°C and 18°C (T)

T	Stage	$\text{NH}_4^+$	SD	IMA	SD	MIX	SD
15	a	59.74	14.32	235.76	19.22	31.57	3.89
18	a	24.79	1.43	230.74	25.64	22.62	7.01
15	j	21.26	15.44	139.30	43.78	23.89	3.45
18	j	16.11	3.4	111.90	20.35	14.75	2.57



**Fig. 1** Box plots showing the median (horizontal lines), the first and third quartiles (box spans) and the smallest and largest observations (box whiskers) of LC50 (mg/L) values at 15°C and 18°C for both adults (A, B, C) and juveniles (D, E, F) of *Eucyclops serrulatus* exposed to  $\text{NH}_4^+$  (A, D), Imazamox (B, E) and mixtures (C, F)

crement of one order of magnitude, comparing the LC50 values for each chemical in the mixture with the LC50 values as assayed alone.

### 3 Discussion

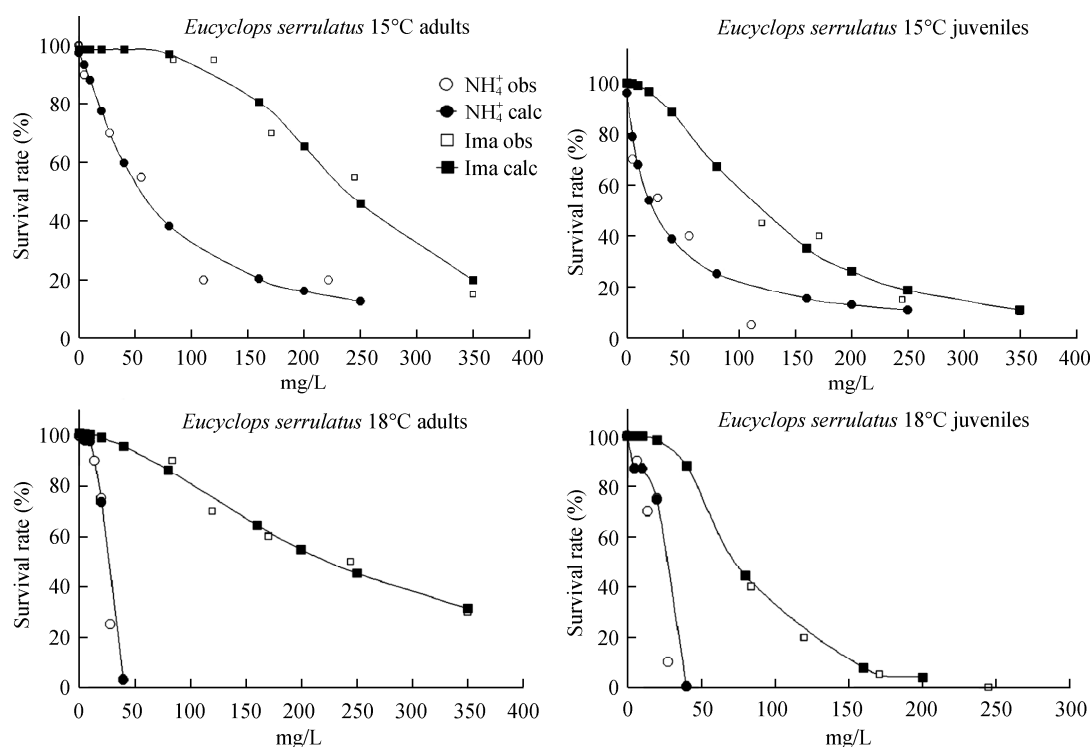
Our results highlighted that *E. serrulatus* was sensitive to ammonia-N at both stages (adults and copepodids). To compare our results with those obtained by Dehedin et al. (2013), we computed the values of LC50 at 96 h for the unionized fraction ( $\text{NH}_3$ ) of ammonia-N applying the equation discussed in Emerson et al. (1975) to the assessed  $\text{NH}_4^+$  LC50 values at 96 h for both adults and juveniles of *E. serrulatus*. According to these LC50 values, at 18°C *E. serrulatus* can be considered even more sensitive to ammonia-N referred to as  $\text{NH}_3$  (96 h LC50 for adults: 0.17 mg/L; 96 h LC50 for juveniles:

0.008 mg/L) than the benthic amphipod *Gammarus pulex* (96 h LC50 for adults at 18°C: 0.25–1.39 mg/L; Dehedin et al., 2013b), an epigeal amphipod usually considered a “pollution-sensitive” species (Whitehurst, 1991; Maltby, 1995). The toxic effects of ammonia-N on aquatic crustaceans involve osmoregulation, immunology, acid/base balance, gas exchange as well as increasing oxidative stress, pathogenic susceptibility and histopathological damage (Romano and Zeng, 2013). An alteration of oxygen consumption through changes of hemolymph pH and, consequently, of respiratory metabolism has been often observed, as well as an alteration of excretion rates (Colt and Armstrong, 1981; Aarset and Aunaas, 1990; Romano and Zeng, 2013). The  $\text{NH}_3$  form is considered more toxic than  $\text{NH}_4^+$  due to its higher permeability and diffusion across the cell

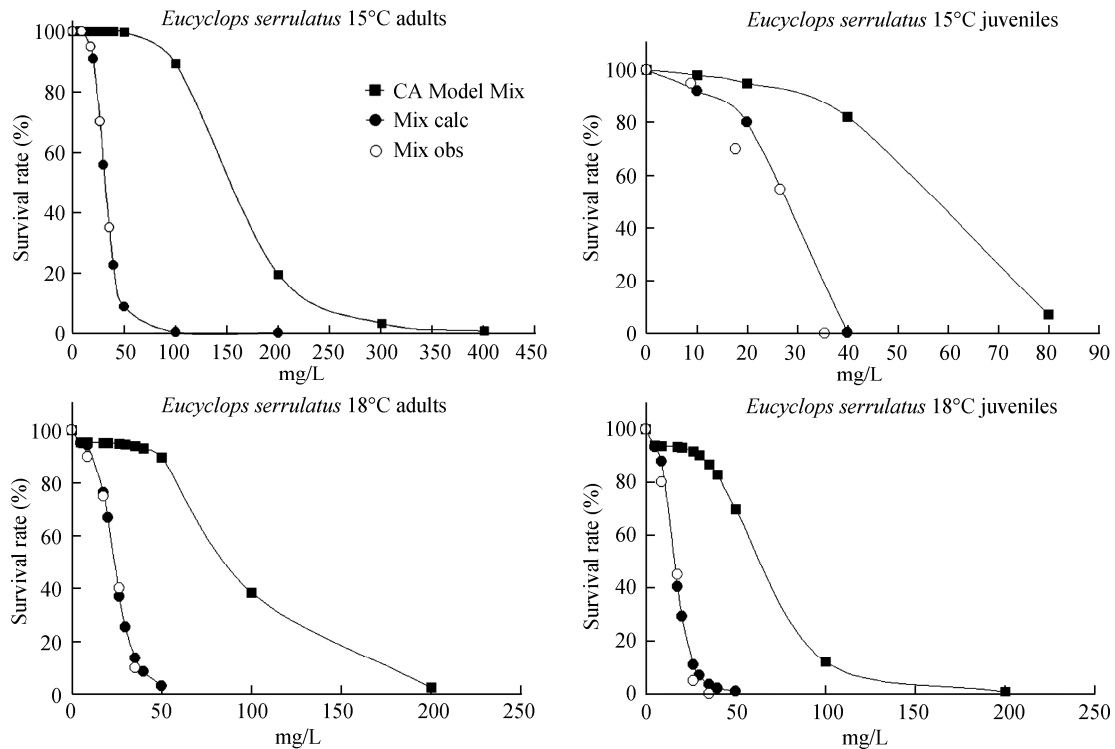
**Table 3** Mixture predictions for CA (concentration addition) expressed in toxic units (TU) according equation 1

	Adults 15°C	Adults 18°C	Juveniles 15°C	Juveniles 18°C
CA	0.21	0.17	0.30	0.23
TU	synergism	synergism	synergism	synergism
$\text{NH}_4^+$ mg/L in LC50 mix	6.2	2.2	3.34	1.84
IT	x9.3	x11.3	x6.36	x8.7
Imazamox mg/L in LC50 mix	18.6	20.42	20.55	12.91
IT	x12.7	x11.3	x6.8	x8.7

IT: increased toxicity comparing the  $\text{NH}_4^+$  or Imazamox concentration in the LC50 of the mixture (mix) with respect to the single-chemical LC50.



**Fig. 2** Observed (obs.) and non-linear adjusted (calc.) survival data - concentrations for single test compounds (ionized ammonia:  $\text{NH}_4^+$ ; Imazamox: IMA) at 15°C and 18°C, for adult and juvenile stages of *Eucyclops serrulatus*



**Fig. 3** Predicted (calc.) and observed (obs.) toxicity values of mixtures (Mix) designed with fixed-ratio tests, for adult and juvenile stages of *Eucyclops serrulatus* at 15°C and 18°C

CA is the toxicity prediction in the mixture according to the concentration addition model.

membrane via the lipid pathway (Golchini and Kurtz, 1988; Wright, 1995). The form  $\text{NH}_4^+$  is less permeable across cell membrane. However, as  $\text{NH}_3$  diffuses across lipid bilayers of the cells, it is protonated to  $\text{NH}_4^+$  which alters the hemolymph pH (Romano and Zeng, 2013). In addition,  $\text{NH}_4^+$  interferes with the activity of  $\text{Na}^+/\text{K}^+$ -ATPase pump which is a major player in ammonia-N excretion in aquatic invertebrates. Furriel et al. (2004) observed that  $\text{NH}_4^+$  substitutes for  $\text{K}^+$  in activation of the ouabain-sensitive branchial  $\text{Na}^+/\text{K}^+$ -ATPase of the freshwater shrimp *Macobrachium olfersii*, suggesting for this species that at high  $\text{NH}_4^+$  concentrations  $\text{Na}^+/\text{K}^+$ -ATPase pump exposes a new binding site for  $\text{NH}_4^+$  which, after binding to  $\text{NH}_4^+$ , modulates the activity of  $\text{Na}^+/\text{K}^+$ -ATPase independently of  $\text{K}^+$  ions. This substitution, hindered by high external ammonia-N concentration, alters the excretion rates in aquatic crustaceans, intoxicating the organisms.

Herbicides are known to affect non-target species, such as fishes and crustaceans, due to alteration of metabolic, hematological and oxidative parameters (Neškovich et al., 1996). A modification of escape behavior has been observed in the copepod species exposed to herbicides (Gutierrez et al., 2012). However, in our trials we observed that *E. serrulatus* was highly tolerant to the herbicide Imazamox; the 96 h LC50 values are more than

three orders of magnitude higher than the concentrations (0.1  $\mu\text{g/L}$ ) normally observed in pristine freshwater ecosystems according to the European Directive 2006/118/CE (EC, 2006). According to the U.S. EPA (2012), at both 15°C and 18°C, Imazamox can be considered non-toxic to *E. serrulatus* since the LC50 values are greater than 100 ppm.

The toxicity scale based on the assessed LC50 values was mixture >  $\text{NH}_4^+$  > Imazamox for both adults and juveniles. Potentiated effects were further highlighted in the case of the herbicide Imazamox in combination with  $\text{NH}_4^+$  because as an individual compound it is considered non-toxic based on its LC50 and on the U.S. EPA classification (U.S. EPA, 2012). However considering its LC50 concentration values in the mixture, we can classify Imazamox as a toxic substance.

This study highlighted that juveniles stages (copepods C3-C4) of *E. serrulatus* were more sensitive than adults to each toxicant. The higher sensitivity of juvenile stages compared to adults in crustacean species is still under debate, since tolerance to toxicants is found to increase across the ontogenetic development in some species (Young-Lai et al., 1991; Suchayo et al., 2008; Strode and Balode, 2013) but not in others (Maltby, 1995). According to Gutierrez et al. (2010), the higher sensitivity of juvenile stages of copepods com-

pared to adults may be associated with the different thickness of the body exoskeleton. In the absence of food, as set for our trials, the main route by which the individuals take up the toxicants is through contact with their body surface; hence, the thinness of juvenile exoskeleton may favor the entrance of the toxicants. Moreover, high metabolic rates are supposed to increase the influx rate of toxicants in aquatic crustaceans (Gutierrez et al., 2010). The metabolic rates of juveniles of *E. serrulatus* are higher than those of adults (Di Lorenzo et al., 2015b); as a consequence, the influx rate of toxicants is likely to be more elevated in juveniles than in adults of *E. serrulatus*, thus explaining the higher sensitivity of the juvenile stages compared to that of the adults. Conversely, the adult stages of aquatic crustaceans show more efficient detoxification mechanisms respect to juveniles, since they are more able to contrast ammonia-N diffusion via cell membrane thus allowing the maintenance of lower ammonia-N levels in the hemolymph (Romano and Zeng, 2013). However, Maltby (1995) reported that the early stages of the amphipod *Gammarus pulex* and the isopod *Asellus aquaticus* are less sensitive to ammonia-N because they are characterized by higher hemolymph concentration with higher affinity to oxygen with respect to adults. On the contrary, Figueroa-Lucero et al. (2012) observed that the juvenile stages of the freshwater prawn *Macrobrachium tenellum* are more sensitive to ammonia-N than the adults because they likely have not mature mechanisms for ammonia-N detoxifying. These results demonstrate that research that includes not only species covering different functional roles, but also various life stages, provides more complete information about the responses of freshwater organisms to pollutants in nature.

Our data suggested that climate change may pose a severe risk for the populations of *E. serrulatus* inhabiting polluted habitats, since the lethal toxicity to ammonia-N and to the mixture of ammonia-N and Imazamox of both adults and juveniles of this species significantly increased with temperature. Our findings highlighted that a small temperature shift (only 3°C) in the hyporheic zone may lead to an increase of sensitivity in *E. serrulatus* to agricultural pollutants. Namely, the sensitivity of the adults to  $\text{NH}_4^+$  increased of 2.4 times, to Imazamox of 1.02 times and to the mixture of 1.39 times at 18°C, with respect to those measured at 15°C; the sensitivity of the juveniles to  $\text{NH}_4^+$  increased of 1.3 times, to Imazamox of 1.2 times and to the mixture of 1.61 times at 18°C, with respect to those measured at 15°C. Indeed, these outcomes were not unexpected

since high temperatures have proved to induce an increase of metabolic rates in aquatic crustaceans, with negative effects on most physiological and biochemical processes (Maazouzi et al., 2011; Cottin et al., 2012). Cottin et al. (2012) observed the appearance of clearly visible spasms in *Gammarus pulex* when habitat temperature increased up to 23°C, 5°C less than the critical thermal maximum for this species (28°C). This observation suggests that *G. pulex* experiences a thermal stress also at lower temperatures, as pointed out by Maazouzi et al. (2011) who measured a strong increase in  $\text{O}_2$  consumption rate for *G. pulex* at temperatures > 20°C. Cruz et al. (2015) observed that respiration rates increased exponentially with temperature (experimental conditions: 8, 13, 19 and 24°C) in both adult males and females of the calanoid copepod *Centropages chierchiae*. Prolonged metabolic acceleration associated with an increased environmental temperature can also shorten the development time (from the naupliar stage N1 to the adult: 20 days at 15°C vs. 14 days at 20°C; Maier, 1990) and the life span (42 days at 20°C vs. 96.5 days at 15°C; Maier, 1990) of *E. serrulatus*. The acceleration of the metabolism due to temperature increasing is likely to enhance the influx rate of the toxicants (Gutierrez et al., 2010), thus increasing the chemical exposure even in the absence of an increase in the environmental chemical concentration. Such an increased rate of exposure might in part explain the elevated toxicity to agricultural pollutants observed at higher temperatures in our current study with *E. serrulatus*, and similarly observed by Willming et al. (2013) who measured alterations in pesticide toxicity under the daily thermal fluctuations in the amphipod *Hyalella azteca* and the water flea *Daphnia magna*. Since ammonia-N interferes in the uptake and transport of oxygen, the lower dissolved oxygen availability at higher temperatures in nitrogen-polluted aquatic habitats is likely responsible for a further stress of aquatic invertebrates (Dehedin et al., 2013b). However, for some species of benthic shredders, Dehedin et al. (2013b) observed a seasonal shift in  $\text{NH}_3$  tolerance which could indicate an in-field acclimation to sublethal concentrations and to gradual thermal increase. Since *E. serrulatus* survives for no more than 96 days at its thermal optimum (15°C; Maier, 1990), it is difficult to test the effects of thermal seasonality on sensitivity to toxicants; however, this hypothesis cannot be refuted without further experimentation.

Freshwater community resilience in benthic habitats of rivers and streams that are eventually affected by anthropogenic disturbances is based primarily on the



ability of the epigeal species to recolonize their original habitats from refuge areas, such as the hyporheic zone (Dole-Olivier, 2011). The hyporheic zone exerts a primary role for both surface and groundwater organisms, serving as refugium or nursery habitat for species unable to face the effects of multiple stressors (Dole-Olivier, 2011 and references therein; Lencioni and Spitale, 2015). This habitat allows aquatic invertebrates to avoid extreme or lethal temperatures and desiccation during dry periods (Dole-Olivier, 2011 and references therein), thus making rivers and streams more resilient and resistant to global change than other types of ecosystems (Stevenson and Sabater, 2010). However, recent studies of subsurface ecosystems have shown that hyporheic zones worldwide are at risk of loss of their functionality due to increased anthropogenic activities (Iepure et al., 2013 and references therein; Dehedin et al., 2013a) affecting the environmental quality of these habitats and their biota (Iepure and Selescu, 2009; Moldovan et al., 2011). True groundwater species normally found in hyporheic sites in the past are dramatically disappearing in both terms of species richness and abundances (Dehedin et al., 2013a). Our results demonstrated that climate change and pollution may interact synergistically to contribute a heightened sensitivity to toxicants in some species occurring in the hyporheic zone. These findings bring to light that in the projected context of global change involving subsurface ecosystems (Kløve et al., 2013), agricultural pollutants could strongly affect the survival of the hyporheic community and consequently have a high impact on the services provided by the dwelling-biota of these aquatic ecotones.

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