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

W.D. Di Marzio[a,b,*], D. Castaldo[c], T. Di Lorenzo[d], A. Di Cioccio[c], M.E. Sáenz[a,b], D.M.P. Galassi[c]

[a] Comisión Nacional de Investigaciones Científicas y Técnicas CONICET, Argentina
[b] Programa de Investigación en Ecotoxicología, Departamento de Ciencias Básicas, Universidad Nacional de Luján, C.C. 221, 6700 Luján B, Argentina
[c] Department of Life, Health and Environmental Sciences, University of L'Aquila, Via Vetoio, Coppito, 67100 L'Aquila, Italy
[d] Istituto per lo Studio degli Ecosistemi, CNR, Via Madonna del Piano 10, 50019 Sesto Fiorentino, Firenze, Italy

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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

* Corresponding author at: Programa de Investigación en Ecotoxicología, Departamento de Ciencias Básicas, Universidad Nacional de Luján, C.C. 221, 6700 Luján B, Argentina. Fax: +54 2323 423171x285.
E-mail addresses: dimarzio@speedy.com.ar, ecotoxicologia@mail.unlu.edu.ar, ecotoxicologi@aae.org.ar (W.D. Di Marzio).

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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 waste-water 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)