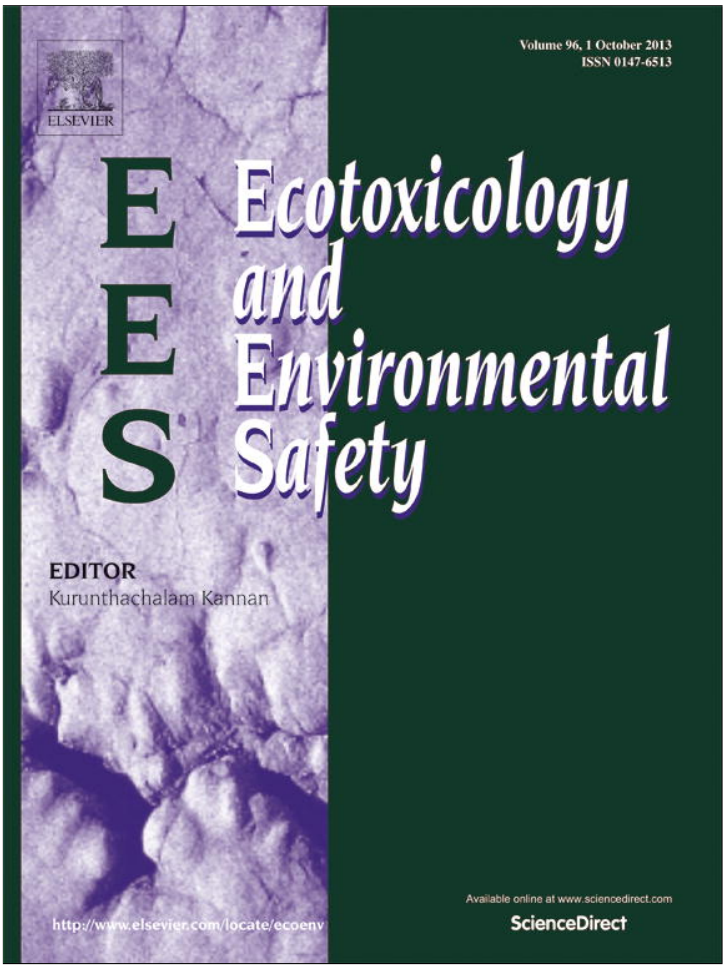

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Developmental endpoints of chronic exposure to suspected endocrine-disrupting chemicals on benthic and hyporheic freshwater copepods

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ABSTRACT

The aims of this study were: (i) to assess if carbamate pesticides and ammonium, widely detected in European freshwater bodies, can be considered ecologically relevant endocrine-disrupting chemicals (EDCs) for benthic and interstitial freshwater copepods; and (ii) to evaluate the potential of copepods as sentinels for monitoring ecosystem health. In order to achieve these objectives, four species belonging to the harpacticoid copepod genus *Bryocamptus*, namely *B.* (*E.*) *echinatus*, *B.* (*R.*) *zschokkei*, *B.* (*R.*) *pygmaeus* and *B.* (*B.*) *minutus*, were subjected to chronic exposures to Aldicarb and ammonium. A significant deviation from the developmental time of unexposed control cultures was observed for all the species in test cultures. Aldicarb caused an increase in generation time over 80% in both *B. minutus* and *B. zschokkei*, but less than 35% in *B. pygmaeus* and *B. echinatus*. Ammonium increased generation time over 33% in *B. minutus*, and 14, 12 and 3.5% for *B. pygmaeus*, *B. zschokkei* and *B. echinatus*, respectively. On the basis of these results it can be concluded that chronic exposure to carbamate pesticides and ammonium alters the post-naupliar development of the test-species and propose their potential role as EDCs, leaving open the basis to search what are the mechanism underlying. A prolonged developmental time would probably produce a detrimental effect on population attributes, such as age structure and population size. These deviations from a pristine population condition may be considered suitable biological indicators of ecosystem stress, particularly useful to compare polluted to unpolluted reference sites. Due to their dominance in both benthic and interstitial habitats, and their sensitivity as test organisms, freshwater benthic and hyporheic copepods can fully be used as sentinel species for assessing health condition of aquatic ecosystems as required by world-wide water legislation.

1. Introduction

Endocrine-disrupting chemicals (EDCs) are compounds that inhibit natural hormones activity, through altering their normal regulatory function in the immune, nervous and endocrine systems, and consequently interfering with hormone-dependent processes, such as development, behaviour, fertility and maintenance of homoeostasis (Crisp et al., 1998; Rodríguez et al., 2007). Several studies have examined the effects of these contaminants on copepods, although focusing mainly on marine species (Kusk and Wollenberger, 2007 and reference herein; Lauritano et al., 2012 and references herein). The response of marine copepods to toxicants, including EDCs, have been widely acknowledged and described in more than 600 papers (e.g. Coull and Chandler, 1992; Di Pinto and Coull, 1997; Forget et al., 1998; Kovatch et al., 1999; Andersen et al., 2001; Hagopian-Schlekat et al., 2001; Bejarano et al., 2004; Fleeger and Carman, 2011; Perez-Landa and Simpson, 2011). Conversely, the effects of pollutants on freshwater copepods have been very poorly investigated (Brown et al., 2003, 2005; Turesson et al., 2007; Garderström et al., 2008; Di Marzio et al., 2009). However, freshwater copepods show many attributes that make them suitable for toxicity bioassays (Turesson et al., 2007; Garderström et al., 2008; Di Marzio et al., 2009). Their small size, short life cycle, ease of rearing under laboratory conditions, and recognisable larval stages make relatively easier the evaluation of their sensitivity to pollutants along their post-embryonic development. Growth and development are considered endocrine endpoints related to invertebrate exposure to EDCs, with reference to

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larval and juvenile developmental rates, larval and adult survival, moult age, exoskeleton growth and development, and sex ratio (EC, 2012). As for other crustaceans, copepod moulting and post-embyonic development from first nauplius to last copepodite stage are controlled by ecdysteroids and juveniles hormones (Zou and Fingerman, 2003; Kusk and Wollenberger, 2007; Hopkins, 2009), allowing their use for laboratory testing of EDC effects.

Freshwater pollution from EDCs, including pesticides, polychlorinated biphenyls, polyaromatic hydrocarbons and dioxin, is particularly severe in Europe (EEA, 2008, 2011; EC, 2003). Several kinds of pesticides have been detected in European freshwater bodies, exceeding standard limits (0.1 μg/L), even in accordance with good agricultural practices (EEA, 2010). The mode of action of pesticides as EDCs is variable. With regards to pesticides that act on the nervous system, such as carbamate pesticides, they can reduce the acetycholinesterase activity, and hence block nerve impulses both in vertebrates and invertebrates (Song et al., 1997; Sturm and Hansen, 1999; Waye and Trudeau, 2011). This effect may be linked to the suppression of hormone release from the nervous system, consequently affecting hormone-dependent physiological processes; however, this aspect is still under scrutiny (EC, 2012). Although it can derive from natural sources, a high concentrations of ammonium in freshwater is mainly due to anthropic sources including use of synthetic fertilizers and wastewater discharges. Ammonium concentrations have decreased in European freshwater bodies from 1992 to 2008; however, 92% of monitoring sites still exceeds the standard limits established by the Water Framework Directive (0.5 mg/L). Unionized ammonium is toxic to aquatic animals, affecting gill epithelium, oxygen-carrying capacity and hepato-pancreas functions (Camargo and Alonso, 2006 and reference herein; Qichen et al., 2012). Ammonium nitrate causes severe breathing abnormalities in terrestrial adults of the amphibian *Triturus boscai* and long-term exposure may limit growth, ability to maintain sexual ornaments, and ultimately breeding success (Griffiths and Mylotte, 1988). However, the question whether ammonium is an ecologically relevant endocrine disruptor in invertebrates remains still uninvestigated.

Water legislation world-wide (e.g. the Water Framework Directive 2000/60/EC, the New South Wales State Groundwater Dependent Ecosystems Policy and the U.S. Clean Water Act) includes the obligation to assess the ecological status of freshwater ecosystems using biological indicators. The Crustacea Copepoda are the most abundant meiofaunal group in benthic and interstitial habitats of streams, springs and lakes (e.g. Dole-Olivier et al., 2000; Galassi, 2001; Galassi et al., 2009a; Di Lorenzo et al., 2013), as well as in groundwater (e.g. Stoch, 1995; Galassi et al., 2009a,b; Hahn and Fuchs, 2009; Malard et al., 2009; Stein et al., 2010; Stoch and Galassi, 2010; Di Lorenzo and Galassi, 2013), being good candidates for identifying quality standards of freshwater bodies. However, they have rarely been used as test species in full or partial life-cycle laboratory tests both in water-only (Brown et al., 2003, 2005) and sediment-associated bioassays (Turesson et al., 2007).

The aim of this study was: (i) to assess if carbamate pesticides and ammonium can be considered ecologically relevant EDCs for benthic and interstitial freshwater copepods; (ii) to evaluate the potential of copepods as sentinels for monitoring ecosystem health. In order to achieve these objectives, four species belonging to the harpacticoid copepod genus *Bryocamptus*, namely *Bryocamptus* (*Rheocamptus*) *zschokkei*, *Bryocamptus* (*Bryocamptus*) *minutus*, *Bryocamptus* (*Echinocamptus*) *echinatus*, and *Bryocamptus* (*Rheocamptus*) *pygmaeus*, were subjected to chronic exposures to Aldicarb and ammonium.

2. Materials and methods

2.1. Sampling and rearing

Copepods were sampled in benthic and interstitial habitats of the River Tirino (Central Italy), in a pristine environment few metres downstream the Presciano spring system, located in the southeastern part of the Gran Sasso Massif, at 330 m a.s.l.; coordinates 42°16′05″ N, 13°46′56″ E, near Capestrano town (L'Aquila, Italy). Quantitative samples were taken by pumping 20 l of water with a Bou-Rouch pump from 50 cm below the streambed, and filtering it through a 60 μm mesh net. Specimens were transported to the laboratory in the same water sampling (pH 7.7, conductivity: 436 μS/cm, total hardness: 190 mg/L as $CaCO_3$, $NH_4^+ < 0.03$ mg/L, NO_3^-: 3.9 mg/L, PO_4^{-3}: 0.01 mg/L, organic N: 0.8 mg/L, Cl^-: 4.3 mg/L, SO_4^{-2}: 15 mg/L, $Cu^{+2} < 0.005$ mg/L and Zn^{+2}: 0.01 mg/L) and kept refrigerated at the environmental temperature of 10 °C. In the laboratory, copepods were reared for 6–8 months in a commercial spring water (pH 7.84, conductivity: 306 μS/cm, hardness: 56 mg/L as $CaCO_3$, $NH_4^+ < 0.03$ mg/L, NO_3^-: 1.1 mg/L, Na^+: 1.23 mg/L, K^+: 0.16 mg/L, SO_4^{-2}: 1.3 mg/L, SiO_2: 1.7 mg/L), at 10 °C and 24 h dark, fed with natural dehydrated organic matter (mean concentration of particulate organic matter (POM): 1 ± 0.5 mg/L) collected at the same place and reared in coarse-fine sand sediment (0.063–2 mm). After acclimation, mono-specific cultures were set up for *Bryocamptus* (*R.*) *zschokkei*, *B.* (*B.*) *minutus*, *B.* (*E.*) *echinatus*, and *B.* (*R.*) *pygmaeus*. These species share key-characteristics: (i) wide geographical distribution in Europe (Dussart, 1969; Rundle et al., 2000); (ii) gonochorism; (iii) high frequency of occurrence in benthic and interstitial habitats (Dole-Olivier et al., 2000); (iv) ease of laboratory rearing; (v) relatively large body size (400–700 μm length). All species show the typical harpacticoid development, moulting through six naupliar (N1–N6) and five copepodid stages (C1–C5), with the sixth copepodid moult corresponding to the adult (A).

2.2. Test design

Chronic toxicity tests were carried out with Aldicarb purchased from Riedel-de Haën—Germany, and ammonium added as nitrate (Fluka—Germany). Both chemicals were analytical reagent-grade.

The experimental design was performed during copepodite life cycle, using the same environmental conditions as described for the cultures. Observation of copepods was done under a stereomicroscope with the light set to darkfield illumination at 100 × magnification. As the transition from the sixth naupliar (N6) to the first copepodite stage (C1) can be difficult to observe, chronic exposures started from copepodite C2 that was exposed (12 replicates, each of 10 individuals) in 5 cm—diameter polystyrene Petri dish containing 10 mL of the appropriate test solution. Acetone was used as pesticide carrier for Aldicarb at a final concentration lower than 500 μL/L. Appropriate controls were designed. Every 48–72 h, each replicate was observed for the presence of dead individuals (no movement after gentle needle stimulation) and every 96 h all individuals were attributed to a moulting stage. Test solution was renewed to ensure 10 mL as final volume.

Copepodite developmental times were assessed per each individual and under controlled temperature and food conditions, starting by an initial cohort of C2 copepodites that were reared to adulthood at sub-lethal concentrations of Aldicarb and ammonium. The tests were terminated when all surviving individuals reached the adult stage. Depending on the species, the duration of the tests varied between 60 and 90 days.

Taking into account the LC50-96 h values for the assayed species (Table 1), chronic exposure was carried out at 0.25 × LC50-96 h of the most sensitive species: 3.65 mg/L for ammonium and 0.65 mg/L for Aldicarb. Experiments were conducted in accordance with national and institutional guidelines for the protection of human subjects and animal welfare.

Table 1
Lethal concentrations 96 h (LC50–96 h) and ±95 % confidence limits (CL) for adults of harpacticoid species exposed to Aldicarb and ammonium. Expressed in mg/L (Di Marzio et al., 2009).

LC50-96 h	*B. pygmaeus*	*B. minutus*	*B. zschokkei*	*B. echinatus*
Aldicarb	2.42 (2.05–2.86)	2.5 (2.19–2.78)	2.47 (1.69–3.6)	2.71 (2.42–3.04)
Ammonium	18.22 (15.37–21.61)	18.22 (15.37–21.61)	18.63 (16.63–20.87)	14.61 (12.76–16.73)

2.3. Chemical analysis

Ammonium and Aldicarb concentrations were measured every week at each reference Petri dish as a mean value for all replicates. Ammonium concentration was measured by Hach method # 8038, adapted from Standard Methods for the Examination of Water and Wastewater 4500-NH_3 B and C, using a DR3900 Hach spectrophotometer. Limit of quantification (LOQ) equal to 20 μg/L. Aldicarb concentration was measured by SPE-GC–MS according to Carabias-Martínez et al. (2003). A 250-mL volume of sample was passed through an SPE cartridge. The analytes retained were eluted with 2 mL of acetonitrile. The eluate collected was evaporated to dryness under a stream of N_2 and the residue was redissolved in 500 μL of acetonitrile; 1 μL was injected directly into the chromatograph. The GC–MS system consisted of a gas chromatograph GC-17A (Shimadzu, Kyoto, Japan) coupled with a quadrupole mass spectrometer QP 5000 (Shimadzu). A capillary column, SPB-5, poly-(5% diphenyl–95% dimethylsiloxane) 30 m × 0.25 mm I.D. and 0.25 μm film thickness (Supelco, Bellafonte, PA) was used. The chromatographic conditions were as follows: injector temperature: 280 °C; oven temperature programme: 50 °C (5 min) to 100 °C (5 min) at a rate of 25 °C/min. The carrier gas was helium at a column flow-rate of 1.2 ml/min. The temperature at the interface was 280 °C. A mass interval of 100.0 to 400.0 a.m.u. and a scanning interval of 0.5 s were used. Spectra were obtained at 70 eV and sample injection was accomplished in splitless mode. The ions selected to identification and quantification the response in SIM mode were 100, 115 and 68 m/z. C-18 was employed as sorbent, obtaining recoveries between 85 ± 15 for Aldicarb. The limit of quantification (LOQ) obtained was 10 μg/L.

2.4. Developmental endpoints and data analysis

Copepods were sampled at intervals of four days to assess the evolving stage structure of the initial cohorts. Time series of the percentage of the cohort that has matured to at least stage i (PC_i) were derived. These PC_i values were fitted against exposure time by least-squares linear regression analysis (LSR), considering a normal distribution of PC_i for at least a restricted portion of data ($5\% < PC_i < 95\%$) according to Peterson (1986). This approach allows the measurement of median development time (MDT) for each stage i (MDT_i) which is defined as the length of time from stage i to stage i+1 until PC_i equals 50% (Campbell et al., 2001). MDT_i for adults is assumed as a median generation time (GT). GT's increase (GT_i) was obtained from $GT_i = GT_x \times 100/GT_c$, GT_x is GT for Aldicarb or Ammonium and GTc is GT for control animals. The difference between the MDT of two successive stages provides a measure of stage duration (SD), being $SD_i = MDT_{i+1} - MDT_i$. Also $MDT_i = SD_1 + SD_2 + \cdots + SD_{i-1}$. According to Gentleman et al. (2008) a linear fit describes the developmental time variability (DTV_i) as the inverse of the slope of the regression line for stage i and equivalent to estimate the time interval during which the entire cohort completes the moult to stage i. Normal distribution of the PC_i also implies that the median matches the mean development time (DT) for an individual to reach stage i (i.e. $MDT_i = \overline{DT_i}$). A least-squares line of best fit with intercept α and slope β gives $MDT_i = (0.5 - \alpha)/\beta$ and $DTV_i = 1/\beta$ when PC_i is equal to 50% (Gentleman et al., 2008). With this approach we obtained the following parameters that describe copepod development from copepodite C2 to adult (A): SD, MDT, DTV, GT. Data of PC_i were fitted by LSR and compared by ANOVA—Dunnet's test using Statistica v8 (StatSoft, Inc., 2007) and ToxStat V 3.5 (WEST Inc., 1996).

3. Results

Development parameters and the slopes of the regression lines fitting developmental time variabilities for each toxicant and moulting stage are reported in Tables 2 and 3, respectively. Individuals belonging to the species *Bryocamptus minutus*, when exposed to ammonium, showed alterations in developmental rate for all stages from C3 to Adult (A), leading to significant changes in GT which was greater than 33% (Table 2). An increase in the number of males produced a reversal of the male/female ratio observed in control groups (Table 2). For the cohorts exposed to Aldicarb, a GT increase of 85% was observed (Table 2). The sex ratio was not reversed but an increase in the percentage of females compared to males was observed. Developmental time slopes were significantly different from control for all moulting stages for both ammonium and Aldicarb (Table 3, Fig. 1). *Bryocamptus pygmaeus* showed a delayed development at the stage C4, under exposure to ammonium, which was offset by a DTV shortening between copepodid stages C4 and C5, leading to a GT increase of only 14% compared to control cohorts (Table 2). Sex ratio was reversed and a significant increase in the proportion of females

Table 2
Development time parameters, in days, for each moulting stage of the assayed species exposed to 3.65 mg ammonium/L and 0.65 mg aldicarb/L.

Species	Control				Aldicarb				Ammonium			
	C3	C4	C5	A	C3	C4	C5	A	C3	C4	C5	A
B. pygmaeus												
MDT	23.23	34.99	43.93	54.31	37.72	48.33	56.47	66.78	30.57	49.33	53.87	61.72
SD	14.23	11.76	8.94	10.38	23.00	10.60	8.14	10.32	14.00	18.77	4.54	7.86
DTV	36.64	43.35	45.45	41.14	43.44	40.42	43.71	44.35	30.63	38.08	44.62	33.55
GT	–	–	–	54.31	–	–	–	66.78	–	–	–	61.72
Male %	–	–	–	55	–	–	–	90	–	–	–	25
Female %	–	–	–	45	–	–	–	10	–	–	–	75
B. minutus												
MDT	17.67	22.11	31.75	44.25	31.61	39.54	57.13	82.00	27.97	38.70	47.51	58.89
SD	10.00	4.44	9.64	12.50	24.00	7.93	17.60	24.87	20.00	10.72	8.81	11.38
DTV	16.92	24.38	23.21	23.09	39.60	33.10	55.31	80.00	38.68	31.92	32.06	34.93
GT	–	–	–	44.25	–	–	–	82	–	–	–	58.89
Male %	–	–	–	35	–	–	–	10	–	–	–	60
Female %	–	–	–	65	–	–	–	90	–	–	–	40
B. echinatus												
MDT	15.31	23.97	29.79	38.91	15.31	27.96	41.75	51.83	18.38	26.35	34.21	40.23
SD	8.00	8.65	5.82	9.12	8.00	12.64	13.79	10.08	10.00	7.98	7.85	6.02
DTV	18.11	23.29	33.50	39.12	18.11	28.51	41.82	45.75	25.42	27.85	33.15	37.11
GT	–	–	–	38.91	–	–	–	51.83	–	–	–	40.23
Male %	–	–	–	40	–	–	–	66	–	–	–	20
Female %	–	–	–	60	–	–	–	34	–	–	–	80
B. zschokkei												
MDT	18.99	28.71	38.95	46.60	32.67	43.37	65.52	85.00	19.60	30.66	41.86	52.18
SD	11.00	9.72	10.24	7.65	16.00	10.70	22.15	19.48	12.00	11.06	11.21	10.32
DTV	20.79	21.79	22.76	20.29	44.50	44.27	44.48	47.46	22.49	30.08	32.68	34.67
GT	–	–	–	46.60	–	–	–	85	–	–	–	52.18
Male %	–	–	–	35	–	–	–	10	–	–	–	90
Female %	–	–	–	65	–	–	–	90	–	–	–	10

MDT: median development time; SD: stage duration; DTV: developmental time variability; GT: generation time.

was observed (Table 2). Developmental time slopes were significantly different from control for moulting transitions C2–C3 and C5-A (Table 3, Fig. 1). For the cohorts exposed to Aldicarb, the developmental rate from one stage to another was the same observed in the control, except for stage C3, which led to an increased 23% GT. Males proportion increased compared to control, although male/female ratio was in favour of males in both exposed and control conditions (Table 2). The developmental time slopes were significantly different from control for moulting stages C2–C3 only (Table 3, Fig. 1). In the case of *Bryocamptus echinatus*, ammonium altered the development at the first two stages, C2–C3 and C3–C4, increasing DTV at these developmental stages (Table 2). However, slopes were modified along the transition C4–C5 and approximated the values observed in the control, giving a slightly higher GT (3.5%). The proportion of females was increased, but the relationship between sexes was maintained (Table 2). Developmental time slopes were significantly different from control for moulting stages C2–C3 and C3–C4 (Table 3; Fig. 2). For the cohorts exposed to Aldicarb, while C2–C3 moulting was not altered in DTV and in developmental rate, the trend abruptly changed from C3 to adult stages. The values of the slopes and DTV showed trend reversal, and a steep increase in generation life time

Table 3
Fit parameters of developmental time for each moulting stage. $\beta \pm$ sd: slope and standard deviation from regression analysis, all significantly different from zero at $p < 0.001$. In bold slopes different from population controls (ANOVA—Dunnet at $p < 0.05$).

Species	Control		Aldicarb		Ammonium	
	$\beta \pm$ sd	R^2	$\beta \pm$ sd	R^2	$\beta \pm$ sd	R^2
B. pygmaeus						
C2–3	2.729 ± 0.151	94.77	**2.302 ± 0.089**	97.04	**3.265 ± 0.126**	97.65
C3–4	2.307 ± 0.069	98.02	2.474 ± 0.079	97.98	2.626 ± 0.080	98.17
C4–5	2.200 ± 0.087	96.63	2.288 ± 0.107	96.2	2.241 ± 0.057	98.72
C5-A	2.431 ± 0.065	98.56	2.255 ± 0.082	97.42	**2.981 ± 0.100**	98.22
B. minutus						
C2–3	5.910 ± 0.676	90.51	**2.525 ± 0.120**	95.68	**2.585 ± 0.070**	98.55
C3–4	4.101 ± 0.304	93.81	**3.021 ± 0.116**	97.66	**3.133 ± 0.153**	96.31
C4–5	4.308 ± 0.222	96.91	**1.808 ± 0.123**	94.7	**3.119 ± 0.194**	94.12
C5-A	4.330 ± 0.217	97.07	**1.250 ± 1.976**	16.67	**2.863 ± 0.183**	93.84
B. echinatus						
C2–3	5.523 ± 0.4651	94.63	5.523 ± 0.4651	94.63	**3.934 ± 0.2349**	95.90
C3–4	4.294 ± 0.1197	99.08	**3.507 ± 0.1819**	96.37	**3.591 ± 0.1231**	98.38
C4–5	2.985 ± 0.08383	98.75	**2.391 ± 0.1000**	96.95	3.017 ± 0.09266	98.51
C5-A	2.556 ± 0.07587	98.44	**2.186 ± 0.07369**	97.78	2.695 ± 0.1277	96.12
B. zschokkei						
C2–3	4.810 ± 0.391	93.79	**2.247 ± 0.080**	97.28	**4.446 ± 0.312**	94.40
C3–4	4.589 ± 0.241	97.31	**2.259 ± 0.066**	98.3	**3.324 ± 0.187**	95.74
C4–5	4.393 ± 0.244	96.99	**2.248 ± 0.098**	96.69	**3.060 ± 0.119**	97.63
C5-A	4.929 ± 0.339	95.46	**2.107 ± 0.158**	94.62	**2.884 ± 0.129**	96.50

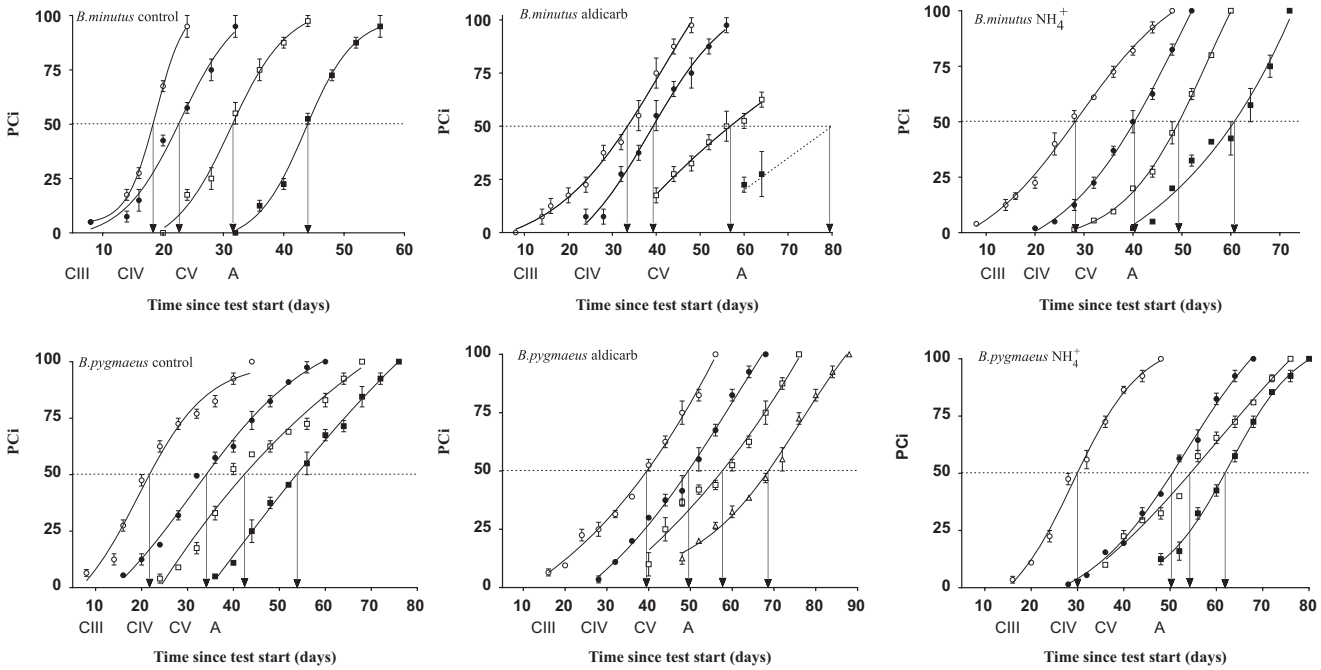

Fig. 1. Development time of *B. minutus* and *B. pygmaeus* for control populations and those exposed to aldicarb and ammonium. PC_i: percentage of the cohort that has matured to at least stage *i*.

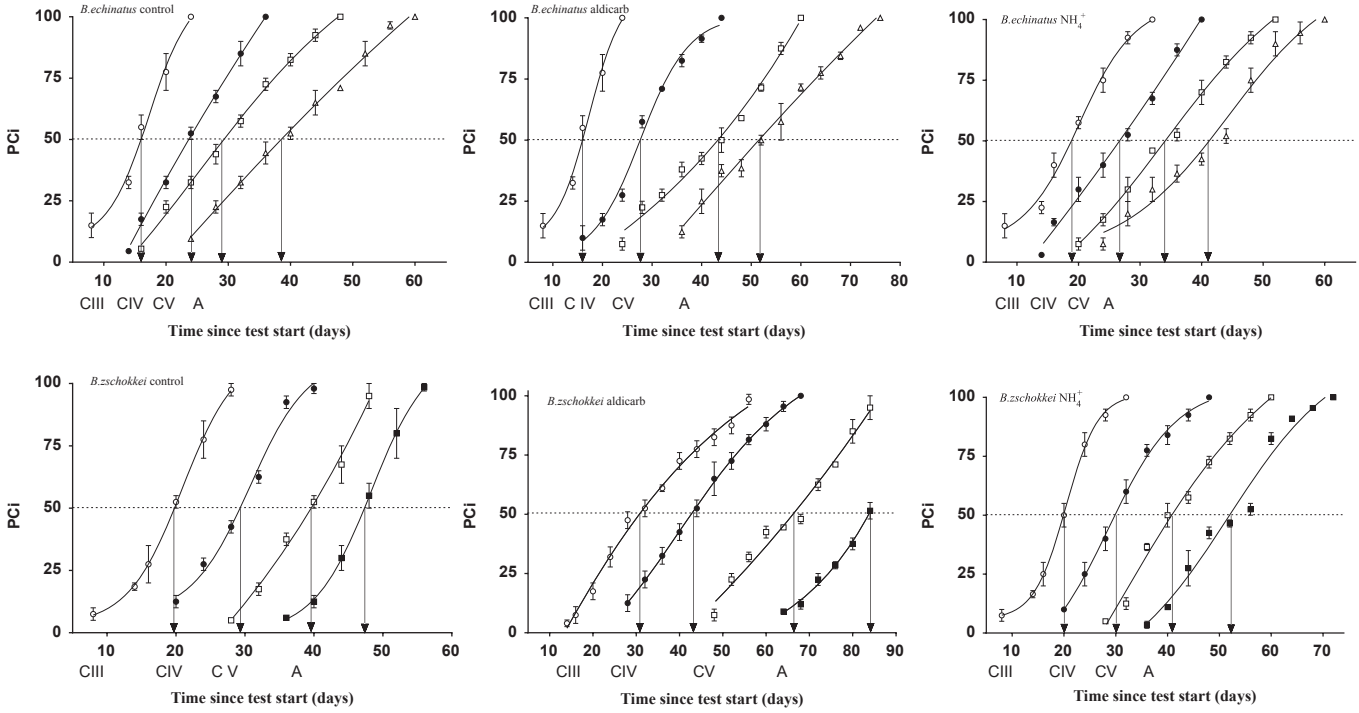

Fig. 2. Development time of *B. echinatus* and *B. zschokkei* for control populations and those exposed to aldicarb and ammonium. PC_i: percentage of the cohort that has matured to at least stage *i*.

over 33% compared to control populations was observed. Reversal of sex ratio derived from an increase in the number of males (Table 2). Developmental time slopes were significantly different from control for all moulting stages, except C2–C3 (Table 3; Fig. 2). The cohorts of *Bryocamptus zschokkei* exposed to ammonium diverged significantly from the control in the developmental rate throughout the life cycle, from C3 to adult stage A. Moult timing among stages was reduced, generating an increase in DTV. Finally, the GT increased by 12% compared to controls. An increase in the number of males occurred, determining sex ratio reversal (Table 2). A similar pattern was observed in the cohorts exposed to Aldicarb. However, the effects on developmental rates were much more impressive and the population showed GT greater than 82%, with a higher percentage of females (Table 2). Developmental time slopes were significantly different from control for all moulting stages for both ammonium and Aldicarb (Table 3; Fig. 2).

4. Discussion

While much information is available on development and life cycles of marine planktonic cyclopoids and harpacticoids, little is known about freshwater benthic and hyporheic copepods, especially from lotic habitats (Sarvala, 1990; Dole-Olivier et al., 2000; Galassi, 2001, Galassi et al., 2009a). However, there are some development traits that are shared by all copepod species (Hart, 1990; Kiørboe and Sabatini, 1995): (i) the rate or slope of development is strongly affected by temperature and food supply; (ii) under conditions of unlimited food resources, development times from egg to adult are independent of adult body size; (iii) males develop to adult more rapidly than females; (iv) copepods go through six naupliar and six copepodite stages, the C6 being the adult (A). At first glance, comparing the acute toxicity values for the toxicants tested, it seems that there are no significant interspecific differences when extrapolated to population effects (Table 1). Considering the assessed LC50s for the species analysed (Di Marzio et al., 2009), the European standard limits for ammonium and Aldicarb in surface water bodies (0.5 mg/L and 0.1 μg/L, respectively) seem to be adequate to protect benthic and hyporheic copepods from acute effects, at least at the adult stage. However, a significant increase in the developmental times, respect to control populations, was observed for the cohorts of all species under chronic exposure, at least in certain moulting stages. These results highlight that carbamate pesticides and ammonium do harm freshwater harpacticoid development, although it remains still unclear if the observed effects can be attributable to endocrine disruption. In fact, in recent years, several studies on marine copepods have demonstrated that larval development, tightly associated to moulting processes, is highly sensitive to a wide range of pollutants, including chemicals that were never suspected to act as EDCs (Kusk and Wollenberger, 2007 and references herein). As far as freshwater copepods are concerned, the matter has been very poorly investigated. Brown et al. (2003) observed that *Bryocamptus zschokkei* showed an increased developmental time to reach the adult stage respect to control after exposure to lindane (insecticide). However, the exposed cohorts did not show any alteration of the proportion of the total developmental time spent at each moulting stage that remained equiproportional as for the control cohorts (Brown et al., 2003). Although lindane is known to be an EDC for invertebrates, acting as ecdysone agonist (Dinan et al., 2001), Brown et al. (2003) reached the conclusion that lindane likely did not disrupt the endocrine function in *B. zschokkei* during moulting since it did not affect the equiproportional development of the species. As previously supposed for other taxa (Maund et al., 1992; Blockwell et al., 1999), Brown et al. (2003) argued that, under lindane exposure, *B. zschokkei* was forced to increase metabolic demands for maintaining homoeostasis, causing the redirection of energy to other physiological processes, such as feeding rates, thus consequently affecting developmental rate.

B. minutus and *B. zschokkei* seemed to be more sensitive to both Aldicarb and ammonium as shown by SD values that were significantly divergent from the control at all stages, while a significant deviation from the developmental time of the control

was observed only in some moulting stages for *B. echinatus* and *B. pygmaeus*. This result suggests that there are differences in how harpacticoid species belonging to the same genus may react to chronic exposure to toxicants, as already observed in marine species (Lauritano et al., 2012 and references herein).

The effect of Aldicarb on the developmental time spent to reach the adult stage is more severe than ammonium. Dissimilarities in the induced effects on development may reside in different reasons: (1) diverse accumulation patterns and detoxification processes that force individuals exposed to Aldicarb to divert more energy for activating a series of cellular defence systems (Lauritano et al., 2012, and references herein) than to development and moulting; (2) the neurotoxic Aldicarb is a more effective EDC than ammonium; (3) a combination of both. Since the mode of action behind the toxic effect of these two chemicals is still unknown at molecular scale, none of the hypotheses can be discarded.

Aldicarb caused an increase in generation time over 80% in both *B. minutus* and *B. zschokkei*, but less than 35% in *B. pygmaeus* and *B. echinatus*. The hypothesis that the observed differences among species may be attributable to different sex ratios cannot be ruled out; male copepods are known to develop more rapidly than females (Hart, 1990; Kriørboe and Sabatini, 1995) and they accounted only for 10% in the cohorts of *B. minutus* and *B. zschokkei* exposed to Aldicarb and for 90% in the cohorts of *B. pygmaeus* and *B. echinatus*. However, sex ratio seemed not to have the same influence on the cohorts exposed to ammonium, whose generation time was increased of no more than 33% for all the species. Nevertheless, many crustaceans have environmental sex determination which may make them prone to changes in sex ratio but in a way still in need to be deeply explored (Ford, 2012).

Although changes in total abundances were not apparent in the chronic bioassays we performed, because all the exposed individuals survived, alteration in the demographic structure may negatively affect individual fitness and population dynamics. When transposing on the field the results obtained from this study, namely in polluted environments, this pattern likely results in population decline after a few generations, if contamination persists, since prolonged developmental rates may lead to a decrease in population reproductive potential, declining in the expected number of offspring due to lower abundances of fertile adults and high abundances of juveniles, even if belonging to the same species. Nevertheless, experimental designs performed on the field for evaluating the effects of pollutants on communities or species assemblages almost always rely on abundances and/or presence/absence of sensitive species (Moldovan et al., 2013 and references herein). Our study suggests caution in placing too much emphasis on specific abundances only, and adds the caveat that low contamination levels may influence age structure of the populations, and lower resilience even under unmodified species abundances.

5. Conclusions

The result of our study highlights that aldicarb and ammonium could to act as EDCs on freshwater benthic and interstitial copepods. Nevertheless, the specific mechanisms by which the endocrine system can be disrupted are scarce in most invertebrates (Pinder et al., 1999; Sumpter, 2002; Oehlmann and Schulte-Oehlmann, 2003) and this aspect is crucial for confirming the role of some chemicals as endocrine disruptors, as well as for predicting the potential of others as EDCs. We propose the potential role of aldicarb and ammonium as EDCs based on developmental parameters, leaving open the basis to search what are the mechanism underlying.

Although the sensitivity to the tested toxicants differs among copepod species, a significant deviation from the developmental time of the control was observed for all the species under investigation. This result indicates that changes in population age classes may be considered a suitable biological indicator of ecosystem stress, particularly useful when species abundances do not vary between polluted and unpolluted reference sites. New approaches, including a combination of field investigations and toxicity bioassays, are crucial for a correct evaluation of the effects of a given pollutant on freshwater ecosystem health. Due to their dominance in both benthic and interstitial habitats, and their sensitivity as test organisms, freshwater benthic and hyporheic copepods can fully be used as sentinel species for assessing health condition of aquatic ecosystems as required by world-wide water legislation.

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