Environ Sci Pollut Res
DOI 10.1007/s11356-013-2390-6

RESEARCH ARTICLE

Sensitivity of hypogean and epigean freshwater copepods to agricultural pollutants

T. Di Lorenzo · W. D. Di Marzio · M. E. Sáenz · M. Baratti · A. A. Dedonno · A. Iannucci · S. Cannicci · G. Messana · D. M. P. Galassi

Received: 13 September 2013 / Accepted: 20 November 2013

Abstract Widespread pollution from agriculture is one of the major causes of the poor freshwater quality currently observed across Europe. Several studies have addressed the direct impact of agricultural pollutants on freshwater biota by means of laboratory bioassays; however, as far as copepod crustaceans are concerned, the ecotoxicological research is scarce for freshwater species and almost nonexistent for the hypogean ones. In this study, we conducted a comparative analysis of the available literature data on the sensitivity of freshwater copepods to agricultural pollutants. We also assessed the acute and chronic sensitivity of a hypogean and an epigean species, both belonging to the Crustacea Copepoda Cyclopoida Cyclopidae, to two N-fertilizers (urea and ammonium nitrate) and two herbicides (ARIANE^{TM} II from Dow AgroSciences LLC, and Imazamox), widely used for cereal agriculture in Europe. According to the literature review, freshwater copepods are sensitive to a range of pesticides and N-fertilizers. Ecotoxicological studies on hypogean species of copepods account only one study. There are no standardized protocols available for acute and chronic toxicity tests for freshwater copepods, making comparisons about sensitivity difficult. From our experiments, ionized ammonia proved to be more toxic than the herbicide Imazamox, in both short and chronic bioassays. Urea was the less toxic chemical for both species. The hypogean species was more sensitive than the epigean one to all chemicals. For both species and for all tested chemicals, acute lethality and chronic lethality were induced at concentrations higher than the law limits of good water body quality in Europe, except for ionized ammonia, which provoked the chronic lethality of the hypogean species at a lower concentration. The hazardous concentration (HC) of unionized ammonia for 5 % of freshwater copepods, obtained by a species sensitivity distribution, was 92 μg l^{-1}, significantly lower than the HC computed for traditional test species from freshwater environments.

Keywords Copepod · Hypogean · Epigean · Groundwater · Sensitivity · Agricultural pollutant · Ammonia · Pesticide

Responsible editor: Henner Hollert

T. Di Lorenzo (✉) · M. Baratti · A. A. Dedonno · A. Iannucci · G. Messana
Istituto per lo Studio degli Ecosistemi—CNR, Via Madonna del Piano 10, Sesto Fiorentino, 50019 Florence, Italy
e-mail: tiziana.dilorenzo@ise.cnr.it

W. D. Di Marzio · M. E. Sáenz
Programa de Investigación en Ecotoxicología, Departamento de Ciencias Básicas, Universidad Nacional de Luján—Comisión Nacional de Investigaciones Científicas y Técnicas CONICET, Buenos Aires, Argentina

A. A. Dedonno · A. Iannucci · S. Cannicci
Department of Biology, University of Florence, Via Madonna del Piano 6, Sesto Fiorentino, 50019 Florence, Italy

D. M. P. Galassi
Department of Life, Health and Environmental Sciences, University of L'Aquila, Via Vetoio, Coppito, 67100 L'Aquila, Italy

Introduction

Widespread pollution from agriculture is still a major cause of poor freshwater quality currently observed across Europe (EEA 2010). Nutrient loads (nitrogen and phosphorous) from fertilizers and pesticides are known to adversely impact freshwater ecosystems, both surface water and groundwater. In particular, nitrate pollution due to N-fertilizer overuse is a main concern in groundwater in the densely cultivated Mediterranean alluvial plains (Di Lorenzo et al. 2012). Several studies have addressed the impact of poor freshwater quality on surface water biota by means of laboratory bioassays (for a review, see Traunspurger and Drews 1996; Camargo et al. 2005; Fleeger and Carman 2011 and

Published online: 19 December 2013

references therein). On the contrary, poor knowledge is available about the ecological effects of toxicants on obligate groundwater-dwelling organisms (Mathews et al. 1977; Bosnak and Morgan 1981a, b; Danielopol 1983; Meinel et al. 1989; Notenboom et al. 1992; Boutin et al. 1995; Mösslacher and Notenboom 1999; Mösslacher 2000; Canivet et al. 2001; Avramov et al. 2013; Marmonier et al. 2013; Reboleira et al. 2013). Although surface water dominant taxa, including insects and vertebrates, are also represented in groundwater, hypogean fauna overwhelmingly comprise crustaceans belonging to several taxonomic groups. Crustacea Copepoda is by far the most abundant and species-rich taxon in groundwater (Galassi et al. 2009a; Di Lorenzo et al. 2013; Di Lorenzo and Galassi 2013). Copepods successfully colonized groundwater habitats with obligate groundwater-dwelling species, called stygobites, that complete their entire life cycle in groundwater and show morphological and physiological adaptations to this environment (Galassi et al. 2009a). Epigean species can also be found, namely, stygoxenes, which enter groundwater occasionally or accidentally and are unable to reproduce in it, and stygophiles, which show incipient adaptation to groundwater life, being able to live in both surface and subsurface environments. Toxicity data for stygobiotic copepods are completely lacking, with the only exception of the harpacticoid copepod *Parastenocaris germanica*, for which acute toxicity tests are the only ones available (Notenboom et al. 1992). The difficulty of rearing and handling hypogean copepods in a laboratory is the reason why so few ecotoxicological studies have been performed to date. Conversely, in an appropriate laboratory setting, mainly determined by food quality and temperature, epigean freshwater copepods can be reared without much effort (Vijverberg 1989; Brown et al. 2005; Di Marzio et al. 2009, 2013). The informative power of acute toxicity tests on copepods has been widely recognized for marine and estuarine environments. Test methodology using copepodid stages of the marine harpacticoid *Tisbe battagliai* was included as an international standard method more than 20 years ago (IOS 1999). However, Hose (2005, 2007) and Humphreys (2007) cautioned against the temptation of inferring the sensitivity of obligate groundwater species to toxicants from the sensitivity values of their surface water relatives. Main concerns arise from differences in physiological traits that groundwater organisms have evolved to cope with different environmental conditions in groundwater ecosystems, striving to reduce energy expenditure in an energy-limited environment. In particular, their metabolic rates are significantly lower than those of phylogenetically related surface water species (Hervant et al. 1996; Wilhelm et al. 2006). They also have greater fat storage and are low energy adapted (Humphreys 2007 and references therein). Such physiological traits can lead to different sensitivities to toxicants (Avramov et al. 2013). True groundwater species have longer life cycles compared to epigean relatives, therefore facing a high risk of multiple exposures along their life span. Hence, Humphreys (2007) cautioned against the uncritical acceptance of groundwater risk assessment based on the sensitivity of epigean taxa with no close phylogenetic relationships with stygobiotic counterparts. In the face of this state of affairs, ecotoxicological tests on stygobiotic taxa are an obligate step toward a more appropriate assessment of groundwater quality and groundwater ecosystem protection.

This study focused on enhancing the knowledge about the sensitivity of freshwater copepod species, both epigean and hypogean, to agricultural pollutants. Our main objectives were (1) to review the available literature data; (2) to perform ecotoxicological experiments in order to assess the toxicity of four toxicants, two fertilizers and two herbicides widely used in Europe for cereal agriculture, on the hypogean cyclopid *Diacyclops belgicus* and the epigean *Eucyclops serrulatus* (Copepoda Cyclopoida Cyclopidae); (3) to assess the hazardous concentration of un-ionized ammonia for freshwater copepods and compare it with the one computed for traditional toxicity test species (algae, macroinvertebrates, and fishes) by a species sensitivity distribution (SSD); and (4) to evaluate whether or not hypogean species are more sensitive than epigean ones to the same pollutants.

Methods

Test organisms and culture conditions

Test specimens were collected from two different groundwater wells (W1 and W2) used for gardening in the campus of Consiglio Nazionale delle Ricerche—CNR in Florence (Italy), 300 m apart from each other. Groundwater samples from both wells were tested for 32 chemicals, including ammonium, nitrites, nitrates, heavy metals, inorganic pollutants, polycyclic aromatic hydrocarbons (PAHs), pesticides, and organochlorine compounds. All chemical concentrations were lower than the limits set by European law, thus indicating the absence of pollution. Both wells (depth < 10 m) are situated in a shallow Quaternary porous aquifer. A phreatobiological net sampler (mesh size 60 μm, modified after Cvetkov 1968) was used to collect copepods from the bottom and the water column of the wells. Samples were immediately carried to the laboratory in a cooling box after collection. Copepods were sorted in a laboratory by a micropipette under a stereomicroscope at ×12 magnification and separated in different groups according to macroscopic differences in morphology. Some specimens of each group were then identified at the species level under an optical microscope at ×100 magnification. Two different species were identified, namely, *Eucyclops serrulatus* (epigean) and *Diacyclops belgicus* (hypogean). Afterward, DNA barcoding analysis was carried out on live specimens collected in both wells, with the aim to verify the

identity of the taxa and to exclude the presence of cryptic species belonging to the same genera. DNA was extracted from pools of individuals (4 for *E. serrulatus* and 20 for *D. belgicus*), randomly chosen, and sampled in different seasons, by using the Qiamp DNA Microkit (Qiagen Inc., USA). Cytochrome OXIdase (COXI) amplification was made using Folmer region primers (LCO-HCO, Folmer et al. 1994), and the Polymerase Chaine Reaction (PCR) products were purified and sequenced. Chromatograms were edited by CHROMAS software v. 2.01 (Technelysium Pty. Ltd.). The sequences, manually corrected, were aligned and translated into amino acids using MEGA v. 5.05 (Tamura et al. 2011). For both *E. serrulatus* and *D. belgicus*, sequences from Barcoding analysis showed a very low intrapopulation variability (1 % of divergence), confirming the taxonomic identity and uniqueness of both species and the absence of cryptic species. The COXI sequences of these species were deposited in GenBank.

Groups of a maximum of 25 individuals of each species were reared in 10-ml vials, with a 2-cm diameter, in unchlorinated rearing water (pH 7.4, electrical conductivity 415 μS 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^{3-} 4.1, K^+ 1, F^-<0.15, all expressed in mg l^{-1}), which was replaced twice a week. Once a week, copepods in each vial were fed on a disc (diameter 3 mm, thickness 2 mm) of a mixture of boiled vegetables and chicken liver, finely minced by a food processor and then hand-pressed. No sediment was added. Cultures were maintained in permanent darkness in a temperature-controlled chamber at 15 °C, corresponding to the average annual temperature in the field that was measured monthly in both wells by a multiparametric probe. Individuals of *D. belgicus* (adult length measured from the tip of the cephalic shield to the end of the caudal rami was about 0.6 mm) survived for more than 21 days in rearing conditions. However, neither molting exuviae nor ovigerous females were found during this period. On the contrary, *E. serrulatus* (adult length measured from the tip of the cephalic shield to the end of the caudal rami was about 1.1 mm) molted and reproduced under laboratory conditions. Taking into account this ecological difference, we sampled individuals in the field and acclimated them in a glass container (500 ml) for only 3 days prior to each ecotoxicological testing. During acclimation, copepods were deprived of food in order to allow emptying guts completely. The digestive tract was clearly visible under stereomicroscope at ×80 magnification. After this procedure, feces were removed, and only actively swimming copepods were selected for each bioassay.

Ecotoxicological testing

The toxic effects of two fertilizers and two commercial herbicides were investigated by means of specifically designed test bioassays. Toxicants were provided by Regione Toscana in the form they are used in the field for cereal crops: (1) ammonium nitrate (pure crystalline solid, Chemical Abstracts Service [CAS]: 6484-52-2); (2) urea (pure crystal prismatic, CAS: 57-13-6); (3) ARIANE™ II from Dow AgroSciences LLC Italy (liquid mixture 21.9 % (4-Chloro-2-methylphenoxy)acetic acid (MCPA) potassium salt, CAS: 5221-16-9; 5.29 % Fluroxypyr, CAS: 81406-37-3; 2.42 % Clopyralid, CAS: 57754-85-5); and (4) BEYOND from BASF Italy (3.7 % Imazamox, CAS: 114311-32-9).

Acute toxicity data (96 h), in absence of food, were obtained for nonovigerous adult females and adult males of both *E. serrulatus* and *D. belgicus*. Experiments were carried out at 15 °C, and the chemicals were added in water solution in order to reproduce the environmental conditions of the natural habitats and the way the chemicals are used in the field. Test solutions were obtained by dilution of stock solutions, prepared with the unchlorinated water used for rearing. For each species, the experimental concentrations were chosen based on preliminary range-finding experiments according to species tolerance (>90 % survivorship in control treatment and 100 % mortality at maximal concentration). For the final tests, only the active ingredients Imazamox and ionized ammonia were retained for subsequent analyses from BEYOND herbicide and ammonium nitrate, respectively. Copepods were exposed to the toxicants by placing them in 5-cm-diameter Petri dishes containing 10 ml of appropriate test solutions. Every 24 h, each replicate was checked for the presence of dead individuals (no movement after gentle stimulation by a sorting needle). Appropriate controls with nonexposed individuals were set up for each test. As proposed in other studies (Brown et al. 2005; Avramov et al. 2013), due to the naturally low abundance of test organisms in the field and the failure of rearing hypogean species in a laboratory, only small numbers of specimens were used for each test at a time. In our study, five specimens of *E. serrulatus* and four specimens of *D. belgicus* were used for each toxicant concentration, respectively. Each test was conducted two times, with test 2 being a replicate of test 1. For each test, copepods were randomly chosen from the same glass container. Only alive and normally moving individuals were selected.

All endpoint estimates were based on nominal values. Based on the results of preliminary tests, the nominal range of toxicant concentrations were selected for the acute bioassays at 96 h. Acute toxicity data (mortality) were analyzed by Probit method whenever the data structure was suitable. If insufficient numbers of partial kills were present in a study, the data were alternatively analyzed by the Trimmed Spearman–Karber method. Validated software and programs used for statistical analyses were USEPA (1994a, b), Probit V1.5 and Trimmed Spearman–Karber V1.5. To estimate the ultimate lethal concentration at 50 % (LC50) shown in Table 1, the mortality data of both tests were used. As the two tests have resulted in similar LC50 values, the mortality responses were pooled in order to enhance the statistical robustness of the

Table 1 Median lethal concentration (96 h±95 % confidence limits) for adults of *Eucyclops serrualtus* (Es) and *Diacyclops belgicus* (Db) exposed to the herbicide mixture ARIANE II, urea, ammonia, and the herbicide Imazamox

	Es		Db	
	LC50—96 h	CL	LC50—96 h	CL
Ariane II (%)	0.004	0.00147	0.002	0.000259
	0.003–0.006	0.0006–0.0026	0.001–0.003	0.0001–0.0005
Urea (g l^{-1})	12.92	4.64	3.14	0.005
	10.20–15.12	3.63–5.95	1.25–6.45	0.001–0.087
Ammonia (mg l^{-1})	52.211	1.196	16.336	0.032
	18.35–154.76	2.35–8.60	11.63–20.36	0.008–0.095
Imazamox (mg l^{-1})	232.44	67.67	199.23	29.52
	186.36–319.52	14.08–112.11	161.20–250.35	5.36–80.40

CL is chronic lethality with confidence intervals at 95 %

dataset, as suggested by Avramov et al. (2013). For each species and each toxicant, chronic lethality (CL) estimations and confidence intervals at 95 % were obtained using the Acute to Chronic Estimation method (ACE V3.0) according to Mayer et al. (1999). ACE is a program included in the Risk Assessment Tools software (Mayer et al. 2010). The method combines two linear regressions in order to estimate low lethal concentrations at each observation time period and regresses these dependent variables against the reciprocal of time (independent variable), with the intercept being the chronic value for lethality. The routine uses Probit and log10 transformations of percent response.

In order to define the concentration of un-ionized ammonia at which freshwater copepod species would not be harmed, we computed a SSD for *D. belgicus* and *E. serrulatus* and other five species of freshwater copepods (data from Di Marzio et al. 2009). We compared the SSD for freshwater copepods to the SSD estimated from traditional toxicity test species (algae: *Ochromonas sociabilis*, invertebrates: *Ceriodaphnia dubia*, vertebrates: *Oncorhynchus mykiss*, *Oncorhynchus nerka*), modeled from values of LC20 and effective concentration at 20 % (EC20), as proposed by the Canadian Council of Ministers of the Environment (CCME) (2010). In order to allow the comparison, acute data of concentrations for ionized ammonia (NH_4^+) were corrected for pH and temperature according to CCME (2010), and after Probit analysis, we obtained the EC20 values for un-ionized ammonia (NH_3) for the freshwater copepod species. Hazardous concentrations for 5 % of assayed species (HC5) were calculated from two-parameter logistic models.

Source of literature data

We carried out a screening of published data available from literature for freshwater copepods using the online query tool of EPA-ECOTOX database crossing the fields Copepoda and freshwater, and the ISI Web of Knowledge SM (© 2010 Thomson Reuters) in the fields of ecology and ecotoxicology. Studies reporting nonsignificant statistical data were not considered, as well as data concerning species living in littoral and brackish environments. In relation to lethal concentrations/doses, in Table 2, we reported LC50 values, when available, in order to allow comparisons with our data. Moreover, we only considered studies dealing with experiments, run both in the laboratory and in the field, where exposition was induced under controlled conditions and specimens identified at least at the genus level.

Results

Ecotoxicological testing

Acute toxicity data are shown in Table 1. The values of LC50 at 96 h, as well as the values of CL, showed a clear pattern of sensitiveness shared by both species: ionized ammonia was the most toxic substance, followed by Imazamox and ultimately by urea. The comparison between the toxicity of ARIANE II and those of other chemicals was not possible, since the concentration of the mixture of the three herbicides was expressed in percentage. The hypogean species *D. belgicus* was more sensitive than the epigean *E. serrulatus* to all toxicants, under both acute and chronic exposure (Table 1). CL estimations of *E. serrulatus* were 1 order of magnitude lower than the values of LC50 at 96 h for this species, for all toxicants. CL estimations of *D. belgicus* were 3 orders of magnitude lower than the values of LC50 at 96 h for ionized ammonia and urea and 1 order for both herbicides. The CL value for ionized ammonia estimated for *D. belgicus* (0.032 mg l^{-1}) was 3 orders of magnitude lower than the actual standard for groundwater quality (0.5 mg l^{-1}) enforced by the European Drinking Water Directive. In Fig. 1 is shown the logistic distribution (solid line) of EC20 toxicity values for *E. serrulatus* and *D. belgicus* and other five species of freshwater copepods (data from Di Marzio et al. 2009) and for standard freshwater species (dashed line) for un-ionized ammonia. The hazardous concentration for 5 % of total traditional test species (HC5) is plotted against the hazardous

Table 2 Sensitivity of freshwater copepods to different agricultural pollutants indicated by CAS (Chemical Abstracts Service) and commercial name

CAS	Commercial name	T	Species	Stage	Loc.	Days	Endp.	Effect	Conc.	L CL 95 %	U CL 95 %	Unit	References
94757	2,4 D (free acid)	H	*Acanthocyclops vernalis*	A	LAB	2	EC50	IMBL	37.42	308080.80	21.70	ppm	Robertson and Bunting 1976
94757	2,4 D (free acid)	H	*Acanthocyclops vernalis*	A	LAB	4	EC50	IMBL	8.72	5.32	11.57	ppm	Robertson and Bunting 1976
2702729	2,4 D sodium salt	H	*Cyclops bohater*	A	LAB	4		MORT	2,430.00			mg l^{-1}	Wierzbicka 1974
2702729	2,4 D sodium salt	H	*Cyclops vicinus vicinus*	A	LAB	7		PHY	15.00			mM	Wierzbicka 1974
95761	3,4-Dichlorobenzenamine	P, I	*Cyclops* sp.	P	FIELDN	>4		ABND	0.025; 0.050			mg l^{-1}	Beyerle-Pfnur et al. 1991
104405	4-Nonylphenol	P, I	*Paracyclops* sp.	A	FIELDN	>4	LOEC	ABND	0.02			mg l^{-1}	O'Halloran et al. 1999
116063	Aldicarb	I	*Attheyella crassa*	A	LAB	4	LC50	MORT	3.17	1.72	5.84	mg l^{-1}	Di Marzio et al. 2009
116063	Aldicarb	I	*Echinocamptus echinatus*	A	LAB	4	LC50	MORT	2.71	2.42	3.04	mg l^{-1}	Di Marzio et al. 2009
116063	Aldicarb	I	*Echinocamptus echinatus*	C–A	LAB	TI		DEV	0.65			mg l^{-1}	Di Marzio et al. 2013
116063	Aldicarb	I	*Bryocamptus minutus*	A	LAB	4	LC50	MORT	2.50	2.19	2.78	mg l^{-1}	Di Marzio et al. 2009
116063	Aldicarb	I	*Bryocamptus minutus*	C–A	LAB	TI		DEV	0.65			mg l^{-1}	Di Marzio et al. 2013
116063	Aldicarb	I	*Bryocamptus pygmaeus*	A	LAB	4	LC50	MORT	2.42	2.05	2.86	mg l^{-1}	Di Marzio et al. 2009
116063	Aldicarb	I	*Bryocamptus pygmaeus*	C–A	LAB	TI		DEV	0.65			mg l^{-1}	Di Marzio et al. 2013
116063	Aldicarb	I	*Bryocamptus zschokkei*	A	LAB	4	LC50	MORT	1.85	1.71	2.00	mg l^{-1}	Di Marzio et al. 2009
116063	Aldicarb	I	*Bryocamptus zschokkei*	C–A	LAB	TI		DEV	0.65			mg l^{-1}	Di Marzio et al. 2013
61825	Amitrole	H	*Acanthocyclops vernalis*	A	LAB	2	EC50	IMBL	58.50	39.76	124.00	ppm	Robertson and Bunting 1976
61825	Amitrole	H	*Acanthocyclops vernalis*	A	LAB	4	EC50	IMBL	22.10	18.54	27.71	ppm	Robertson and Bunting 1976
7664417	Ammonia	F	*Cyclops* sp.	P	FIELDN	>4		ABND	0.04; 22.00			mg l^{-1}	Ramaprabhu et al. 1986
6484522	Ammonia as NH_4NO_3	F	*Attheyella crassa*	A	LAB	4	LC50	MORT	17.80	15.89	19.83	mg l^{-1}	Di Marzio et al. 2009
6484522	Ammonia as NH_4NO_3	F	*Echinocamptus echinatus*	A	LAB	4	LC50	MORT	14.61	12.76	16.73	mg l^{-1}	Di Marzio et al. 2009
6484522	Ammonia as NH_4NO_3	F	*Echinocamptus echinatus*	C–A	LAB	TI		DEV	3.65			mg l^{-1}	Di Marzio et al. 2013
6484522	Ammonia as NH_4NO_3	F	*Bryocamptus minutus*	A	LAB	4	LC50	MORT	18.22	15.37	21.61	mg l^{-1}	Di Marzio et al. 2009
6484522	Ammonia as NH_4NO_3	F	*Bryocamptus minutus*	C–A	LAB	TI		DEV	3.65			mg l^{-1}	Di Marzio et al. 2013
6484522	Ammonia as NH_4NO_3	F	*Bryocamptus pygmaeus*	A	LAB	4	LC50	MORT	18.22	15.37	21.61	mg l^{-1}	Di Marzio et al. 2009
6484522	Ammonia as NH_4NO_3	F	*Bryocamptus pygmaeus*	C–A	LAB	TI		DEV	3.65			mg l^{-1}	Di Marzio et al. 2013
6484522	Ammonia as NH_4NO_3	F	*Bryocamptus zschokkei*	A	LAB	4	LC50	MORT	18.63	16.63	20.87	mg l^{-1}	Di Marzio et al. 2009
6484522	Ammonia as NH_4NO_3	F	*Bryocamptus zschokkei*	C–A	LAB	TI		DEV	3.65			mg l^{-1}	Di Marzio et al. 2013
11141176	Azadirachtin	H	*Mesocyclops leuckarti*	A	LAB	3	LC50	MORT	0.39			ppm	Mukherjee et al. 1990
12789036	Chlordan	I	*Cyclops strenuus*	A	LAB	4		MORT	0.05; 1.00			mg l^{-1}	Ludemann and Neumann 1960
88040	Chloroxylenol	I	*Thermocyclops oblongatus*	A	LAB	1	LC50	MORT	0.17			mg l^{-1}	Chippaux et al. 1996
2921882	Chlorpyrifos	I	*Acanthocyclops vernalis*	P	FIELDN	>4		ABND	0.01; 0.05; 0.01; 1.00			lb $acre^{-1}$	Hurlbert et al. 1970
12069691	Cutrine-plus	A	*Eucyclops* sp.	A	LAB	2	LC50	MORT	11.40			ppm	Naqvi and Hawkins 1989
68359375	Cyfluthrin	I	*Thermocyclops oblongatus*	A	LAB	1	LC50	MORT	0.05			mg l^{-1}	Chippaux et al. 1996
52315078	Cypermethrin	I	*Cyclops* sp.	P	FIELDA	1	LC50	ABND	0.00018	<0.0001	0.002563	mg l^{-1}	Wendt-Rasch et al. 2003

Table 2 (continued)

CAS	Commercial name	T	Species	Stage	Loc.	Days	Endp.	Effect	Conc.	L CL 95 %	U CL 95 %	Unit	References
52315078	Cypermethrin	I	*Thermocyclops oblongatus*	A	LAB	1	LC50	MORT	0.03			mg l^{-1}	Chippaux et al. 1996
50293	DDT	I	*Cyclops* sp.	A	LAB	1	LC50	MORT	515.00			ppb	Sharma and Dattagupta 1983
52918635	Deltamethrin	I	*Cyclops vicinus*	A	LAB	4		GHIS	$(2.4; 4.8)\times 10^{-5}$			mg l^{-1}	Musko 1987
52918635	Deltamethrin	I	*Thermocyclops oblongatus*	A	LAB	1	LC50	MORT	0.02			mg l^{-1}	Chippaux et al. 1996
8065483	Demeton	I	*Cyclops strenuus*	A	LAB	1		MORT	5.0; 10.0			mg l^{-1}	Ludemann and Neumann 1962
60571	Dieldrin	I	*Diacyclops thomasi*	A	LAB	4	EC50	IMBL	0.1; 1.0			mg l^{-1}	Novak et al. 1980
35367385	Diflubenzuron	I	*Cyclops* sp.	P	FIELDN	>4		ABND	28; 56			kg AI ha^{-1}	Ali and Lord 1980
55389	Fenthion	I	*Macrocyclops albidus*	A	LAB	1		MORT	0.01			mg l^{-1}	Johnson 1978
55389	Fenthion	I	*Mesocyclops* sp.	A	LAB	2	LC50	MORT	3.16			µl l^{-1}	Kaur and Ansal 1996
55389	Fenthion	I	*Mesocyclops* sp.	A	LAB	1	LC50	MORT	5.37			µl l^{-1}	Kaur and Ansal 1996
76879	Fentin	Fu, H	*Acanthocyclops vernalis*	A	NR	>4	NOEC	REP	0.003			mg l^{-1}	Becker 1992
120068373	Fipronil	I	*Acanthocyclops robustus*	A	LAB	2	LC50	MORT	194.20	143.90	262.10	nmol	Chaton et al. 2002
120068373	Fipronil	I	*Diaptomus castor*	A	LAB	2	LC50	MORT	7.90	6.40	9.90	nmol	Chaton et al. 2002
1563662	Furadan	I	*Megacyclops viridis*	A	LAB	2	LC50	MORT	0.62			mg l^{-1}	Konar and Ghosh 1981
76448	Heptachlor	I	*Cyclops* sp.	A	LAB	>4	LC100	MORT	0.10			mg l^{-1}	Konar 1970
58899	Lindane	I	*Eucyclops serrulatus*	P	FIELDN	>4	EC50	ABND	0.002			mg l^{-1}	Peither et al. 1996
121755	Malathion	I	*Megacyclops viridis*	A	LAB	2	LC50	MORT	1.30			mg l^{-1}	Konar and Ghosh 1981
121755	Malathion	I	*Macrocyclops albidus*	A	LAB	1	LD50	MORT	0.76	0.38	0.27	ppm	Marten et al. 1993
16752775	Methomyl	I	*Cyclops strenuus*	A	LAB	4	LC50	MORT	0.19			mg l^{-1}	Aboul-Ela and Khalil 1987
40596698	Methoprene	I	*Macrocyclops albidus*	A	LAB	1	LD50	MORT	0.67	0.63	0.73	ppm	Marten et al. 1993
19937598	Metoxuron	H	*Cyclops strenuus*	A	LAB	2	LC50	MORT	122.00			mg l^{-1}	Svobodova et al. 1986
12125029	NH_4Cl	F	*Mesocyclops leuckarti*	A	LAB	4		IMBL	75.00			mg l^{-1}	Anderson et al. 1948
50657	Niclosamide	I	*Thermocyclops oblongatus*	A	LAB	1	LC50	MORT	1.06			mg l^{-1}	Chippaux et al. 1996
7757791	Nitric acid potassium salt	F	*Acanthocyclops vernalis*	A	LAB	1	LC50	MORT	1,985.00	1,780.00	2,306.00	mg l^{-1}	Mösslacher 2000
7757791	Nitric acid potassium salt	F	*Diacyclops bicuspidatus*	A	LAB	2	LC50	MORT	1,460	1,228	2,344	mg l^{-1}	Mosslacher 2000
87865	PCP	I	*Boeckella delicata*	A	LAB	1	LC50	MORT	0.14			mg l^{-1}	Willis 1999
87865	PCP	I	*Boeckella delicata*	N	LAB	1	LC50	MORT	0.25			mg l^{-1}	Willis 1999
87865	PCP	I	*Boeckella delicata*	A	LAB	2	LC50	MORT	0.13			mg l^{-1}	Willis 1999
87865	PCP	I	*Boeckella delicata*	N	LAB	2	LC50	MORT	0.23			mg l^{-1}	Willis 1999
87865	PCP	I	*Calamoecia lucasi*	A	LAB	1	LC50	MORT	0.14			mg l^{-1}	Willis 1999
87865	PCP	I	*Calamoecia lucasi*	N	LAB	1	LC50	MORT	0.09			mg l^{-1}	Willis 1999
87865	PCP	I	*Calamoecia lucasi*	A	LAB	2	LC50	MORT	0.11			mg l^{-1}	Willis 1999
87865	PCP	I	*Calamoecia lucasi*	N	LAB	2	LC50	MORT	0.05			mg l^{-1}	Willis 1999

Table 2 (continued)

CAS	Commercial name	T	Species	Stage	Loc.	Days	Endp.	Effect	Conc.	L CL 95 %	U CL 95 %	Unit	References
87865	PCP	I	*Mesocyclops leuckarti*	A	LAB	1	LC50	MORT	0.370			mg l^{-1}	Willis 1999
87865	PCP	I	*Mesocyclops leuckarti*	N	LAB	1	LC50	MORT	0.389			mg l^{-1}	Willis 1999
87865	PCP	I	*Mesocyclops leuckarti*	A	LAB	2	LC50	MORT	0.173			mg l^{-1}	Willis 1999
87865	PCP	I	*Mesocyclops leuckarti*	N	LAB	2	LC50	MORT	0.138			mg l^{-1}	Willis 1999
87865	PCP	I	*Parastenocaris germanica*	A	LAB	2	LC50 ^{Hy}	MORT	0.07	0.07	0.08	mg l^{-1}	Notenboom et al. 1992
87865	PCP	I	*Parastenocaris germanica*	A	LAB	4	LC50 ^{Hy}	MORT	0.03	0.03	0.04	mg l^{-1}	Notenboom et al. 1992
87865	PCP	I	*Parastenocaris germanica*	A	LAB	2	LC50 ^{No}	MORT	0.08	0.07	0.09	mg l^{-1}	Notenboom et al. 1992
87865	PCP	I	*Parastenocaris germanica*	A	LAB	4	LC50 ^{No}	MORT	0.04	0.03	0.04	mg l^{-1}	Notenboom et al. 1992
131522	PCP sodium salt	Fu, H	*Mesocyclops leuckarti*	P	FIELDA	>4		ABND	0.30			mg l^{-1}	Feind et al. 1988
52645531	Permethrin	I	*Acanthocyclops vernalis*	A	LAB	1	LD50	MORT	1.80	1.60	2.00	ppm	Marten et al. 1993
52645531	Permethrin	I	*Macrocyclops albidus*	A	LAB	1	LD50	MORT	0.29	0.26	0.31	ppm	Marten et al. 1993
52645531	Permethrin	I	*Mesocyclops ruttneri*	A	LAB	1	LD50	MORT	1.90	1.60	2.30	ppm	Marten et al. 1993
122145	Phenitrothion	I	*Mesocyclops* sp.	A	LAB	2	LC50	MORT	1.86			µl l^{-1}	Kaur and Ansal 1996
122145	Phenitrothion	I	*Mesocyclops* sp.	A	LAB	1	LC50	MORT	4.01			µl l^{-1}	Kaur and Ansal 1996
13171216	Phosphamidon	I	*Mesocyclops* sp.	A	LAB	2	LC50	MORT	1.69			µl l^{-1}	Kaur and Ansal 1996
13171216	Phosphamidon	I	*Mesocyclops* sp.	A	LAB	1	LC50	MORT	4.34			µl l^{-1}	Kaur and Ansal 1996
96489713	Pyridaben	I	*Acanthocyclops* sp.	P	FIELDA	>4	LOEC	ABND	0.0034			mg l^{-1}	Rand et al. 2000
10453868	Resmethrin	I	*Macrocyclops albidus*	A	LAB	1	LD50	MORT	0.094	0.091	0.097	ppm	Marten et al. 1993
3383968	Temephos	I	*Cyclops* sp.	P	FIELDN	>4		ABND	0.0092			ppm	Ali and Mulla 1978
3383968	Temephos	I	*Cyclops* sp.	P	FIELDN	3	NOEL	ABND	0.10			ppm	Rettich 1979
3383968	Temephos	I	*Macrocyclops albidus*	A	LAB	1	LD50	MORT	0.011	0.006	0.001	ppm	Marten et al. 1993
3383968	Temephos	I	*Thermocyclops hyalinus*	N	LAB	1	LC50	MORT	0.23			ppm	Samman and Thomas 1978
3383968	Temephos	I	*Thermocyclops hyalinus*	C–A	LAB	1	LC50	MORT	0.20			ppm	Samman and Thomas 1978
3383968	Temephos	I	*Thermocyclops oblongatus*	A	LAB	1	LC50	MORT	0.07			mg l^{-1}	Chippaux et al. 1996
59669260	Thiodicarb	I	*Cyclops* sp.	P	FIELDA	>4		ABND	0.055; 0.11			ppm	Ali and Stanley 1982
1582098	Trifluralin	H	*Eucyclops* sp.	A	LAB	2	LC50	MORT	0.05			ppm	Naqvi and Hawkins 1989
900958	Triphenyltin acetate	F	*Acanthocyclops venustus*	P	LAB	4	LC50	MORT	0.0008	0.0003	0.002	mg l^{-1}	Roessink et al. 2006
900958	Triphenyltin acetate	F	*Acanthocyclops venustus*	P	LAB	2	LC50	MORT	0.0069	0.0055	0.0088	mg l^{-1}	Roessink et al. 2006
76879	Triphenyltin hydroxide	F	*Mesocyclops leuckarti*	N	LAB	2	LC50	MORT	0.0339			mg l^{-1}	Kulkarni et al. 2013a
76879	Triphenyltin hydroxide	F	*Mesocyclops leuckarti*	C	LAB	2	LC50	MORT	0.0968			mg l^{-1}	Kulkarni et al. 2013a
76879	Triphenyltin hydroxide	F	*Mesocyclops leuckarti*	A♀	LAB	2	LC50	MORT	0.081	0.0513	0.1449	mg l^{-1}	Kulkarni et al. 2013a
76879	Triphenyltin hydroxide	F	*Mesocyclops leuckarti*	A♂	LAB	2	LC50	MORT	0.0861	0.0734	0.1026	mg l^{-1}	Kulkarni et al. 2013a
76879	Triphenyltin hydroxide	F	*Mesocyclops leuckarti*	N	LAB	4	LC50	MORT	0.0103	0.0082	0.0125	mg l^{-1}	Kulkarni et al. 2013a
76879	Triphenyltin hydroxide	F	*Mesocyclops leuckarti*	C	LAB	4	LC50	MORT	0.051	0.0184	0.1431	mg l^{-1}	Kulkarni et al. 2013a
76879	Triphenyltin hydroxide	F	*Mesocyclops leuckarti*	A♀	LAB	4	LC50	MORT	0.0448	0.0214	0.0966	mg l^{-1}	Kulkarni et al. 2013a

Table 2 (continued)

CAS	Commercial name	T	Species	Stage	Loc.	Days	Endp.	Effect	Conc.	L CL 95 %	U CL 95 %	Unit	References
76879	Triphenyltin hydroxide	F	*Mesocyclops leuckarti*	A♂	LAB	4	LC50	MORT	0.0498	0.0198	0.1459	mg l^{-1}	Kulkarni et al. 2013a
137304	Ziram	Fu	*Thermocyclops oblongatus*	A	LAB	1	LC50	MORT	0.02			mg l^{-1}	Chippaux et al. 1996
115297	α-Endosulfan	I	*Attheyella crassa*	A	LAB	4	LC50	MORT	0.25	0.19	0.43	mg l^{-1}	Di Marzio et al. 2009
115297	α-Endosulfan	I	*Echinocamptus echinatus*	A	LAB	4	LC50	MORT	0.095	0.048	0.120	mg l^{-1}	Di Marzio et al. 2009
115297	α-Endosulfan	I	*Bryocamptus minutus*	A	LAB	4	LC50	MORT	0.20	0.19	0.21	mg l^{-1}	Di Marzio et al. 2009
115297	α-Endosulfan	I	*Bryocamptus pygmaeus*	A	LAB	4	LC50	MORT	0.20	0.19	0.21	mg l^{-1}	Di Marzio et al. 2009
115297	α-Endosulfan	I	*Bryocamptus zschokkei*	A	LAB	4	LC50	MORT	0.07	0.06	0.09	mg l^{-1}	Di Marzio et al. 2009
115297	α-Endosulfan	I	*Cyclops strenuus*	A	LAB	1	LC100	MORT	1.0			mg l^{-1}	Gorbach and Knauf 1971

PCP Pentachlorophenol; *T* type of pollutant (*P* pesticide, *I* insecticide, *H* herbicide, *Fu* fungicide, *F* fertilizer); *Stage* stage of development (*A* adults, *N* nauplii, *C* copepodites, *P* all stages together [population approach], ♂ male, ♀ female); *Loc.* place of experimentation (*LAB* laboratory, *FIELDA* outdoors in artificial conditions, *FIELDN* outdoors in natural conditions, *NR* not reported); *Days* duration of experiments in days (*TI* time-independent test); *Endp.* endpoint (*LCXX* lethal concentration, *ECXX* effective concentration, *LDXX* lethal dose, *LOEC* lowest observable effective concentration, *NOEC* no observed effect concentration, *NOEL* no observable effect level, *Hy* hypoxic conditions, *No* normoxic conditions); *Effect* (*MORT* mortality, *ABDN* changes in abundance, *REP* changes in reproduction, *GHIS* changes in general histology, *PHY* changes in physiology, *IMBL* immobilization); *Conc.* toxicant concentration; *L CL95 %* lower confidence limits 95 %; *U CL95 %* upper confidence limits 95 %; *Unit* (*kg AI ha*$^{-1}$ kilograms of active ingredient per hectare)

concentration for 5 % of the copepod species (HC5c). For traditional test species, the HC5 value was 120 μg l^{-1} (95 % confidence limits 105–137 μg l^{-1}), and for copepods, it was 92 μg l^{-1} (95 % confidence limits 81–104 μg l^{-1}). HC5 values were significantly different ($p < 0.05$).

Review of literature data

According to the information we derived from published data, 41 studies dealt with sensitivity of freshwater copepods to pesticides and N-fertilizers, from 1948 up to now. Only one paper involved a hypogean copepod, *Parastenocaris germanica*. The studies, involving 30 copepod taxa (21 cyclopoids, 6 harpacticoids, and 3 calanoids), 5 of which identified only at the genus level, are consistently fewer than those concerning marine, brackish, and littoral copepods exposed to plant protection products (reviewed by Kulkarni et al. 2013b). The experiments were carried out in a laboratory (29 out of 39), outdoors in artificial and/or simulated environments (4), and outdoors in natural settings (8). Bioassays predominantly dealt with short-term (≤4 days) tests. Overall, the effects investigated were mainly mortality (21 out of 39), the others concerning variation in population abundances (12), reproductive alterations (1), histological changes (1), physiological modifications (1), and immobility (3). Only four published studies have addressed N-fertilizer toxicity, the remnants concerning herbicides and fungicides and mainly insecticides and insecticide precursors. Considering short-term bioassays and comparing endpoints of LC50s with mortality effects (Table 2), the sensitivity scale for freshwater

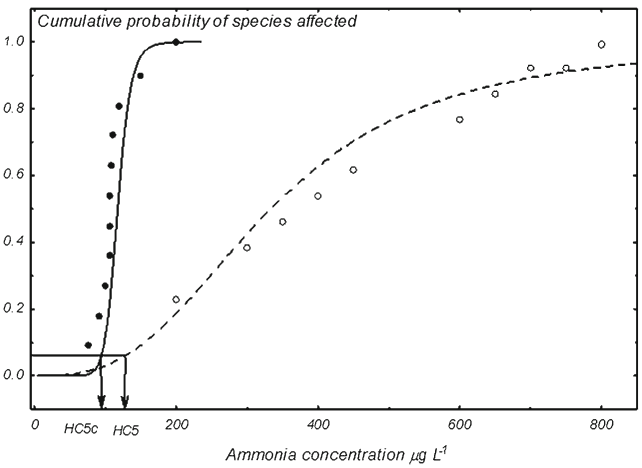

Fig. 1 Aquatic community risk model for un-ionized ammonia (NH_3) using EC20 values of *Eucyclops serrulatus* and *Diacyclops belgicus* and other five species of freshwater copepods from Di Marzio et al. (2009) (*solid line*) compared with EC20/LD20 data (*dashed line*) from standard test species (algae: *Ochromonas sociabilis*, invertebrates: *Ceriodaphnia dubia*, vertebrates: *Oncorhynchus mykiss*, *Oncorhynchus nerka*). HC5c: hazardous concentration at 5 % of protection based on copepods, HC5: hazardous concentration at 5 % of protection based on standard test species according to CCME

copepods is insecticides and fungicides>herbicides>fertilizers. Sensitivity to N-fertilizers is at least 1 order of magnitude lower than sensitivity to pesticides (Table 2). Taking into account the studies concerning acute exposures of 24 h, the insecticides Temephos and Fenthion are the most toxic chemicals, inducing 50 % mortality at concentrations of about 0.01 mg l^{-1} (Table 1). The insecticide Cypermethrin significantly reduced abundances of *Cyclops* sp. populations after an exposure to a concentration of <0.001 mg l^{-1} (Wendt-Rasch et al. 2003). Concerning acute exposures of <24 h, the fungicide Triphenyltin hydroxide was the most noxious chemical at low concentrations (<0.001 mg l^{-1}). The influence of time in short-term bioassays was considered in five studies, where sensitivity to pesticides was shown to increase with time of exposure (Ludemann and Neumann 1960; Robertson and Bunting 1976; Notenboom et al. 1992; Willis 1999; Kulkarni et al. 2013a). Acute sensitivity to the insecticide Temephos has been investigated in three different studies up to now (Table 2): besides differences in the experimental conditions, data indicated that the cyclopoid *Macrocyclops albidus* and *Thermocyclops oblongatus* are more sensitive than *Thermocyclops hyalinus* at 24 h of exposure (Samman and Thomas 1978; Chippaux et al. 1996; Marten et al. 1993). Notenboom et al. (1992) and Willis (1999) tested the effect of Pentachlorophenol, respectively, on the hypogean species *Parastenocaris germanica* and on three surface freshwater species, namely, the calanoid *Boeckella delicata* and *Calamoecia lucasi* and the cyclopoid *Mesocyclops leuckarti*. Only data derived from 96 h of exposure of adult stages in normoxic conditions were comparable, highlighting that LC50 at 96 h of the hypogean species was <0.01 mg l^{-1}, whereas the LC50s of the surface water species were higher than 0.1 mg l^{-1}. Acute sensitivity of different species to the same toxicant under the same experimental conditions has been investigated in five studies: Chaton et al. (2002) observed that the calanoid species *Diaptomus castor* is 2 orders of magnitude more sensitive than the cyclopoid *Acanthocyclops robustus* to the insecticide Fipronil. Likewise, Marten et al. (1993) observed that *Macrocyclops albidus* is 1 order of magnitude more sensitive than *Acanthocyclops vernalis* and *Mesocyclops ruttneri* to the insecticide Permethrin. Conversely, other authors observed a comparable sensitivity of different species to insecticides and fertilizers (Willis 1999; Mösslacher 2000; Di Marzio et al. 2009).

The influence of age was investigated by Willis (1999) in relation to the sensitivity to Pentachlorophenol of two calanoid and one cyclopoid species and by Kulkarni et al. (2013a) for the cyclopoid *Mesocyclops leuckarti* exposed to Thiphenyltin hydroxide. Naupliar stages were more sensitive than adults for *Calamoecia lucasi* and *Mesocyclops leuckarti* at both 24 h and 48 h of exposure to Pentachlorophenol, whereas nauplii of *Boeckella delicata* were less sensitive than adults (Willis, 1999). Similarly, LC50 values after 48 h and 96 h of exposure to Thiphenyltin hydroxide for nauplii of *Mesocyclops leuckarti* were significantly lower compared to the other stages; on the contrary, there were no significant differences between the LC50 values at 96 h for the copepodids, adult males, and adult females (Kulkarni et al. 2013a).

Regarding N-fertilizers, the only long-term bioassay, conducted outdoors in natural settings, highlighted the negative effect of ionized ammonia on population abundances (Ramaprabhu et al. 1986). However, in this study the taxon was identified at the genus level only (*Cyclops* sp.). Data from long-term exposure to insecticides, under either natural or artificial settings, are hardly comparable. However, besides differences in experimental conditions, low toxicant concentrations (<0.1 mg l^{-1}) seemed to significantly decrease population abundances (Table 2). Although only few studies addressed this topic, concentrations of <1 mg l^{-1} of insecticides and <5 mg l^{-1} of ionized ammonia seemed to determine a significant delayed development of freshwater interstitial copepods (Di Marzio et al. 2013).

Discussion

Sensitivity of freshwater copepods

The importance of freshwater copepods is underestimated in ecotoxicological research (Kulkarni et al. 2013b). According to the literature review, studies on hypogean species are almost nonexistent. Freshwater planktonic cyclopoids and calanoids have been involved more often than harpacticoids. This is likely due to ecological attributes of the former groups that make their collection and rearing in laboratory easier. Calanoids are predominantly planktonic, and cyclopoids can either be planktonic (also in subterranean lakes and pools) or reside in interstitial habitats, however preferring medium-coarse sediments. Harpacticoids are almost exclusively benthic organisms, either inhabiting the minute voids among the sediment particles or living as epiphytic in the benthos (e.g., in mosses or macrophytes, swimming or gliding on the leaf surfaces in proximity of the benthic surface) or as hyperbenthic, swimming in the few millimeters above the sediment surface or alternatively in phytotelmata (Reid 2001; Galassi et al. 2011). In groundwater, they are exclusively linked to benthic habitats (Galassi et al. 2009a). In contrast with marine copepods, there are no standardized protocols available for acute and chronic toxicity tests for freshwater copepods, making any consistent comparison about sensitivity almost impossible. However, the ecotoxicological studies summarized earlier in this paper, as well as data derived from our experiments, showed that freshwater copepods are sensitive to a range of pesticides and N-fertilizers. For both

hypogean and epigean species, acute and chronic mortality was induced by exposition to pesticides and N-fertilizers in concentrations that are higher than the law limits of good water body quality in Europe (pesticides 0.1 μg l^{-1} in both surface and groundwater; ionized ammonia 0.5 mg l^{-1} in surface and groundwater; 2000/60/EC, 2006/118/EC, and 98/83/EC Directives). As a matter of fact, the presence of hypogean fauna in alluvial aquifers deeply affected by agricultural pollutants has been observed in various ecological surveys (Galassi et al. 2009b; Di Lorenzo and Galassi 2013 and references therein). However, the extinction of less tolerant species due to long-term exposure to fertilizers and pesticides at concentrations lower than law limits cannot be neglected. The effect of chronic exposures at the maximum concentration of ionized ammonia enforced by the European Drinking Water Directive (0.5 mg l^{-1}) might be hazardous for some hypogean taxa, as observed for *D. belgicus*. According to literature data and the results from our own tests, N-fertilizers, mainly urea, nitric acid potassium salt, and ammonium chloride, are less toxic to freshwater copepods than pesticides, both in short-term and long-term exposures. This is likely due to differences in the detoxification systems activated at molecular level on which no information has been provided yet. For aquatic invertebrates, the toxic effects of the reactive forms of inorganic nitrogen, ammonia, nitrite, and nitrate, are mediated by damages to the respiratory surfaces and by changes in hemolymph pH (Colt and Armstrong 1981; Camargo et al. 2005; Camargo and Alonso 2006). Conversely, pesticides are mostly recognized to act as endocrine disruptors (Crisp et al. 1998). The toxic effects of pesticides have been observed to be more severe than respiratory damages: they interfere with synthesis, secretion, transport, binding, and activity of natural hormones in the body, thus affecting development, behavior, fertility, and maintenance of homeostasis (Crisp et al. 1998). However, the assumption that the forms of inorganic nitrogen can act as endocrine disruptor chemicals in freshwater copepods cannot be discarded, as recently observed by Di Marzio et al. (2013).

Some authors observed significant (2 or 3 orders of magnitude) interorder or even intergeneric differences in sensitivity to the same toxicant under the same conditions (Marten et al. 1993; Chaton et al. 2002). Consequently, an SSD should be considered with a view to establishing thresholds of toxicants, which would be protective of most freshwater copepod species. For un-ionized ammonia, we estimated that 95 % of the freshwater copepod species that we considered in the analysis would be protected if the concentration does not exceed 92 μg l^{-1}. The estimation of HC5 for standard species was higher (120 μg l^{-1}). Although this difference makes little sense from a chemical point of view, a concentration of un-ionized ammonia in groundwater higher than 92 μg l^{-1} is hazardous for the taxon that dominates groundwater habitats, thus affecting the groundwater ecosystem as a whole. With the scarce information available to date, the extrapolation of toxicity data to natural populations requires caution, as there are numerous interacting factors, abiotic (e.g., temperature and substrate) and biotic (e.g., competition, predation, and nutritional status), usually not considered in experimental designs. Increase in temperature likely enhances metabolic rates with negative effects on sensitivity to pollutants and survival rates, as it has been observed for the ionized form of ammonia on a species of freshwater amphipods and two isopod species (Dehedin et al. 2013).

Hypogean versus epigean species sensitivity

Stygobiotic copepods have mostly been ignored in ecotoxicological research. This is likely due to unfavorable attributes as their long generation times, low fecundity, scarce information about metabolism, and extreme difficulty in rearing them in a laboratory. For this reason, no other studies, aimed at comparing the sensitivity of a hypogean versus an epigean copepod species, are available. According to our results and comparing data from Notenboom et al. (1992) and Willis (1999), stygobiotic copepods seem to be more sensitive to pesticides and N-fertilizers than epigean ones, under both acute and chronic exposures. Moreover, the difference in sensitivity is more evident if CL values are considered. However, there are no studies that provide an explanation for such dissimilarities. Whereas some data exist on morphological adaptations to groundwater life, very little is known about physiological and behavioral strategies in stygobiotic copepods. Indeed, information about metabolic adaptation is missing for most groundwater invertebrates. However, a few studies have demonstrated that some stygobiotic species have metabolic rates lower than those exhibited by their surface relatives (Hüppop 1986; Hervant et al. 1996; Malard and Hervant 2001; Hervant 1996; Hervant and Renault 2002). Wilhelm et al. (2006) observed that the oxygen consumption rate in stygobiotic species is fairly lower than those of epigean taxa. Concerning copepods, evidence of the lower metabolic rate of stygobiotic species, compared to that of epigean relatives, is apparent in a longer developmental time observed for some stygobiotic cyclopoids by Lescher-Moutoué (1973). The same pattern was observed also by Bjornberg and Por (1986) in a comparative study of an epigean species of *Bryocyclops* and a stygobiotic species of *Speocyclops*. Hypogean cyclopoids and harpacticoids develop more slowly (although they have a longer life span) than epigean relatives, with one or more generations per year (Galassi 2001 and references therein). However, the question whether reduced metabolic rate may have a role in determining the observed difference in sensitivity to agricultural toxicants is a matter of discussion. The lower metabolic rate assumed for most stygobiotic taxa might lead to a delayed onset of defense mechanisms compared to epigean relatives, as supposed by Avramov et al. (2013). This

could explain the difference in sensitivity observed in our work. However, this statement remains to be proven.

Conclusion

Ecotoxicological research on freshwater copepods is a growing concern for standardizing experimental protocols and identifying appropriate representative species in freshwater ecosystems, as it happened for marine environments. To date, the extrapolation of sensitivity data from laboratory bioassay to natural populations requires experimental settings that more closely resemble field conditions. As far as agricultural impact on freshwater is concerned, N-fertilizer and pesticide mixtures should be certainly considered in further ecotoxicological studies. Moreover, although acute toxicity tests have the advantage to allow a rapid assessment of environmental threats, sublethal measures in time-independent tests may better represent the biological response of groundwater species to toxicants. Considering their dominance in the hyporheic zone and their holobenthic lifestyle, freshwater harpacticoids deserve a key role in the evaluation of the effects of contaminants associated with alluvial sediments. The few data concerning the sensitivity of larval stages to pollutants require additional studies. Even if further information is needed, it is apparent that groundwater ecosystem safety may be guaranteed only by assessing the sensitivity of hypogean taxa to pollutants, no longer by extrapolating information from epigean planktonic surrogates. On the other hand, the use of an SSD approach, which considers the sensitivity of both hypogean and epigean species, would provide more suitable results in monitoring some groundwater-dependent ecosystems, such as springs and the hyporheic habitat.

Acknowledgments This research was granted by Regione Toscana under POR-FESR 2007–2013 (project acronym RIPARI: Riduzione Impatto Pressioni Agricole sulla Risorsa Idrica). We are indebted to Luigi Fabbrini, Director of the Agricultural Farm of the Regione Toscana at Cesa (Arezzo, Italy). We are much indebted to two anonymous reviewers for their useful comments that improved an earlier version of the manuscript.

References

Aboul-Ela IA, Khalil MT (1987) The acute toxicity of three pesticides on organisms of different trophic levels as parameters of pollution in Lake Wadi El Rayan, El Fayoum, Egypt. Proc Zool Soc A R Egypt 13:31–36

Ali A, Lord J (1980) Impact of experimental insect growth regulators on some nontarget aquatic invertebrates. Mosq News 40(4):564–571

Ali A, Mulla MS (1978) Effects of chironomid larvicides and diflubenzuron on nontarget invertebrates in residential–recreational lakes. Environ Entomol 7(1):21–27

Ali A, Stanley BH (1982) Effects of a new carbamate insecticide, Larvin (UC-51762), on some nontarget aquatic invertebrates. Fla Entomol 65(4):477–483

Anderson BG, Chandler DC, Andrews TF, Jahoda WJ (1948) The evaluation of aquatic invertebrates as assay organisms for the determination of the toxicity of industrial wastes. Final Report 51, American Petroleum Institute Project, Ohio State University, Columbus, OH

Avramov M, Schmidt SI, Griebler C (2013) A new bioassay for the ecotoxicological testing of VOCs on groundwater invertebrates and the effects of toluene on *Niphargus inopinatus*. Aquat Toxicol 130–131:1–8

Becker E (1992) Ableitung von Qualitatszielenzum Schutz Oberirdischer Binnengewasser fur Organozinnverbindungen: Dibutylzinnverbindungen, Tetrabutylzinn, Tributylzinnverbindungen, Triphenylzinnverbindungen. Umweltbundesamt, Entwurf fur den BLAK QZ, Stand 26.3.1992

Beyerle-Pfnur MR, Burkhardt G, Peither A (1991) Chronic ecotoxicity of 3,4-dichloroaniline to freshwater ecosystems. Toxicol Environ Chem 31–32:367–373

Bjornberg TKS, Por FD (1986) Comparative notes on the development of two species of *Bryocyclops* (Copepoda, Cyclopoida). In: Schriever G, Schminke HK, Shih CT (eds) Proceedings of the Second International Conference on Copepoda. Syllogeus 58:229–231

Bosnak AD, Morgan EL (1981a) Acute toxicity of cadmium, zinc, and total residual chlorine to epigean and hypogean isopods (Asellidae). NSS Bull 43:12–18

Bosnak AD, Morgan EL (1981b) Comparison of acute toxicity for Cd, Cr, and Cu between two distinct populations of aquatic hypogean isopods. 8th International Congress of Speleology 1:72–74

Boutin C, Boulanouar M, Yacoubi-Khebiza M (1995) Un test biologique simple pour apprécier la toxicité de l'eau et des sédiments d'un puits. Toxicité comparée, in vitro, de quelques metaux lourds et de l'ammonium, vis-à-vis de trois genres de crustacés de la zoocenose des puits. HydroEcologie Appliquée 7:91–109

Brown EJ, Rundle SD, Hutchinson TH, Williams TD, Jones MB (2005) A microplate freshwater copepod bioassay for evaluating acute and chronic effects of chemicals. Environ Toxicol Chem 24(6):1528–1531

Camargo JA, Alonso A (2006) Ecological and ecotoxicological effects of inorganic nitrogen pollution in aquatic ecosystems: a global assessment. Environ Int 32:931–849

Camargo JA, Alonso A, Salamanca A (2005) Nitrate toxicity to aquatic animals: a review with new data for freshwater invertebrates. Chemosphere 58:1255–1267

Canivet V, Chambon P, Gibert J (2001) Toxicity and bioaccumulation of arsenic and chromium in epigean and hypogean freshwater macroinvertebrates. Arch Environ Contam Toxicol 40(3):345–354

CCME—Canadian Council of Ministers of the Environment (2010) Canadian water quality guidelines for the protection of aquatic life: Ammonia. In: Canadian environmental quality guidelines. Canadian Council of Ministers of the Environment, Winnipeg

Chaton PF, Ravanel P, Tissut M, Meyran JC (2002) Toxicity and bioaccumulation of fipronil in the nontarget arthropodan fauna associated with subalpine mosquito breeding sites. Ecotoxicol Environ Saf 52(1):8–12

Chippaux JP, Yovo KP, Soakoude Y, Akogbeto M (1996) Efficacy of selected compounds on *Thermocyclops oblongatus*, one of the main intermediate hosts of guinea worm in Africa. Acta Hydrochim Hydrobiol 24(6):283–286

Colt JE, Armstrong DA (1981) Nitrogen toxicity to crustaceans, fish, and mollusks. Bio-Eng Symp Fish Cult 1:34–47

Crisp TM, Clegg ED, Cooper RL, Wood WP, Anderson DG, Baetcke KP, Hoffmann JL, Morrow MS, Rodier DJ, Schaeffer JE, Touart LW, Zeeman MG, Patel YM (1998) Environmental endocrine disruption: an effects assessment and analysis. Environ Health Perspect 106:11–56

Cvetkov L (1968) Un filet phreatobiologique. Bull Inst Zool Mus Acad Bulg Sc 7:215–218

Danielopol D (1983) Bekämpfungsmöglichkeiten der Grundwasserassel *Proasellus strouhali* Karaman nim Trinkwasserversorgungsnetz der Stadt Linz. Internal Report, Limnological Institute Mondsee, ÖAW, unpubl., 1–26

Dehedin A, Piscart C, Marmonier P (2013) 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 DMP (2013) Agricultural impact in Mediterranean alluvial aquifers: do groundwater communities respond? Fund Appl Limnol 182(4):271–282

Di Lorenzo T, Brilli M, Del Tosto D, Galassi DMP, Petitta M (2012) Nitrate source and fate at the catchment scale of the Vibrata River and aquifer (central Italy): an analysis by integrating component approaches and nitrogen isotopes. Environ Earth Sci 67(8):2383–2398

Di Lorenzo T, Stoch F, Galassi DMP (2013) Incorporating the hyporheic zone within the river discontinuum: longitudinal patterns of subsurface copepod assemblages in an Alpine stream. Limnologica 43:288–296

Di Marzio WD, Castaldo D, Pantani C, Di Cioccio A, Di Lorenzo T, Sáenz ME, Galassi DMP (2009) Relative sensitivity of hyporheic copepods to chemicals. Bull Environ Contam Toxicol 82(4):488–491

Di Marzio WD, Castaldo D, Di Lorenzo T, Di Cioccio A, Sáenz ME, Galassi DMP (2013) Developmental endpoints of chronic exposure to suspected endocrine-disrupting chemicals (EDCs) on benthic and hyporheic freshwater copepods. Ecotox Environ Safe 96:86–92

EEA—European Environmental Agency (2010) The European Environment. State and outlook 2010. Freshwater quality. Publications Office of the European Union, Luxembourg. doi: 10.2800/60214

Feind D, Zieris FJ, Huber W (1988) Effects of sodium pentachlorophenate on the ecology of a freshwater model ecosystem. Environ Pollut 50:211–223

Fleeger JW, Carman KR (2011) Experimental and genetic studies of meiofauna assess environmental quality and reveal mechanisms of toxicity. Vie Milieu 61:1–26

Folmer O, Black M, Hoe W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotech 3(5):294–299

Galassi DMP (2001) Groundwater copepods: diversity patterns over ecological and evolutionary scales. Hydrobiologia 453(454):227–253

Galassi DMP, Huys R, Reid JW (2009a) Diversity, ecology and evolution of groundwater copepods. Freshwater Biol 54:691–708

Galassi DMP, Stoch F, Fiasca B, Di Lorenzo T, Gattone E (2009b) Groundwater biodiversity patterns in the Lessinian Massif of northern Italy. Freshwater Biol 54:830–843

Galassi DMP, De Laurentiis P, Fiasca B (2011) Systematics of the Phyllognathopodidae (Copepoda, Harpacticoida): re-examination of *Phyllognathopus viguieri* (Maupas, 1892) and *Parbatocamptus jochenmartensi* Dumont and Maas, 1988, proposal of a new genus for *Phyllognathopus bassoti* Rouch, 1972, and description of a new species of *Phyllognathopus*. ZooKeys 104:1–65002E doi: 10.3897/zookeys.104.763

Gorbach S, Knauf W (1971) Endosulfan und Umwelt das Ruckstandsverhalten von Endosulfan in Wasser und seine Wirkung auf Organismen, die im Wasser leben. Schriftner ver Wasser Boden Lufthyg, Dahlem 34:85 pp

Hervant F (1996) The activities of enzymes associated with the intermediary and energy metabolism in hypogean and epigean crustaceans. C R Acad Sci III 319:1071–1077

Hervant F, Renault D (2002) Long-term fasting and realimentation in hypogean and epigean isopods: a proposed adaptive strategy for groundwater organisms. J Exp Biol 205(14):2079–2087

Hervant F, Mathieu J, Garin D, Fréminet A (1996) Behavioral, ventilatory and metabolic responses of the hypogean amphipod *Niphargus virei* and the epigean isopod *Asellus aquaticus* to severe hypoxia and subsequent recovery. Physiol Zool 69:12771300

Hose CG (2005) Assessing the need for groundwater quality guidelines for pesticides using the species sensitivity distribution approach. Hum Ecol Risk Assess 11:951–966

Hose CG (2007) Response to comments on assessing the need for groundwater quality guidelines for pesticides using the species sensitivity distribution approach. Hum Ecol Risk Assess 13(1):241–246

Humphreys WF (2007) Comment on assessing the need for groundwater quality guidelines for pesticides using the species sensitivity distribution approach by Hose (2005). J Econ Entomol 13:236–240

Hüppop C (1986) The role of metabolism in the evolution of cave animals. NSS Bull 47:136–146

Hurlbert SH, Mulla MS, Keith JO, Westlake WE, Dusch ME (1970) Biological effects and persistence of Dursban in freshwater ponds. J Econ Entomol 63:43–62

IOS—International Organization for Standardization (1999) Water quality-determination of acute lethal toxicity to marine copepods (Crustacea Copepoda). ISO 14669. 1st edn. Geneva, Switzerland

Johnson CR (1978) The effect of five organophosphorus insecticides on survival and temperature tolerance in the copepod, *Macrocyclops albidus* (Copepoda: Cyclopidae). Zool J Linn Soc 64(1):59–62

Kaur K, Ansal MD (1996) Sensitivity of selected zooplankton exposed to phosphamidon, fenitrothion, and fenthion. Bull Environ Contam Toxicol 57(2):199–203

Konar SK (1970) Toxicity of heptachlor to aquatic life. J Water Pollut Control Fed 42(8):299–303

Konar SK, Ghosh TK (1981) Effects of furadan on fish, plankton, worm and water quality. IAWPC Tech Annu 9:128–139

Kulkarni D, Daniel B, Preuss TG (2013a) Life-stage-dependent sensitivity of the cyclopoid copepod *Mesocyclops leuckarti* to triphenyltin. Chemosphere 92:1145–1153

Kulkarni D, Gergs A, Hommen U, Ratte HR, Preuss TG (2013b) A plea for the use of copepods in freshwater ecotoxicology. Environ Sci Pollut R 20(1):75–85

Lescher-Moutoué F (1973) Sur la biologie et l'écologie des copépodes cyclopides hypogés (crustacés). Ann Spéléol 28:429–502

Ludemann D, Neumann H (1960) Studies on the acute toxicity of modern contact insecticides in fresh water. Z Angew Zool 47:303–321

Ludemann D, Neumann H (1962) Über die Wirkung der Neuzeitlichen Kontaktinsektizide auf die Tiere des Subwassers. Anz Schaedlingskd Pflanzenschutz 35:5–9

Malard F, Hervant F (2001) Oxygen supply and the adaptation of animals in groundwater. Freshwater Biol 41(1):1–30

Marmonier P, Mazouzi C, Foulquier A, Simon N, François C, Hervant F, Mermillod-Blondin F, Vieney A, Barraud S, Togola A, Piscart C (2013) The use of crustaceans as sentinel organisms to evaluate groundwater ecological quality. Ecol Eng 57:118–132

Marten GG, Che W, Bordes ES (1993) Compatibility of cyclopoid copepods with mosquito insecticides. J Am Mosq Control Assoc 9(2):150–154

Mathews RC, Bosnak AD, Tennant DS, Morgan EL (1977) Mortality curves of blind crayfish (*Orconectes australis australis*) exposed to chlorinated stream water. Hydrobiologia 53:107–111

Mayer FL, Sun K, Lee G, Ellersieck MR, Krause GF (1999) User guide: acute to chronic estimation. Report nr EPA/600/R-98/152, U.S. Environmental Protection Agency, Washington DC. http://cfpub.epa.gov/si/si_public_file_download.cfm?p_download_id=506783. Accessed July 2013

Mayer FL, Ellersieck MR, Asfaw A (2010) Risk assessment tools: software and user's guide. SETAC, Pensacola, FL

Meinel W, Krause R, Musko J (1989) Experimentezur pH-Wert-abhängigen Toxizität von Kadmium bei einigen Grundwasser organismen. Z Angew Zool 75:159–182

Mösslacher F (2000) Sensitivity of groundwater and surface water crustaceans to chemical pollutants and hypoxia: implications for pollution management. Arch Hydrobiol 149(1):51–66

Mösslacher F, Notenboom J (1999) Groundwater biomonitoring. In: Gerhardt (ed) Biomonitoring of polluted water—reviews on actual topics. Trans Tech Publications, Switzerland, pp 119–140

Mukherjee SN, Deshpande P, Sharma RN (1990) Effect of azadirachtin on *Mesocyclops leuckarti* sensu lato, vector of dracunculiasis. Indian J Med Res 91:461–463

Musko IB (1987) Effect of pesticides on the ultrastructure of the alimentary canal of two freshwater copepods, Crustacea. Eur J Cell Biol 43(18):24

Naqvi SM, Hawkins RH (1989) Responses and LC50 values for selected microcrustaceans exposed to Spartan, Malathion, Sonar, Weedtrine-D, and Oust pesticides. Bull Environ Contam Toxicol 43(3):386–393

Notenboom J, Cruys K, Hoekstra J, van Beelen P (1992) Effect of ambient oxygen concentration upon the acute toxicity of chlorophenols and heavy metals to the groundwater copepod *Parastenocaris germanica* (Crustacea). Ecotox Environ Safe 24: 131–143

Novak A, Walters BS, Passino DRM (1980) Toxicity of contaminants to invertebrate food organisms. Progress in fisheries research 1980. Great Lakes Fisheries Laboratory, US Fish and Wildlife Services, Ann Arbor, MI, 2 pp

O'Halloran SL, Liber K, Gangl JA, Knuth ML (1999) Effects of repeated exposure to 4-nonylphenol on the zooplankton community in littoral enclosures. Environ Toxicol Chem 18(3):376–385

Peither A, Juttner I, Kettrup A, Lay JP (1996) A pond mesocosm study to determine direct and indirect effects of lindane on a natural zooplankton community. Environ Pollut 93(1):49–56

Ramaprabhu T, Ghose SR, Das MS, Mohanti AN (1986) Some observations on the use of ammonia as a fish toxicant. Indian J Comp Anim Physiol 4(1):39–48

Rand GM, Clark JR, Holmes CM (2000) Use of outdoor freshwater pond microcosms: II. Responses of biota to pyridaben. Environ Toxicol Chem 19(2):396

Reboleira AS, Abrantes N, Oromì P, Gonçalves F (2013) Acute toxicity of copper sulfate and potassium dichromate on stygobiont *Proasellus*: general aspects of groundwater ecotoxicological and future perspective. Water Air Soil Pollut 224:1550

Reid J (2001) A human challenge: discovering and understanding continental copepod habitats. Hydrobiologia, 453/454:201–226

Rettich F (1979) Laboratory and field investigations in Czechoslovakia with fenitrothion, pirimiphos-methyl, temephos and other organophosphorous larvicides. Mosq News 39(2):320–328

Robertson EB, Bunting DL (1976) The acute toxicity of four herbicides to 0–4 hour nauplii of *Cyclops vernalis* Fisher (Copepoda, Cyclopoida). Bull Environ Contam Toxicol 16(6):682–688

Roessink I, Belgers JDM, Crum SJH, Van der Brink PJ, Brock TMC (2006) Impact of triphenyltin acetate in microcosms simulating floodplains lakes. II Comparison of species sensitivity distribution between laboratory and semifield. Ecotoxicology 15:411–424

Samman J, Thomas MP (1978) Changes in zooplankton populations in the White Volta with particular reference to the effect of abate. Int J Environ Stud 12:207–214

Sharma MP, Dattagupta AK (1983) Response of some freshwater micrometazoans to DDT. Proc Indian Acad Sci Anim Sci 92(1): 19–29

Svobodova Z, Machova J, Faina R, Kocova A, Kunce P (1986) Acute toxicity of selected pesticides to aquatic organisms. Bull V U R H Vodnany 1:8–20

Tamura K, Peterson D, Peterson N, Stecher G, Nei N, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28(10):2731–2739. doi:10.1093/molbev/msr121

Traunspurger W, Drews C (1996) Toxicity analysis of freshwater and marine sediments with meio- and macrobenthic organisms: a review. Hydrobiologia 328:215–261

USEPA (1994a) US Environmental Protection Agency (USEPA). User's guide, Probit V. 1.5. USEPA, Cincinnati, OH

USEPA (1994b) US Environmental Protection Agency (USEPA). User's guide, Trimmed Spearman–Karber (TSK) V. 1.5. USEPA, Cincinnati, OH

Vijverberg J (1989) Culture techniques for studies on the growth, development and reproduction of copepods and cladocerans under laboratory and in situ conditions—a review. Freshwater Biol 21:317–373

Wendt-Rasch L, Friberg-Jensen U, Woin P, Christoffersen K (2003) Effects of the pyrethroid insecticide cypermethrin on a freshwater community studied under field conditions. Direct and indirect effects on the species composition. Aquat Toxicol 63(4):373–389

Wierzbicka M (1974) Influence of 2,4-D sodium salt on the survival of some Copepoda species. Pol Arch Hydrobiol 21(2):275–282

Wilhelm FM, Taylor SJ, Adams GL (2006) Comparison of routine metabolic rates of the stygobite, *Gammarus acherondytes* (Amphipoda: Gammaridae) and the stygophile, *Gammarus troglophilus*. Freshwater Biol 51:1162–1174

Willis KJ (1999) Acute and chronic bioassays with New Zealand freshwater copepods using pentachlorophenol. Environ Toxicol Chem 18(11):2580–2586