



Bioenergetic cost of living in polluted freshwater bodies: respiration rates of the cyclopoid *Eucyclops serrulatus* under ammonia-N exposures

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With 2 figures and 3 tables

Abstract: Ammonia-N ($\text{NH}_3+\text{NH}_4^+$) is considered one of the most important pollutants of freshwater bodies worldwide due to its high toxicity and ubiquity in freshwater ecosystems. The aim of this study was to assess if short-term exposures to sublethal ammonia-N concentrations affect the metabolic rates of the cyclopoid *Eucyclops serrulatus*. This is a target species for routine biomonitoring due to its world-wide distribution, wide ecological niche, short life-cycle and suitability to be easily reared in the laboratory. We measured the oxygen consumption as a proxy of possible metabolic reaction to stress under a 3-day exposure to two different ammonia-N concentrations, dosed as NH_4^+ , at 15 °C. We also measured the respiration rates of juveniles (C1-C5 copepodids) separately from those of adults in order to assess whether the metabolic cost differed between the two stages. The respiration rates of both adults and copepodids increased with increasing NH_4^+ concentrations. However, the adults of *E. serrulatus* experienced a significant energetic stress under exposure to 12 mg L⁻¹ NH_4^+ . On the contrary, the copepodid respiration rates significantly varied under exposure to 1 mg L⁻¹ NH_4^+ , just one order of magnitude greater than the current European threshold value for freshwater bodies. According to these outcomes, it is prudent to consider river monitoring sites not contaminated by ammonia-N under the current legislation to represent a risk for the juveniles of this species.

Key words: ammonia-N; freshwater; copepod; oxygen; metabolism; respirometric

Introduction

Ammonia-N ($\text{NH}_3+\text{NH}_4^+$; Camargo & Alonso 2006) is considered one of the most important pollutants of freshwater bodies worldwide due to its high toxicity and ubiquity in freshwater ecosystems (U.S. EPA 2013). It occurs in very small amounts in pristine freshwater bodies, as a result of the microbial reduction of

nitrogen-containing compounds (EPA 2001). A sewage or agricultural contamination is indicated whenever ammonia-N concentrations exceed 0.129 mg L⁻¹ NH_4^+ in freshwater (EPA 2001). Although the average ammonia-N concentration in freshwater bodies has been decreasing in Europe since 1998, thus reflecting to some extent the effectiveness of the European Directives, it still remains higher than the natural level

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in several freshwater bodies because of agricultural practices and underpowered wastewater treatments (EEA 2010; EEA 2015). In freshwater bodies, the ammonia-N in excess contributes to eutrophication and is oxidized to ammonium hydroxide, which is extremely toxic to aquatic life at elevated pH levels (Ward 2013).

Ammonia-N itself has strong impacts on aquatic invertebrates (Camargo & Alonso 2006; Romano & Zeng 2013; U.S. EPA 2013; EEA 2015), as it alters the hemolymph pH, thus reducing hemolymph oxygen-carrying capacity due to progressive acidosis (Cheng et al. 2013). Moreover, it interferes with the activity of the Na^+/K^+ -ATPase pump, which is a major player in ammonia-N excretion in aquatic invertebrates (Furriel et al. 2004). Osmoregulatory and circulatory activities of invertebrates, as well as the normal metabolic functioning, are altered under exposure to high ammonia-N concentrations (Romano & Zeng 2013).

Freshwater copepods represent the most diverse taxon in groundwater and dependent ecosystems, such as springs (Galassi et al. 2014; Stoch et al. 2016), hyporheic zones (Di Lorenzo et al. 2013; Caschetto et al. 2014), alluvial (Di Lorenzo & Galassi 2013) and karstic aquifers (Gibert et al. 1994; Malard et al. 1994; Brancelj & Culver 2005; Dole-Olivier et al. 2009; Meleg et al. 2011). In surface water bodies copepods are a key group, linking primary production with upper trophic levels (Perlmutter et al. 1991; Schmid-Araya & Schmid 2000; Transpurger et al. 2006; Muschiol et al. 2008a; Muschiol et al. 2008b). An acute exposure to high concentrations of ammonia-N has proven to affect the survival of these crustaceans (Di Lorenzo et al. 2014). Under chronic exposure to sub-lethal ammonia-N concentrations, laboratory reared freshwater copepods have showed delayed developmental rates (Di Marzio et al. 2013), as well as lower taxonomic richness and population abundances in the field (Di Lorenzo et al. 2015a). However, no metabolic studies have been performed yet to investigate the effect of ammonia-N on freshwater copepods.

The cyclopoid *Eucyclops serrulatus* (Fisher 1851) has a wide ecological niche overlapping with that of many other freshwater taxa (Alekseev et al. 2005). Until recently, *E. serrulatus* has been thought to be one of the few copepods truly distributed worldwide, with the exception of Antarctica. However, Alekseev & Defaye (2011) recently formulated a new hypothesis on the actual distribution of this species. The distribution range of *E. serrulatus sensu stricto* is limited to Eastern Siberia in the East, to the Iberian Peninsula in the West, to the islands of the Arctic seas in the North, and to North Africa, Middle Asia, Turkey,

Iran, Afghanistan, Kazakhstan, Uzbekistan and Mongolia in the South (Alekseev & Defaye 2011). The species seems not to be present either in the Far East or in South-East Asia. As suggested by Karanovic (2005), several records of this species outside this area, such as in Australia, Canada and U.S.A., are likely the result of recent introductions, possibly in relation to human activities. It mostly lives in surface water bodies, including pelagic and benthic habitats of lakes and streams. However, it is also commonly found in groundwater, in alluvial (Di Lorenzo & Galassi 2013; Di Lorenzo et al. 2015a) and karstic aquifers, in springs (Galassi et al. 2014; Stoch et al. 2016) and in hyporheic zones (Fischer 1851; Alekseev et al. 2005; Di Lorenzo et al. 2013). Its wide distribution and ecological niche, short life-cycle, suitability to be easily reared in the laboratory (Nandini & Sarma 2007) and the availability of genetic resources (Baratti et al. 2015) make *E. serrulatus* a good target species for routine biomonitoring.

The aim of this study was to assess if short-term exposures to different sub-lethal concentrations of ammonia-N affect the metabolic rates of individuals of *E. serrulatus*. We measured oxygen consumption as a proxy of a metabolic response to stress (Calow 1991). We also measured the respiration rates of the juveniles separately from those of the adults in order to assess whether the metabolic cost differed between the two stages.

Methods

Collection and stock culture

We collected individuals of *E. serrulatus* in a shallow bore (coordinates: 43° 49' 02.61" N, 11° 11' 59.79" E; mean water table depth: 3 m), located in the Quaternary alluvial aquifer of Medio Valdarno (Tuscany, Italy), in March 2014. The bore was provided with a creek casing opened at the top. No contaminants were detected in the bore water (see Di Lorenzo et al. 2015b for details about the bore water chemistry). A basket-sampler provided with a 60 μm -mesh net was used to collect the copepods from the bottom and the water column of the bore. After collection, samples were transported to the laboratory within 15 minutes and sorted under a stereomicroscope at 12 \times magnification. Ovigerous females were picked up one by one with a glass pipette and each loaded in a 100 mL glass chamber. The chambers were filled with the bore water and maintained at room temperature. No additional food was offered in order to let the ovigerous females maintain the same diet as it was in the field. After the eggs hatched, each female was dissected on a slide and identified to species level under an optical microscope at 100 \times magnification. Offspring of the females belonging to species other than *E. serrulatus* were brought back to the bore. The hatched nauplii of *E. serrulatus* were pooled in a 2 L plastic beaker. The beaker was filled with the bore water and

maintained at room temperature. The bore water was changed weekly. Neither aeration nor additional food were provided. The offspring of the parental generation of the stock culture were used as the experimental pool of conspecific individuals.

Test solutions

Ammonia-N was dosed as NH_4^+ from ammonium nitrate (pure crystalline solid, CAS: 6484-52-2), provided by Regione Toscana in the form used for crop fertilization. A stock solution ($50 \text{ mg L}^{-1} \text{ NH}_4^+$) was prepared dissolving the appropriate weights of ammonium nitrate in a standard water (pH: 7.4, electrical conductivity: $415 \mu\text{S cm}^{-1}$, HCO_3^- : 301, Ca^{2+} : 48.6, Mg^{2+} : 28.2, SiO_2 : 15.2, NO_3^- : 8.5, Na^+ : 5.8, SO_4^{2-} : 4.1, K^+ : 1, $\text{F}^- < 0.15$, $\text{NH}_4^+ = 0$, all expressed in mg L^{-1}) and stored at 4°C . The stock solution concentration was measured by the method UNICHIM M.U. 941: 1995 (the limit of quantification was equal to $20 \mu\text{g L}^{-1}$). Nominal working solutions were prepared from the stock solution by appropriate dilutions.

Respiration trials

Respiration tests were carried out at 15°C , because that is the mean annual temperature of the bore water (Di Lorenzo et al. 2015b). The trials were performed on two different developmental stages, namely adults and juveniles (C1-C5 copepodids). Two different test concentrations (TC) and a control were used for each stage. According to US EPA guidelines (US EPA 2012), TC were designated on the basis of the MATC (maximum acceptable toxicant concentration; Rand 1995) so that:

$$\text{TC} = \text{MATC} = \text{LC50}/\text{ACR}$$

where LC50 is the lethal concentration to 50% of the exposed individuals of *E. serrulatus* at 15°C after 96 h reported in Di Lorenzo et al. (2014; 2015c) and ACR is the acute-to-chronic ratio (Kenaga 1982). In detail, the highest working concentrations (HWC) were set as:

$$\text{HWC} = \text{LC50}/5$$

and the lowest working concentrations (LWC) were set as:

$$\text{LWC} = \text{LC50}/20$$

The ACR equal to 5 and 20 were chosen arbitrarily in the range 1–20 assessed by Kenaga (1982) for aquatic species and organic chemicals. To ease the comparisons with data from other papers and studies, in Table 1 we expressed the working concentrations as $\text{mg L}^{-1} \text{ NH}_4^+$ and as $\text{mg L}^{-1} \text{ TAN}$ (total ammonia nitrogen: $\text{NH}_4^+-\text{N}+\text{NH}_3-\text{N}$).

Table 1. Test concentrations (LWC: lowest working concentration; HWC: highest working concentration) expressed in mg L^{-1} of NH_4^+ and mg L^{-1} of TAN (total ammonia nitrogen) for adults and copepodids of *Eucyclops serrulatus*.

		NH_4^+	TAN ($\text{NH}_4^+-\text{N}+\text{NH}_3-\text{N}$)
adults	LWC	3	2.31 ± 0.016
	HWC	12	9.24 ± 0.063
copepodids	LWC	1	0.77 ± 0.005
	HWC	4	3.08 ± 0.021

The individuals required for the respirometric trials were picked up with a glass pipette from the stock culture, pooled in a 500 mL glass baker and acclimated for three days at 15°C in the appropriate medium (standard water or test solutions) prior to measurements. No food was offered during acclimation in order to allow gut emptying (see Di Lorenzo et al. 2015b for details). After acclimation, only the actively swimming individuals were selected for testing. The copepodids were picked up on day 25 and the adults on day 35 from the start of the stock culture.

Two treatment and one control (standard water without toxicant) trials were set up for each developmental stage, for a total of six trials (Table 2), with 8 replicates each, in June 2014. In each trial, standard respiration rates (SRRs) were measured simultaneously in 8 sealed glass respirometric chambers, each containing 2 mL of the appropriate oxygen-saturated medium (standard water or test solutions). SRRs were measured by PSt3 (PreSens Regensburg, Germany) optical oxygen sensors. Each sensor was glued to the inside wall of the respirometer and connected to a single-channel oxygen transmitter Fibox 3 (PreSens) via an optical fibre. Data were recorded using the FibSoft v.1.0 software (Loligo Systems ApS, Tjele, Denmark). More information about the oxygen sensor device, data recording and calibration, are given in Di Lorenzo et al. (2015b). The chambers were placed in a 15°C water bath and kept in the dark. Each glass respirometer contained 25 *E. serrulatus* copepodids or 20 adults, according to Di Lorenzo et al. (2015b). A procedural control chamber filled with standard water or test solutions without animals was run at each trial to account for background oxygen depletion. For each replicate in a trial, the oxygen consumed in each respirometer was corrected by the oxygen depletion in the empty chamber. Measurements were taken every 30 minutes during 3-h-incubations, after discarding an initial 30 minutes stabilization period. At the end of each trial, the chambers were opened and copepods were counted

Table 2. Respiration rates and size of adults and copepodids of *Eucyclops serrulatus* at 15°C for each trial (c: copepodids; a: adults). C: control; TC: test concentration; LWC: lowest working concentration; HWC: highest working concentration. Values are represented as mean \pm SD (for $n = 8$).

Trial C or TC	copepodids			adults		
	1c C	2c LWC	3c HWC	1a C	2a LWC	3a HWC
SRR ($\mu\text{g O}_2 \text{ ind}^{-1} \text{ h}^{-1}$)	0.0153 ± 0.0084	0.0380 ± 0.0159	0.0718 ± 0.0431	0.0418 ± 0.0134	0.0366 ± 0.0095	0.0791 ± 0.0122
Lm (mm)	0.4151 ± 0.0951	0.5184 ± 0.0230	0.4072 ± 0.1547	0.7060 ± 0.0557	0.6856 ± 0.1043	0.6748 ± 0.0646
DM (mg)	0.0031 ± 0.0004	0.0033 ± 0.0003	0.0022 ± 0.0014	0.0049 ± 0.0017	0.0055 ± 0.0004	0.0051 ± 0.0009
Wm (mm)	0.2075 ± 0.0476	0.2901 ± 0.0115	0.2333 ± 0.0774	0.3530 ± 0.0279	0.3428 ± 0.0521	0.3374 ± 0.0323

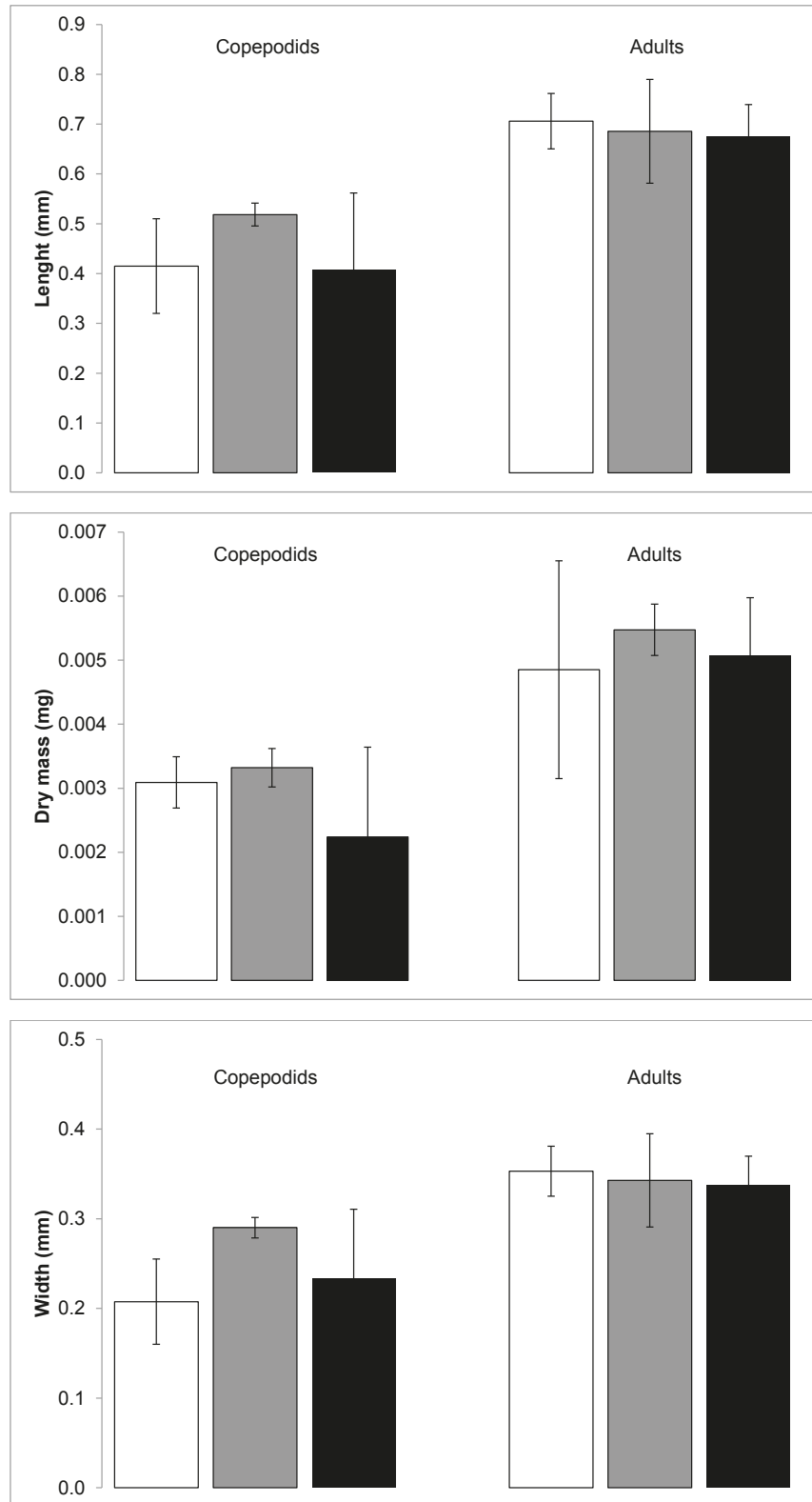


Fig. 1. Mean \pm SD length, width and dry mass of copepodids and adults of *Eucyclops serrulatus* in the control (white bars), LWC (light grey bars) and HWC (black bars) treatment. Length, width and dry mass were significantly different between copepodid and adult stages (respectively: Pseudo- $F_{1,40}$: 73.72, p -value = 0.0001; Pseudo- $F_{1,40}$: 73.77, p -value = 0.0001; Pseudo- $F_{1,40}$: 53.69, p -value = 0.0001) but there were no significant differences in size within each developmental stage for each treatment.

under a stereomicroscope at 12× magnification and checked for mortality (no movement after gentle stimulation by means of a sorting needle). No dead individuals were detected at the end of the trials. Hence, the oxygen depletion recorded in each chamber was divided by the initial number of individuals in the respirometer in order to obtain SRRs in $\mu\text{g O}_2 \text{ ind}^{-1} \text{ h}^{-1}$. At the end of each trial, the prosome length (L, mm), width (W, mm) and the dry mass (DM, mg) of each individual, for a total of 1215 specimens, were measured (see Di Lorenzo et al. 2015 b for details).

Statistical analyses

To test for differences in size (L, W and DM) among trials for each stage and between the two developmental stages, we used three two-way permutational analyses of variance (permutational ANOVA; Anderson 2001), with crossed design, namely with “treatment” (three levels: C = control, LWC = lowest working concentration, HWC = highest working concentration) and “developmental stage” (two levels: adults, copepodids), as the grouping factors, both fixed and orthogonal. The PERMANOVA was done on the basis of the Euclidean Distance similarity matrix and using non-transformed data, after performing a Levene test on the original dataset. To test for differences in the SRRs among trials for each stage and between the two developmental stages, we used a two-way permutational analysis of covariance (permutational ANCOVA; Anderson 2001), with individual DM as the covariate, and “treatment” and “developmental stage” as the grouping factors, both fixed and orthogonal. The permutational analysis was performed on the basis of the Euclidean Distance similarity matrix and using

square root transformed data, applied after performing a Levene test on the original dataset. Post hoc t-tests were applied when appropriate to test for differences between levels within factors.

Prior to all the statistical analyses, the outliers with the absolute values of z-score, corrected by the median absolute deviation, greater than 3.5 were excluded according to Iglewicz & Hoaglin (1993). The distributions of the remaining data were checked by a Levene test for equality of variances. When required, data were square root transformed.

All the statistical tests were performed using PRIMER v.6 and PERMANOVA+routines for PRIMER (Anderson et al. 2008). The level of significance for all of the critical ranges was set to $p < 0.05$.

Results

Respirometric trials

L, W and DM (Table 2, Fig. 1) were significantly different between copepodid and adult stages (“developmental stage” factor; Table 3). The factor “treatment” and its interaction with the factor “developmental stage” were not significant (Table 3).

The SRRs did not vary with the dry mass (Pseudo- $F_{1,36} = 1.12$, $p = 0.3022$). A significant difference was found in SRRs according to the interaction between “treatment” and “developmental stage” fac-

Table 3. PERMANOVA results table for length, dry mass and width. df: degrees of freedom; SS: sum of squares; MS: mean sum of squares; Pseudo-F: F value by permutation, P(perm): p -values based on more than 9000 permutations (the lowest possible p -value is 0.0001); Perms: number of permutations; St: stage; Tr: treatment.

Length						
Source	df	SS	MS	Pseudo-F	P(perm)	Perms
St	1	0.66518	0.66518	73.772	0.0001	9834
Tr	2	2.83 E-02	1.41 E-02	15.668	0.2159	9945
StxTr	2	3.12 E-02	1.56 E-02	17.314	0.1897	9960
Res	40	0.36067	9.02 E-03			
Total	45	1.1233				
Dry mass						
Source	df	SS	MS	Pseudo-F	P(perm)	Perms
St	1	5.75 E-05	5.75 E-05	53.694	0.0001	9811
Tr	2	4.04 E-06	2.02 E-06	1.8889	0.1616	9953
StxTr	2	2.36 E-06	1.18 E-06	1.1041	0.3456	9949
Res	40	4.28 E-05	1.07 E-06			
Total	45	1.09 E-04				
Width						
Source	df	SS	MS	Pseudo-F	P(perm)	Perms
St	1	0.1663	0.1663	73.772	0.0001	9837
Tr	2	7.06 E-03	3.53 E-03	1.5668	0.2242	9950
StxTr	2	7.81 E-03	3.90 E-03	1.7314	0.1958	9952
Res	40	9.02 E-02	2.25 E-01			
Total	45	0.28083				

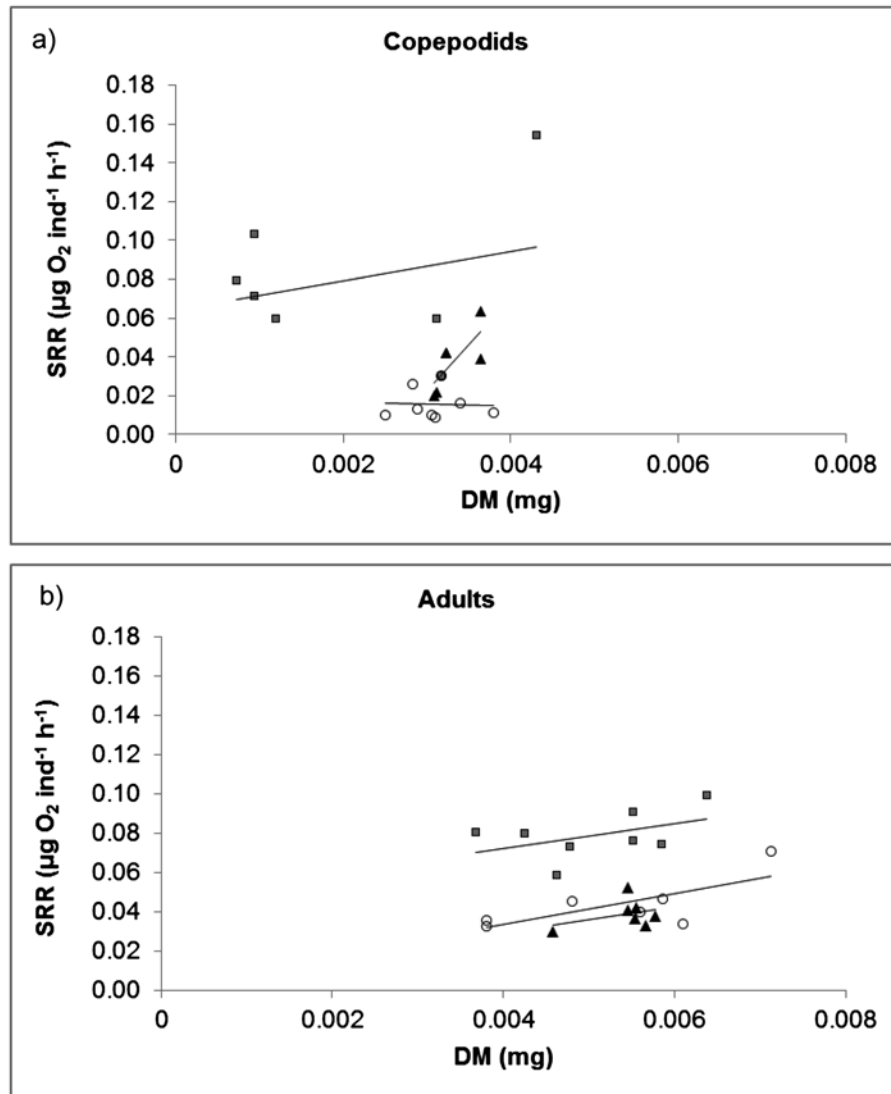


Fig. 2. Standard respiration rates (SRR) of copepodids (a) and adults (b) of the freshwater cyclopoid *Eucyclops serrulatus*, recorded in the laboratory at 15 °C, in the control (white dots) and under LWC (black triangles) and HWC (grey squares) treatments. The SRRs of copepodids were significantly different at the three treatments, increasing with NH_4^+ concentration. The SRRs of adults were significantly different between the control and the HWC treatment and were significantly higher under HWC than under LWC treatment. However, the SRRs of adults under LWC treatment were not significantly different from those under control.

tors (Pseudo- $F_{2,36} = 3.57$, $p = 0.0331$). The SRRs of copepodids were significantly different at the three treatments (C,HWC: $t = 4.34$, $p = 0.0018$; C,LWC: $t = 2.88$, $p = 0.0144$; LWC,HWC: $t = 2.54$, $p = 0.0236$), increasing with NH_4^+ concentration (Fig. 2a). The SRRs of adults (Fig. 2b) were significantly different between the control and the HWC treatment ($t = 6.78$, $p = 0.0005$) and between LWC and HWC treatments ($t = 8.32$, $p = 0.0001$). However, the SRRs of adults under LWC treatment were not significantly different from the control ones ($t = 1.32$, $p = 0.2050$; Fig. 2b).

Discussion

As a general prediction, the metabolic rate of an organism, measured as respiration rate, varies under a toxicant exposure (Calow 1989; Calow 1991; Calow & Sibly 1990; Rowe et al. 1998; Hopkins et al. 1999). Moreover, respiration rates are supposed to increase with an increasing exposure time and/or toxicant concentration, at least until repairing mechanisms occur (Calow 1989; Hopkins et al. 1999). Few exceptions to this rule are known. For instance, Knops et al. (2001)

observed that an 8-day exposure to the heavy metals cadmium and copper or to the cationic surfactant, cetyltrimethylammonium bromide, did not change the metabolic rates of exposed daphnids as it would be expected. This was likely because the metabolic demand occurred early during the exposure, before the start of the respirometric measurements. In another study, Frontera et al. (2014) observed that the juveniles of the crayfish *Procambarus clarkii* showed lower metabolic rates than those measured in the control (tap water) after 60-day exposures to glyphosate, polyoxyethylene amine or a combination of both. On the contrary, in our trials the respiration rates of both copepodids and adults of the freshwater cyclopoid *E. serrulatus* significantly increased after a 3-day exposure to sublethal concentrations of ammonia-N, according to the general prediction. The relationship between increasing ammonia-N concentration and the SRRs of *E. serrulatus* supports the prediction that the exposure to ammonia-N is energetically costly to this species.

Both environmental NH_3 and NH_4^+ diffuse across the cell membrane via the lipid pathway (Golchini & Kurtz 1988; Wright 1995). As a dissolved gas, NH_3 diffuses faster than NH_4^+ across the lipid bilayers of the cell. Therefore, it is considered more toxic than the ionized ammonia (Romano & Zeng 2013). However, NH_3 is protonated to NH_4^+ once inside the cells, thus triggering the variation of the hemolymph pH (Romano & Zeng 2013). An alteration of the oxygen consumption due to changes of hemolymph pH has been observed in several marine crustacean species (Colt & Armstrong 1981; Aarset & Aunaas 1990; Romano & Zeng 2013). The outcomes of our study highlighted that the exposure to sublethal ammonia-N concentrations triggered an increasing of SRRs in the individuals of *E. serrulatus*. The SRRs of both adults and copepodids increased under ammonia-N exposure. For each stage, the size of the individuals was not different among treatments proving that the trials were not biased by the size of the individuals and that the methodology applied in this study is suitable for respiratory routine experiments. However, adults of *E. serrulatus* experienced a significant energetic stress only at $12 \text{ mg L}^{-1} \text{NH}_4^+$. On the contrary, the copepodid SRRs increased under the exposure to $1 \text{ mg L}^{-1} \text{NH}_4^+$; that is at a concentration of just one order of magnitude greater than the current EEA threshold value (0.129 mg L^{-1}). Moreover, under the exposure to $3 \text{ mg L}^{-1} \text{NH}_4^+$ the SRRs of the copepodids were comparable to the SRRs of the adults exposed to $12 \text{ mg L}^{-1} \text{NH}_4^+$. The higher metabolic sensitivity of copepodids compared to adults is likely due to the different thickness

of the body exoskeleton (Gutierrez et al. 2010; Di Lorenzo et al. 2015c). Given the absence of food, as set for our trials, the main route of intoxication is through contact with the body surface (Gutierrez et al. 2010), as the thinness of juvenile exoskeleton may favor the diffusion of NH_3 and NH_4^+ across the lipid bilayers of the cells. Moreover, Romano & Zeng (2013) observed that the adult stages of aquatic crustaceans show more efficient detoxification mechanisms than juveniles, thus allowing the maintenance of lower ammonia-N levels in the hemolymph compared to those measured in the juveniles.

Both the inhibition and the stimulation of the metabolism are adverse to copepod physiology (Ylönen et al. 2004). Inhibition of metabolic activities could threaten survival while an increase in metabolism could significantly reduce the energetic reserve available for growth, reproduction and immunity (Ylönen et al. 2004). A 2-day exposure to ammonia-N up to $80 \text{ mg L}^{-1} \text{NH}_3 + \text{NH}_4^+$ triggered the accumulation of NH_4^+ in the hemolymph of the juvenile crab *Eriocheir sinensis*, resulting in an increase in the energy demand and in a depression of the immune capacity (Hong et al. 2007). A 2-day exposure to an ambient ammonia-N concentration higher than $5 \text{ mg L}^{-1} \text{NH}_3 + \text{NH}_4^+$ interrupted coagulation and down-regulated the TG gene expression in the shrimp *Litopenaeus vannamei*, which caused immune deficiencies, thus contributing to the increased susceptibility to infection by pathogens (Chang et al. 2015). A significant deviation from the post-naupliar developmental time of unexposed control cohorts was observed for copepodids of freshwater copepod species exposed to sub-lethal concentrations of ammonia-N (Di Marzio et al. 2013). The developmental time went up from 3.5 % to 33 % with respect to the controls, affecting the age structure and size of the exposed experimental cohorts (Di Marzio et al. 2013). The assemblages of freshwater crustaceans inhabiting freshwater bodies contaminated by ammonia-N were significantly different from those of pristine reference sites within the same freshwater bodies of France and Italy (Dehedin et al. 2012; Di Lorenzo & Galassi 2013; Di Lorenzo et al. 2014). Moreover, both field surveys on amphipod natural populations and laboratory experiments with *E. serrulatus* have demonstrated that additional stressors, such as insecticides and herbicides, significantly enhanced the detrimental effect of ammonia-N on these taxa (Dehedin et al. 2012; Dehedin et al. 2013; Di Lorenzo et al. 2015c).

Proteomic studies highlighted that an oxidative stress could enhance the production of reactive oxygen species (ROS), accelerating senescence in sev-

eral taxa (Monaghan et al. 2009). Considering that a female of *E. serrulatus* produces an average number of three clutches over 35 days after becoming adult at 15 °C (personal observation), the senescence acceleration could result in a drastic reduction of reproduction rates. Moreover, Rubolini et al. (2006) suggested that there could be a connection between the maternal oxidative balance and the offspring quality in birds, since females allocate diverse antioxidants to eggs that protect the embryo from oxidative stress. This maternal heritage has a positive effect on offspring development and growth (Rubolini et al. 2006). Accordingly, Vehmaa et al. (2015) observed that the females of the marine copepod *Acartia bifilosa* with the highest antioxidant capacity produced eggs with the highest hatching success. Given that this could also apply to freshwater copepods, the oxidative stress could significantly affect *E. serrulatus* populations, as the hatching success of this species ranges between 70 % to 75 % in non-stressed conditions and when fed *ad libitum* (personal observation).

According to WATERBASE, which is the European Environmental Agency database of the quantitative status and quality of European rivers, lakes, groundwater bodies and transitional, coastal and marine waters (www.eea.europa.eu/data-and-maps/data/waterbase-transitional-coastal-and-marine-waters-8), 67% (2788) of river monitoring sites in Europe can be considered not contaminated by ammonia-N from 2000 to 2011. Our results indicate that *E. serrulatus* adults would not be expected to suffer a metabolic stress, at least under short exposures, if they occur in these sites, since SRRs did not vary when compared to those of the control when adults were exposed to 3 mg L⁻¹ NH₄⁺. However, this favorable scenario might not apply to copepodids of *E. serrulatus*. Copepodid SRRs at 1 mg L⁻¹ NH₄⁺ were significantly higher (2 times) than those in the control, and we did not measure copepodid SRRs at concentrations ranging from 0 to 1 mg L⁻¹ NH₄⁺. According to these outcomes, the river monitoring sites, which are not contaminated by ammonia-N in Europe upon the current legislation, should be prudently considered at risk to the juveniles of this species.

According to the provisions of Section 304(a) of the Clean Water Act, U.S. EPA has recently updated the freshwater ammonia aquatic life ambient water quality criteria, through revising scientific papers published from 1985 to 2012 (EPA 2013). The new criteria have set the thresholds at 17 mg L⁻¹ TAN for one-hour acute exposure and at 1.9 mg L⁻¹ TAN for 30-days rolling average chronic exposure, corresponding to 16.93 mg

L⁻¹ NH₄⁺-N+0.07 mg L⁻¹ NH₃-N and to 1.89 mg L⁻¹ NH₄⁺-N+0.008 mg L⁻¹ NH₃-N respectively, at 20 °C and pH 7.0. According to the results of this study, it is evident that EPA's criteria are not sufficient to protect the adults of *E. serrulatus* from respiratory stress under acute exposures and the juveniles of this species from both acute and chronic exposures to ammonia-N.

Conclusions

Our results revealed that coping with sublethal ammonia-N concentrations has an energetic cost for the copepod species *E. serrulatus*. Further investigations are needed to assess whether the copepodids, and even more importantly, the naupliar stages of this species, suffer from metabolic stress at ammonia-N concentrations near to or even lower than the threshold value set by the EEA. Additional testing is needed with other copepod species to determine how common the increase of the metabolic demand in contaminated habitats is. Moreover, the introduction of emerging contaminants or complex mixtures of pollutants into freshwater ecosystems, as well as the rise in temperature due to climate change, may subject freshwater copepods to additional physiological stress. Since copepods are a link between primary producers and higher consumers, variation in their population dynamics will affect the whole freshwater ecosystem due to a metabolic stress under sub-lethal conditions. Therefore, the effects of ammonia-N pollution, which affects about 40 % of the rivers and streams in Europe, should be taken into greater consideration for proper and effective environmental management strategies.

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References

- Aarset, V. & Aunaas, T., 1990: Effects of osmotic stress on oxygen consumption and ammonia excretion of the Arctic sympagic amphipod *Gammarus wilkitzkii*. – Mar. Ecol. Prog. Ser. **58**: 217–224.
- Alekseev, V. & Defaye, D., 2011: Taxonomic differentiation and world geographical distribution of the *Eucyclops serrulatus* group (Copepoda, Cyclopidae, Eucyclopinæ). – In: Defaye, D., Suárez-Morales, E. & Von Vaupel Klein, C. (eds): Studies on Freshwater Copepoda: a Volume in Honour of Bernard Dussart. Crustaceana Monographs 16. – Brill EJ, Amsterdam, pp. 41–72.

- Alekseev, V., Dumont, J. H., Pensaert, J., Baribwegure, D. & Vanfleteren, J. R., 2005: A redescription of *Eucyclops serrulatus* (Fischer, 1851) (Crustacea: Copepoda: Cyclopoida) and some related taxa, with a phylogeny of the *E. serrulatus*-group. – *Zool. Script.* **35**: 123–147.
- Anderson, M. J., Gorley, R. N. & Clarke, K. R., 2008: PERMANOVA+for PRIMER: Guide to software and statistical Methods. – PRIMER-E Ltd, Plymouth, UK.
- Anderson, M. J., 2001: A new method for non-parametric multivariate analysis of variance. – *Austral. Ecol.* **26**: 32–46.
- Baratti, M., Cattonaro, F., Di Lorenzo, T., Galassi, D. M. P., Iannilli, V., Iannucci, A., Jensen, J., Larsen, P. F., Nielsen, R. O., Pertoldi, C., Postolache, D., Pujolar, J. M., Randi, E., Ruiz-Gonzalez, A., Thirstrup, J. P., Vendramin, G. G. & Zalewski, A., 2015: Genomic Resources Notes Accepted 1 October 2014–30 November 2014. – *Mol. Ecol. Resour.* **15** (2): 458–459.
- Brancelj, A. & Culver, D. C., 2005: Epikarst communities. – In: Culver, D. C. & White, W. B. (eds): *Encyclopedia of caves*. – Elsevier Academic Press, Amsterdam, pp. 223–229.
- Calow, P., 1991: Physiological costs of combating chemical toxicants: ecological implications. – *Comp. Biochem. Physiol. C.* **100**: 3–6.
- Calow, P., 1989: Physiological ecotoxicology: theory, practice and application. – In: Lokke, H., Tyle, H. & Bro-Rasmussen, F. (eds): *Proceedings of the First European Conference on Ecotoxicology*. – Conference Organizing Committee, Lyngby, Denmark, pp. 23–25.
- Calow, P. & Sibly, R. M., 1990: A physiological basis of population processes: Ecotoxicological implications. – *Funct. Ecol.* **4** (3): 283–288.
- Camargo, J. A. & Alonso, A., 2006: Ecological and ecotoxicological effects of inorganic nitrogen pollution in aquatic ecosystems: a global assessment. – *Environ. Int.* **32**: 831–849.
- Caschetto, M., Barbieri, M., Galassi, D. M. P., Mastrotrillo, L., Rusi, S., Stoch, F., Di Cioccio, A. & Petitta, M., 2014: Human alteration of groundwater–surface water interactions (Sagittario River, Central Italy): implication for flow regime, contaminant fate and invertebrate response. – *Environ. Earth Sci.* **71**: 1791–1807.
- Chang, Y. L., Hsieh, M. H., Chang, W. W., Wang, H. Y., Lin, M. C., Wang, C. P., Lou, P. J. & Teng, S. C., 2015: Instability of Succinate Dehydrogenase in SDHD Polymorphism Connects Reactive Oxygen Species Production to Nuclear and Mitochondrial Genomic Mutations in Yeast. – *Antioxid. & Redox Signal* **22** (7): 587–602.
- Cheng, S. Y., Shieh, L. W. & Chen, J. C., 2013: Changes in hemolymph oxyhemocyanin, acid-base balance, and electrolytes in *Marsupenaeus japonicus* under combined ammonia and nitrite stress. – *Aquat. Toxicol.* **130–131**: 132–138.
- Colt, J. E. & Armstrong, D. A., 1981: Nitrogen toxicity to crustaceans, fish and molluscs. – In: Allen, L. J. & Kinney, E. C. (eds): *Proceeding of the Bio-Engineering Symposium for Fish Culture*, Fish Culture Section. – American Fisheries Society, Northeast Society of Conservation Engineers, Bethesda, pp. 34–47.
- Dehedin, A., Maazouzi, C., Puijalon, S., Marmonier, P. & Piscart, C., 2013: The combined effects of water level reduction and an increase in ammonia concentration on organic matter processing by key freshwater shredders in alluvial wetlands. – *Glob. Chang. Biol.* **19**: 763–774.
- Dehedin, A., Piscart, C. & Marmonier, P., 2012: Seasonal variations of the effect of temperature on lethal and sublethal toxicities of ammonia for three common freshwater shredders. – *Chemosphere* **90**: 1016–1022.
- Di Lorenzo, T. & Galassi, D. M. P., 2013: Agricultural impacts on Mediterranean alluvial aquifers: do invertebrates respond? – *Fundam. Appl. Limnol.* **182** (4): 271–281.
- Di Lorenzo, T., Stoch, F. & Galassi, D. M. P., 2013: Incorporating the hyporheic zone within the river discontinuum: Longitudinal patterns of subsurface copepod assemblages in an Alpine stream. – *Limnologica* **43**: 288–296.
- Di Lorenzo, T., Di Marzio, W. D., Sáenz, M. E., Baratti, M., Dedonno, A. A., Iannucci, A., Cannicci, S., Messina, G. & Galassi, D. M. P., 2014: Sensitivity of hypogean and epigean freshwater copepods to agricultural pollutants. – *Environ. Sci. Pollut. Res.* **21**: 4643–4655.
- Di Lorenzo, T., Cifoni, M., Lombardo, P., Fiasca, B. & Galassi, D. M. P., 2015a: Ammonium threshold values for groundwater quality in the EU may not protect groundwater fauna: evidence from an alluvial aquifer in Italy. – *Hydrobiologia* **743** (1): 139–150.
- Di Lorenzo, T., Di Marzio, W. D., Spigoli, D., Baratti, M., Messana, G., Cannicci, S. & Galassi, D. M. P., 2015b: Metabolic rates of a hypogean and an epigean species of copepod in an alluvial aquifer. – *Freshw. Biol.* **60**: 426–435.
- Di Lorenzo, T., Di Marzio, W. D., Cifoni, M., Fiasca, B., Baratti, M., Sáenz, M. E. & Galassi, D. M. P., 2015c: Temperature effect on the sensitivity of the copepod *Eucyclops serrulatus* (Crustacea, Copepoda, Cyclopoida) to agricultural pollutants in the hyporheic zone. – *Curr. Zool.* **61** (4): 629–640.
- Di Marzio, W. D., Castaldo, D., Di Lorenzo, T., Di Cioccio, A., Sáenz, M. E. & Galassi, D. M. P., 2013: Developmental endpoints of chronic exposure to suspected endocrine-disrupting chemicals on benthic and hyporheic freshwater copepods. – *Ecotox. Environ. Safe.* **96**: 86–92.
- Dole-Olivier, M. J., Castellarini, F., Coineau, N., Galassi, D. M. P., Martin, P., Mori, N., Valdecasas, A. & Gibert, J., 2009: Towards an optimal sampling strategy to assess groundwater biodiversity: comparison across six European regions. – *Freshw. Biol.* **54**: 777–796.
- EEA-European Environmental Agency, 2010: The European environment-state and outlook 2010: synthesis report. – Publications Office of the European Union, European Environment Agency, Copenhagen, Luxembourg.
- EEA-European Environmental Agency, 2015: The European environment-state and outlook 2015: synthesis report. – Publications Office of the European Union, European Environment Agency, Copenhagen, Luxembourg.
- EPA-Environmental Protection Agency of Europe, 2001: Parameters of water quality. Interpretation and standards. – Environmental Protection Agency, Ireland, pp. 1–132. https://www.epa.ie/pubs/advice/water/quality/Water_Quality.pdf. Accessed 24 January 2015
- Fisher, S., 1851: Beiträge zur Kenntniss der in der Umgegend von St Petersburg sich findenden Cyclopiden. – *Bull. Soc. Nat. Moscou.* **24**: 409–438.
- Frontera, J. L., Gilette, N., Hull, B., Korang, J., Amorim, C., Vatnick, I. & Rodríguez, E. M., 2014: Effects of Glyphosate and Polyoxyethylene Amine on Metabolic Rate and Energy Reserves of *Procambarus clarkii* Juveniles. – *Open Environ. Sci.* **8**: 49–53.
- Furriel, R. P., Masur, D. C., McNamara, J. C. & Leone, P. A., 2004: Modulation of gill Na⁺, K⁺-ATPase activity by ammonium ions: putative coupling of nitrogen excretion and ion uptake in the freshwater shrimp *Macrobrachium olfersii*. – *J. Exp. Zool. A.* **301**: 63–74.

- Galassi, D. M. P., Lombardo, P., Fiasca, B., Di Cioccio, A., Di Lorenzo, T., Petitta, M. & Di Carlo, P., 2014: Earthquakes trigger the loss of groundwater biodiversity. – *Sci. Rep.* **4**: 1–8. doi: 10.1038/srep06273.
- Gibert, J., Vervier, P., Malard, F., Laurent, R. & Reygrobellet, J.-L., 1994: Dynamics of communities and ecology of karst ecosystems: example of three karsts in eastern and southern France. – In: Gibert, J., Danielopol, D. L. & Stanford, J. A. (eds): *Groundwater Ecology*. – Academic Press, Inc., San Diego, pp. 425–450.
- Golchini, K. & Kurtz, I., 1988: NH₃ permeation through the apical membrane of MDCK cells via a lipid pathway. – *Am. J. Physiol.* **255**: 135–141.
- Gutierrez, F. M., Gagneten, A. M. & Paggi, J. C., 2010: Copper and chromium alter life cycle variables and the equiproportional development of the freshwater copepod *Notodiptomus conifer* (SARS). – *Water Air Soil Poll.* **23** (1–4): 275–286.
- Hong, S., Lu, Y., Yang, R., Gotlinger, K. H., Petasis, N. A. & Serhan, C. N., 2007: Resolvin D1, protectin D1, and related docosahexaenoic acid-derived products: analysis via electrospray/low energy tandem mass spectrometry based on spectra and fragmentation mechanisms. – *J. Am. Soc. Mass Spectrom.* **18**: 128–144.
- Hopkins, W. A., Rowe, C. L. & Congdon, J. D., 1999: Elevated trace element concentrations and standard metabolic rate in banded water snakes, *Nerodia fasciata*, exposed to coal combustion wastes. – *Environ. Toxicol. Chem.* **18**: 258–263.
- Iglewicz, B. & Hoaglin, D., 1993 (eds): *How to detect and handle outliers*. – American Society of Quality, Statistics Division 16, Quality Press, Milwaukee, Wisconsin.
- Karanovic, T., 2005: Two new genera and three new species of subterranean cyclopoids (Crustacea, Copepoda) from New Zealand, with redescription of *Goniocyclops silvestris* Harding, 1958. – *Contrib. Zool.* **74**: 223–254.
- Kenaga, E. E., 1982: Predictability of chronic toxicity from acute toxicity of chemicals in fish and aquatic invertebrates. – *Environ. Toxicol. Chem.* **1** (4): 347–358.
- Knops, M., Altenburger, R. & Segner, H., 2001: Alterations of physiological energetics, growth and reproduction of *Daphnia magna* under toxicant stress. – *Aquat. Toxicol.* **53**: 79–90.
- Malard, F., Reygrobellet, J.-L., Mathieu, J. & Lafont, M., 1994: The use of invertebrate communities to describe groundwater flow and contaminant transport in a fractured rock aquifer. – *Arch. Hydrobiol.* **131**: 93–110.
- Meleg, I. N., Fiers, F. & Moldovan, O. T., 2011: Assessing copepod (Crustacea: Copepoda) species richness at different spatial scales in northwestern Romanian caves. – *Subterr. Biol.* **9**: 103–112.
- Monaghan, P., Metcalfe, N. B. & Torres, R., 2009: Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. – *Ecol. Lett.* **12** (1): 75–92.
- Muschiol, D., Markovic, M., Threis, I. & Traunspurger, W., 2008a: Predator-prey relationship between the cyclopoid copepod *Diacyclops bicuspidatus* and a free-living bacterivorous nematode. – *Nematology* **10**: 55–62.
- Muschiol, D., Markovic, M., Threis, I. & Traunspurger, W., 2008b: Predatory copepods can control nematode populations: A functional response experiment with *Eucyclops subterraneus* and bacterivorous nematodes. – *Fundam. Appl. Limnol.* **172**: 317–324.
- Nandini, S. & Sarma, S. S. S., 2007: Effect of algal and animal diets on life history of the freshwater copepod *Eucyclops serrolatus* (Fischer, 1851). – *Aquat. Ecol.* **41**: 75–84.
- Perlmutter, D. G. & Meyer, J. L., 1991: The impact of a streamdwelling harpacticoid copepod upon detritally associated bacteria. – *Ecology* **72**: 2170–2180.
- Romano, N. & Zeng, C., 2013: Toxic effects of ammonia, nitrite, and nitrate to decapod crustaceans: A review on factors influencing their toxicity, physiological consequences, and coping mechanisms. – *Rev. Fish. Sci.* **21** (1): 1–21.
- Rand, G. M., 1995 (eds): *Fundamentals of aquatic toxicology: Effects, environmental fate, and risk assessment*. 2nd edition. – Taylor & Francis, Washington, DC, pp. 1–1125.
- Rowe, C. L., Kinney, O. M., Nagle, R. D. & Congdon, J. D., 1998: Elevated maintenance costs in an anuran (*Rana catesbeiana*) exposed to a mixture of trace elements during the embryonic and early larval periods. – *Physiol. Zool.* **71**: 27–35.
- Rubolini, D., Romano, M., Bonisoli-Alquati, A. & Saino, N., 2006: Early maternal, genetic and environmental components of antioxidant protection, morphology and immunity of yellow-legged gull (*Larus michahellis*) chicks. – *J. Evol. Biol.* **19** (5): 1571–1584.
- Schmid-Araya, J. M. & Schmid, P. E., 2000: Trophic relationships: integrating meiofauna into a realistic food web. – *Freshw. Biol.* **44**: 149–163.
- Stoch, F., Fiasca, B., Di Lorenzo, T., Porfirio, S., Petitta, M. & Galassi, D. M. P., 2016: Exploring copepod distribution patterns at three nested spatial scales in a spring system: habitat partitioning and potential for hydrological bioindication. – *J. Limnol.* **75** (1): 1–13.
- Traunspurger, W., Bergtold, M., Ettemeyer, A. & Goedkoop, W., 2006: Effects of copepods and chironomids on the abundance and vertical distribution of nematodes in a freshwater sediment. – *J. Freshwater Ecol.* **21**: 81–90.
- U.S. EPA, 2012: *Technical Overview of Ecological Risk Assessment: Risk Characterization*. – US Environmental Protection Agency [online]. Available at: <http://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/technical-overview-ecological-risk-assessment-risk>. Accessed 23 February 2016.
- U.S. EPA, 2013: *Aquatic Life Ambient Water Quality Criteria for Ammonia – Freshwater 2013*. – Office of Water, Office of Science and Technology, Washington D.C., EPA-822-R-13-001. <http://water.epa.gov/scitech/swguidance/standards/criteria/aqlife/ammonia/upload/AQUATIC-LIFE-AMBIENT-WATER-QUALITY-CRITERIA-FOR-AMMONIA-FRESHWATER-2013.pdf>. Accessed 27 June 2015.
- Vehmaa, A., Almén, A.-K., Brutemark, A., Paul, A., Riebesell, U., Furuhaugen, S. & Engström-Öst, J., 2015: Ocean acidification challenges copepod reproductive plasticity. – *Biogeosci. Discuss.* **12**: 18541–18570.
- Ward, B. B., 2013: How Nitrogen Is Lost. – *Science* **341** (6144): 352–353.
- Wright, P. A., 1995: Nitrogen excretion: three and products, many physiological roles. – *J. Exp. Biol.* **273**: 273–281.
- Ylönen, I., Heikkilä, J. & Karjalainen, J., 2004: Metabolic depression in UV-beta exposed larval coregonids. – *Ann. Zool. Fenn.* **41**: 577–585.