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Test procedures for measuring the (sub)chronic effects of chemicals on the freshwater cyclopoid *Eucyclops serrulatus*

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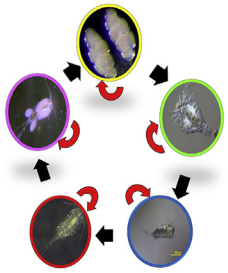
HIGHLIGHTS

- A full life-cycle protocol with the cyclopoid *Eucyclops serrulatus* is proposed.
- The best performance was reached on an mixed algal diet, 2 mL vials and at 18 °C.
- The control full life-cycle trial lasted 51 days at 18 °C.
- The chronic test with a mixed algal diet, in 2 mL vials, at 25 °C lasted 42 days.
- Subchronic tests at the conditions of the chronic tests are available options.

GRAPHICAL ABSTRACT



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ABSTRACT

The purpose of this study has been to describe test procedures for measuring the (sub)chronic effects of chemicals on the freshwater cyclopoid *Eucyclops serrulatus*. To this end we have adapted the setting of the standard full life-cycle protocol of the marine harpacticoid *A. tenuiremis* to *E. serrulatus*. We have tested the effects of 4 different diets, two temperatures and two rearing volumes on the survival, development, reproduction and population growth rates of this species. Our results have highlighted that full life-cycle tests can be run using 2 mL-glass vials, a diet consisting of a mixture of living cells of *Chlorella sorokiniana* and *Scenedesmus quadricauda*, at either 25 °C (test duration: 42 days) or 18 °C (test duration: 51 days). However, the best performance in terms of survival, development, reproducibility and population growth rates with this species was obtained at 18 °C, albeit with significantly longer test duration. Subchronic tests in 2 mL-glass vials with the mixture microalgal diet at both temperatures are available options if considered appropriate for the objectives of a given study. In particular, developmental tests from nauplius to copepodid may profitably be performed in about 11 days at 18 °C and in 6 days at 25 °C. Under the same test conditions, subchronic tests from copepodid to adult may be run in 19 days at 18 °C and in 16 days at 25 °C.

1. Introduction

In the last fifty years, chemical pollution originating from anthropic activities have led to severe changes in the composition of freshwater communities (Vörösmarty et al., 2000; Allan and

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Castillo, 2007; Woodward et al., 2012). ERA (environmental risk assessment) is the process used to identify and evaluate the adverse effects of chemicals on ecosystems (Fairman et al., 1999). The risk posed to freshwater taxa is usually estimated by means of toxicological bioassays (European Commission, 2003; European Medicines Agency, 2006; Defra, 2011). Among chronic tests, full life-cycle bioassays are widely considered the best tools to study the effect of chemicals (Forbes and Calow, 2002; European Commission, 2003; Godoy et al., 2015).

Copepod species are priority crustacean taxa, with regard to toxicity tests (Kulkarni et al., 2013a). Copepods are the most abundant meiofaunal group of crustaceans in benthic and interstitial habitats of streams, springs and lakes (Dole-Olivier et al., 2000; Galassi, 2001; Galassi et al., 2009a; Di Lorenzo et al., 2013), as well as in groundwater (Galassi et al., 2009a,b; Hahn and Fuchs, 2009; Malard et al., 2009; Stoch and Galassi, 2010). They form an important energy link in aquatic food webs as they feed on bacteria, algae, detritus, rotifers, crustaceans, dipteran larvae and even larval fish (Reid and Williamson, 2010). Copepods also serve as prey for larger crustaceans and larval fish (Dole-Olivier et al., 2000; Galassi, 2001). The attention to copepods in chronic tests has so far concerned marine, estuarine or brackish species (Chandler and Green, 2001; Breitholtz et al., 2003; Chandler et al., 2004; Lundström et al., 2010; OECD, 2014). The full life-cycle test with the marine benthic harpacticoid *Amphiascus tenuiremis* is currently the only chronic standard for copepods (ASTM, 2004; OECD, 2014). In contrast, the small number of chronic bioassays with freshwater copepods performed over the recent years (Brown et al., 2005; Turesson et al., 2007; Di Marzio et al., 2013; Kulkarni et al., 2013b; Marus et al., 2015) have been run with different, non-standard, methods (Kulkarni et al., 2013a).

ERA preferentially requires toxicological data following standard tests (US EPA, 1998; OECD, 1999; European Commission, 2003; European Medicines Agency, 2006). However, since the development of standard tests with new species is costly and may take from 10 to 15 years, adaptation of current standard tests to new species is a potential way forward (Breitholtz et al., 2011). The choice of food, temperature and rearing volume is a critical issue in adapting a standard test to a new copepod species, because these factors affect copepod survival, development and reproduction (Maier, 1990; Hart, 1998; Twombly et al., 1998; Nandini and Sarma, 2007; Koussoroplis et al., 2014; Suárez-Morales, 2015).

The purpose of this study has been to describe test procedures for measuring the (sub)chronic effects of chemicals on the freshwater cyclopoid *Eucyclops serrulatus*. To this end we have adapted the setting of the standard full life-cycle test with the marine copepod species *A. tenuiremis* (OECD, 2014) to *E. serrulatus,* so to meet the test validity criteria. We have tested the effects of 4 different diets, two temperatures and two rearing volumes on the survival, development, reproduction and population growth rates of this species. Specifically, we tested: (1) three microalgal diets, namely two microalgae species used in the standard reproduction test of *Daphnia magna* (OECD, 2012), the mixture of the two microalgae (equal ratio 1:1), and a non-algal diet; (2) 18° C and 25 °C; and (3) coated plastic microwells (300 μL) used in the standard full life-cycle test of *A. tenuiremis* (OECD, 2014), and glass vials (2 mL). The non-algal diet consisted in 45 μm-filtered water collected from the habitat from which we sampled *E. serrulatus* individuals. We have selected 18 °C and 25 °C because 18 °C is the minimum temperature allowed in the standard reproduction test of *Daphnia magna* (OECD, 2012) and 25 °C is the temperature used in the standard full life-cycle test of *A. tenuiremis* (OECD, 2014). Ultimately, we have selected *E. serrulatus* because the Cyclopidae is the largest freshwater copepod family, including more than 800 acknowledged species (Boxshall and Defaye, 2008). In addition, *E. serrulatus* has a wide ecological niche overlapping with that of many other freshwater taxa (Alekseev et al., 2005). It is distributed worldwide, with the exception of Antarctica, the Far East and South-East Asia (Alekseev and Defaye, 2011). It lives primarily in benthic habitats of lakes and streams, but it is frequently found even in alluvial (Di Lorenzo and Galassi, 2013; Di Lorenzo et al., 2014) and karstic aquifers, in springs (Galassi et al., 2014; Stoch et al., 2016) and in hyporheic zones (Alekseev et al., 2005; Di Lorenzo et al., 2013). It has a short life-cycle and it is suitable for rearing in the laboratory (Nandini and Sarma, 2007). Lastly, its respirometric metabolism have been fully investigated (Di Lorenzo et al., 2015a, 2016) and its transcriptome resources are available (Baratti et al., 2015).

2. Material and methods

2.1. Principles of the test with Amphiascus tenuiremis

The standard full life-cycle test with *A. tenuiremis* is fully described in OECD (2014). Briefly, newly hatched nauplii are exposed individually in 300 μL-microwell test chambers (60–120 nauplii are allocated over at least three replicate microplates per treatment). It is recommended that no more than 20–40 nauplii be assayed per microplate. The test animals are fed on a mixture of microalgae (1:1:1) and kept at 25 °C. About 90% of the total test medium volume is renewed using a Hamilton gas syringe at least every three days. Copepod life-cycle endpoints are monitored daily in each microplate. When 50% of the copepods can be sexed visually, mature virgin males and females are paired randomly. Each mating pair is then loaded into a new or unused microwell in the same treatment microplate from which it came. Observations are made daily for the presence of females carrying their first and subsequent egg sacs. Numbers of successfully hatching nauplii (i.e., called total viable clutch, or brood, or fecundity) is recorded for the first and second successive clutches of eggs. The duration of the full life-cycle test is 36 days. For the test to be valid, the following validity criteria are applied: 1) the average survival of the parent generation in the control should be at least 70%; 2) sex ratio at maturity in the control should on average be between 35% and 65% = %male:%female or %female:%male; 3) at least 75% of the control mating pairs are able to produce offspring by the end of the test; 4) the average number of viable offspring through two clutches in the controls is at least eight individuals; 5) the average number of days to extrusion of the first control egg clutch at a temperature of 25 °C should be less than or equal to 25 days in this 36 day test. As the criteria 4) and 5) are species specific, they were not considered in this study. We applied the 70% average survival criterion to subchronic tests as well (from nauplius to copepodid and from copepodid to adult).

2.2. Specimens collection

Details on collection and set up of the stock culture of *E. serrulatus* are provided in Di Lorenzo et al. (2016). Ovigerous females of *E. serrulatus* were picked up by a glass micropipette from the stock culture under a stereomicroscope (Leica M80) at 12× magnification. One hundred and twenty ovigerous females (each bearing 14–16 eggs) were pooled in four 25-mL glass beakers (30 ovigerous females per beaker; Fig. 1). One beaker was filled with a bore water (pH: 7.6, electrical conductivity: 524 μS/cm, DOC: 1.1, HCO_3^-: 437, Ca^{2+}: 85.6, Mg^{2+}: 17.0, NO_3^-: 11.4, Na^+: 6.8, SO_4^{2-}: 7.4, K^+: 3.0, all expressed in mg/L; 1.8×10^6 prokaryotic cells/mL; microalgae: absent). In order to exclude macro and meiofaunal species that could prey on the ovigerous females of *E. serrulatus*, the bore water was previously 45 μm-filtered. The remaining three