



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



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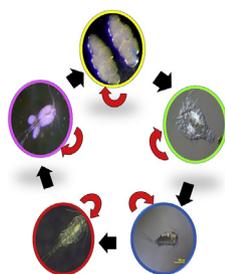
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HIGHLIGHTS

- A full life-cycle protocol with the cyclopid *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 cyclopid *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.

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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, HCO₃⁻: 437, Ca²⁺: 85.6, Mg²⁺: 17.0, NO₃⁻: 11.4, Na⁺: 6.8, SO₄²⁻: 7.4, K⁺: 3.0, all expressed in mg/L; 1.8 × 10⁶ 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

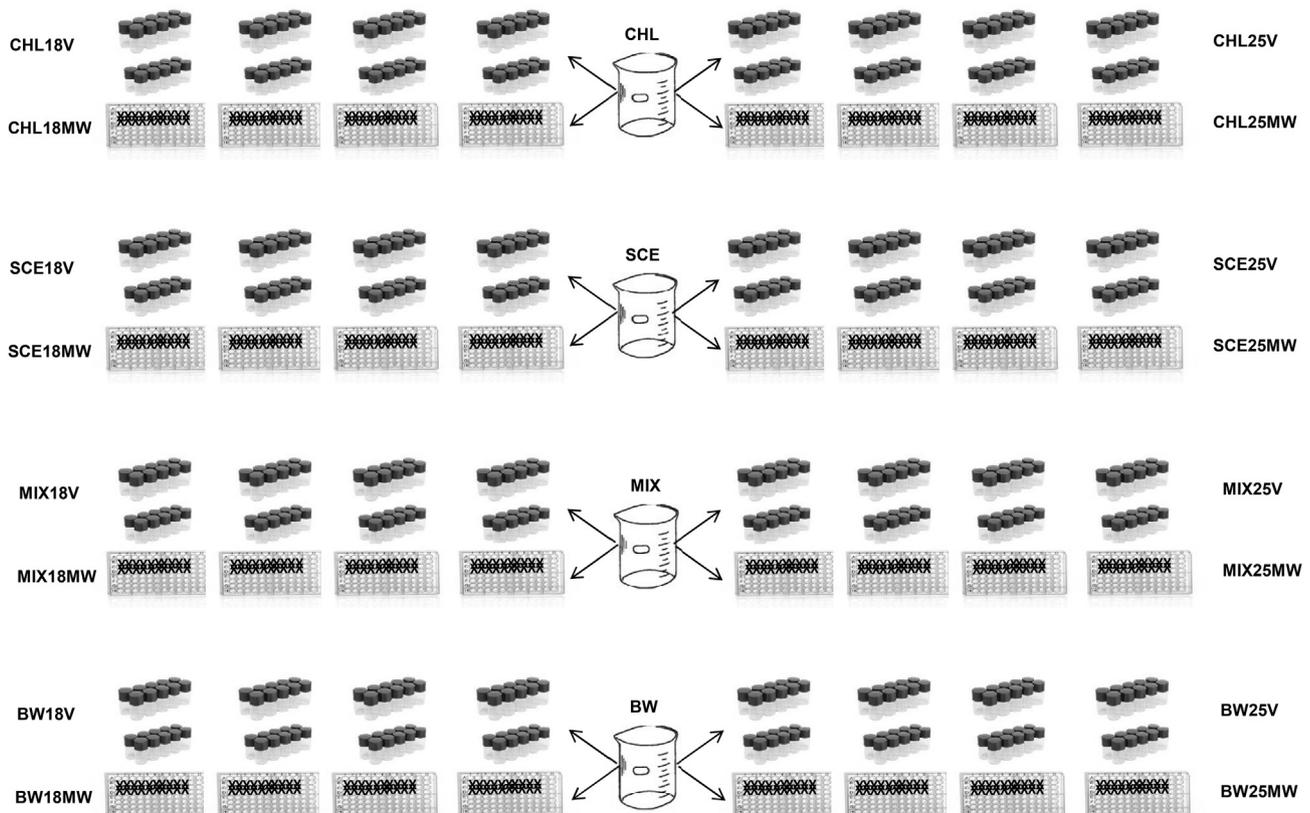


Fig. 1. Experimental design scheme showing the four 25 mL-glass beakers containing 30 ovigerous females of *Eucyclops serrulatus* each. CHL: *Chlorella sorokiniana*, SCE: *Scenedesmus quadricauda*, MIX: mixture of the two microalgae, BW: bore water. After 24 h, 320 nauplii (NI) were picked up from each beaker and arranged either in 300 μ L-microwells or in 2-mL glass vials. In the microwells, NI were arranged in four replicates, with 20 NI loaded into 20 individual microwells disposed on two rows in 4 different microplates per treatment. In the 2-mL glass vials, NI were arranged in four replicates with 20 NI loaded in 20 vials for each replicate. The NI were kept either at 18 °C (vessels on the left) or at 25 °C (vessels on the right). Finally, the NI were fed on CHL, SCE, MIX and BW to set up 16 different treatments. Treatment abbreviations as in Table 1.

beakers were filled with standard water (1 L MILLIPORE® MILLI-Q® deionised water remineralized with the following reagent grade chemicals: 0.06 g of $MgSO_4$, 0.096 g of $NaHCO_3$, 0.004 g KCl, 0.06 g $CaSO_4 \cdot 2H_2O$; US EPA, 2002). According to US EPA (2002) the water chemistry variables measured during the test medium preparation were pH (7.4), hardness (80 mg/L) and alkalinity (30 mg/L). We measured these variables to check if the reconstituted water was moderately hard as recommended (US EPA, 2002). Dissolved oxygen (9.2 mg/L) was measured by the oxygen sensor Hamilton Oxygens 120. No food was offered to the ovigerous females kept in the bore water, in order to allow them to feed on the non-algal diet coming from the field. Living cells of two chlorophytes, namely 4×10^3 microalgal cells/individual of either *Chlorella sorokiniana* or *Scenedesmus quadricauda*, and a mixture of the two microalgae (1:1), were dispensed in the three beakers with standard water. All the beakers were kept in permanent darkness and in a laboratory thermostatic cabinet (Pol-Eko-Aparatura Mod. ST 3) at the field temperature (14.8 °C) for 24 h.

2.3. Test arrangements

After 24 h, the tests were started as follows: hatched nauplii (NI) were picked up by a glass pipette at 40 \times magnification from the beaker containing *C. sorokiniana*. Eighty NI were randomly loaded in individual 300 μ L-microwells; 4 replicates were arranged, with 20 NI loaded into 20 individual microwells disposed on 2 rows in 4 different microplates (Fig. 1). The NI were then fed on *C. sorokiniana* and kept at 18 °C for the test duration, so to accomplish the setting for the treatment CHL18MW (Fig. 1; Table 1). An additional 80 NI

were arranged in microwells and fed on *C. sorokiniana* as well, but kept at 25 °C (treatment CHL25MW; Fig. 1). Further 80 NI were randomly loaded in individual 2-mL glass vials, fed on *C. sorokiniana* and kept at 18 °C for the test duration, so to accomplish the treatment CHL18V (Fig. 1, Table 1). Four replicates were used, with 20 NI loaded in 20 2 mL-glass vials for each replicate (Fig. 1). Finally, an additionally 80 NI were arranged and fed similarly, but kept at 25 °C (treatment CHL25V; Fig. 1). The whole procedure was repeated with the NI coming from the other three beakers, respectively containing *S. quadricauda*, the mixture of the two microalgae and the bore water (Fig. 1). The NI fed on the microalgal diets were reared in standard water. Those fed on the non-algal diet were kept in the bore water (Fig. 1). Overall, 1280 NI, less than 48 h-aged, were used in this study. Throughout the test, the specimens were held in darkness at the appropriate testing temperatures at all times when not handled. The animals were monitored daily using the stereomicroscope.

2.4. Feeding procedure

C. sorokiniana and *S. quadricauda* were grown on BG11 medium (Rippka et al., 1979), in 250 mL Erlenmeyer flask, on a rotary shaker, under continuous illumination at light intensity of 100 μ mol photons $m^{-2} s^{-1}$, in a thermostatic room at 28 °C. For each microalgal strain the suspension was prepared with the cultures harvested in the logarithmic phase of growth by centrifugation at 2500g for 5 min and suspended in fresh medium in order to have a final cell density of 1×10^6 cells/mL. The microalgal mixture was prepared at the final concentration of 1×10^6 cells/mL of each strain. The cells

Table 1
Treatment abbreviations and experimental design factors. CHL: *Chlorella sorokiniana*; SCE: *Scenedesmus quadricauda*; MIX: mixture of the two microalgae; BW: 45 μm -filtered bore water; MW: 300 μL -microwell and V: 2 mL-glass vials.

Treatment	Diet	Volume (mL)	Temperature ($^{\circ}\text{C}$)	Treatment	Diet	Temperature ($^{\circ}\text{C}$)	Volume (mL)
MIX25MW	MIX	0.3	25	MIX18MW	MIX	18	0.3
SCE25MW	SCE	0.3	25	SCE18MW	SCE	18	0.3
CHL25MW	CHL	0.3	25	CHL18MW	CHL	18	0.3
BW25MW	BW	0.3	25	BW18MW	BW	18	0.3
MIX25V	MIX	2.0	25	MIX18V	MIX	18	2.0
SCE25V	SCE	2.0	25	SCE18V	SCE	18	2.0
CHL25V	CHL	2.0	25	CHL18V	CHL	18	2.0
BW25V	BW	2.0	25	BW18V	BW	18	2.0

were counted on triplicate samples in a Bürker hemocytometer (Assistant, Sondheim-Rhön, Germany). The microalgal cultures were maintained in the logarithmic phase of growth by removing part of the cultures and adding new fresh medium every 3 days. *C. sorokiniana* and *S. quadricauda* were dispensed in each test vessel (either 2 mL-glass vials or 300 μL -microwells) by adding 2 μL -drops of the appropriate microalgal suspension every 3 days. In the treatments with the mixture of the two microalgae, 1 μL -drop of *C. sorokiniana* and 1 μL -drop of *S. quadricauda* were dispensed in each test vessel every 3 days. Finally, the dispensed microalgal cell density was about 2×10^3 cells/test vessel. In the treatments with the non-algal natural diet, 45 μm -filter bore water was used as medium and no other food was offered. The dispensed prokaryotic cell density of the bore water was about 7.2×10^4 prokaryotic cells/test vessel.

2.5. Test medium renewal

In the treatments with 300 μL -microwell plates, 90% of the medium were aspirated by a Hamilton syringe (SIRINGA 1710, 100 μL , GA22s/51mm/pst2) from the bottom of each microwell to pick up the faecal pellets, every 3 days. In the treatments with 2 mL-glass vials, 90% of the test medium was renewed by a glass pipette, every 3 days. The aspirated medium volumes were observed under a stereomicroscope at $16\times$ magnification to verify that no test organisms had been picked up during the aspiration procedure. When it happened, the relative vessel was excluded from the bioassay. This event only occurred in the treatments with the microwells. After water renewal, the vessels were refilled with either standard water or the bore water. Dissolved oxygen, pH and temperature of the fresh medium were measured each time the test medium was renewed (OECD, 2014). Temperature was measured via direct immersion thermometry in 500-mL glass beakers, filled with either standard water or bore water and placed in the thermostatic cabinet near the test vessels. The 3-days old medium removed from the test vessels was pooled from each treatment and the pH was measured each time the test medium was renewed. If the pH does not remain in the range 6–9 or vary more than 1.5 units, then a second test should be carried out in accord to OECD (2012). However, this event has never occurred.

2.6. Observations and mating cohorts set up

Development and survival from NI to copepodids (CI), from CI to adult (A) and from NI to A were observed under a stereomicroscope daily and analysed over time per each treatment. Development was defined as the number of days spent by 50% of the specimens in a treatment to pass to the next stage (from NI to CI, from CI to A and from NI to A). Survival was defined as the percentage of surviving animals, on each separate day. The animals that died were excluded from the development counts (Lundström et al., 2010).

The sex of virgin copepods was recorded at the reproductive maturity. When >50% of the animals were sexually mature, mating cohorts were set up. One cohort was constructed for each of the 4 replicates of a treatment, so to arrange 4 cohorts per treatment and 64 cohorts overall (Supplementary File 1). For each replicate, the relative cohort was arranged as follows: sexually mature males and females were removed from the individual test vessels by using a glass pipette and pooled into a Petri dish (5 cm in diameter), containing 15 mL of the appropriate test medium (either standard water or bore water). The newly arranged cohorts received fresh food (4×10^3 microalgal cells/individual in the treatments with microalgal diets) every 3 days. Twice the concentration (2×10^3 microalgal cells/individual) that was provided in the previously described development test was delivered to avoid the cannibalistic behaviour that was observed during preliminary experiments. Test medium was renewed as often. In the treatment with the non-algal diet, 45 μm -filtered bore water was used as medium and renewed every 3 days and no other food was offered. The number of individuals that were pooled in each cohort is given in the Supplementary File 1. The cohorts were checked daily for ovigerous females, that were then picked up and loaded in individual test vessels (either 300 μL -microwell or 2 mL-glass vial), one ovigerous female each, and kept with the appropriate food (2×10^3 microalgal cells/individual in the treatments with microalgal diets) and temperature. The test vessels were re-arranged into four replicates per treatment and checked daily. The percentage of females extruding one and two viable clutches and the percentage of hatched nauplii/female were recorded over time per each treatment. We set the end of the reproduction experiments at 21 days from the cohort starting at both temperatures, because after that time male individuals started dying. At the end of the tests, specimens of each cohort were sacrificed by transferring them to a 70% ethanol solution. The sex of preserved specimens was examined at a later stage and was used to calculate sex ratios for each cohort. In order to follow the standard protocol of *A. tenuiremis*, we had established *a priori* to discard cohorts with sex ratios that did not fall between 35:65 = %females:%males or %males:%females (OECD, 2014). However, this event has never occurred.

2.7. Statistical analyses

The statistical design was set in order to test the effect of the interaction of three fixed factors (diet, volume and temperature) on the survival, development and reproduction of *E. serrulatus*. The diet factor consisted of 4 levels: 45 μm -filtered bore water (BW), *Chlorella sorokiniana* (CHL), *Scenedesmus quadricauda* (SCE) and the mixture of the two microalgae (MIX). The volume factor consisted of two levels: 300- μL microwell plates (MW) and 2 mL-glass vials (V). The temperature factor consisted of two levels: 18 $^{\circ}\text{C}$ and 25 $^{\circ}\text{C}$.

The variables survival (%), development (days) and reproduction

success (% of females producing one and two clutches and % of hatching) were analysed using permutational (perms: 9999) univariate analyses of variance (permutational ANOVA; Anderson, 2001). A permutational ANOVA was preferred to a regular multi-factorial univariate ANOVA due to the non-normal distribution of the variables, which was checked by Shapiro's tests (Shapiro and Wilk, 1965). Permutational ANOVA works with any distance measure that is appropriate to the data, and uses permutations so that it is essentially distribution free (Anderson, 2001). Following the standard test of *A. tenuiremis*, the nested model was applied because the microwells and the glass vials were nested within their particular replicate in each treatment (OECD, 2014). The permutational ANOVAs were performed on the basis of the Bray-Curtis similarity matrix, applied after performing Levene's tests (Levene, 1960) on the original or transformed (development: $\log(x+1)$); percentages: arcsine square root) datasets. Whenever the Levene's test results indicated heterogeneity of data dispersion, the multi-factorial models were reduced excluding one or more factors from the permutational ANOVA. The excluded factors were investigated individually by means of Friedman's tests (Wayne, 1990). Pairwise *post-hoc* *t*-tests were applied when appropriate to test for differences between levels within the factors. The level of significance was set to $p < 0.05$ for the ANOVAs, Friedman's and *post-hoc* *t*-tests, while $p > 0.05$ was set for Shapiro's and Levene's tests. No additional corrections for multiple comparisons were made, according to Anderson et al. (2008). All the permutational ANOVAs were performed using PRIMER v.6 and PERMANOVA + routines for PRIMER (Anderson et al., 2008). Shapiro's and Friedman's tests were performed using R software v. 3.2.3 (R Core Team, 2015).

2.8. Population modelling

Growth rates (λ) of the populations of *E. serrulatus* in each treatment were modelled by a matriarchal 5 stage-structured Leslie matrix, that assumes simple exponential growth and no density dependence (Leslie, 1945; Lefkovich, 1965; Caswell, 2001). The following five-stage (embryos, nauplius, copepodid, female, ovigerous female) matriarchal matrix model (Equation (1)) was created.

$$\begin{pmatrix} Ne \\ Nn \\ Nc \\ Nv \\ Ng \end{pmatrix} t + 1 = \begin{pmatrix} Pee & 0 & 0 & 0 & F \\ Pen & Pnn & 0 & 0 & 0 \\ 0 & Pnc & Pcc & 0 & 0 \\ 0 & 0 & Pcv & Pvv & 0 \\ 0 & 0 & 0 & Pvg & Pgg \end{pmatrix} \times \begin{pmatrix} Ne \\ Nn \\ Nc \\ Nv \\ Ng \end{pmatrix} t \quad (1)$$

N represents the number of individuals in a certain stage class and *P* the life stage transition proportion. *F* represents the number of offspring per female. Indices represent the different stage classes: *e* = embryo; *n* = nauplius; *c* = copepodid; *v* = virgin female; *g* = gravid female. Hence, the proportion of embryos not developing to nauplii is P_{ee} , the proportion of embryos developing to nauplii is P_{en} , the proportion of nauplii not developing to copepodids is P_{nn} , the proportion of nauplii developing into copepodids is P_{nc} , the proportion of copepodids not developing into virgin females is P_{cc} , the proportion of copepodids developing into virgin females is P_{cv} , the proportion of females not producing one (or two) viable clutch(es) is P_{vv} , the proportion of females producing one (or two) viable clutch(es) is P_{vg} . All proportions were exclusive of mortality, i.e., dead individuals were not scored as developmental failures, according to OECD (2014).

For each treatment, Monte Carlo simulations were used to generate 1000 matrices with random proportions and fecundities obtained from the distributions defined by the replicates within each treatment (Manly, 1991; Lundström et al., 2010). Proportions were assumed to be beta distributed, while fecundity was assumed

to be normally distributed (Lundström et al., 2010). The expected short-term (16 time steps) λ of an initial F0 cohort of 80 individuals (i.e. the number of nauplii in each treatment at the time *t*) were projected for each treatment. Mean values and 95% confidence intervals (CIs) were calculated automatically. If lambda values < 1 , populations will go extinct, while if λ values are close to 1, population abundances will stay on a stable level (Lundström et al., 2010). The mean λ and 95% CIs values were compared among the treatments. If the CIs did not overlap, we assumed that there was a significant difference between the compared λ values among treatments (Environment Canada, 2005; Lundström et al., 2010). Monte Carlo simulations were performed using the free Microsoft Excel™ Plug-in Pop Tools 3.0 (<http://www.cse.csiro.au/poptools>, build 6, released 2008-09-01) and Microsoft Excel™ 2007.

2.9. Sensitivity power tests

Two sensitivity power tests (Cohen, 1988) were performed to compute the expected effect size given an alpha = 0.05, power = 0.8 and a sample size constrained by the design described in this study (N = 80 individuals per treatment). Assuming that *E. serrulatus* could be used as model species to investigate life-cycle effects of chemicals in a future study with a design that includes a control and 5 treatments with increasing chemical concentrations, we performed the following two sensitivity tests: 1) a test for a one-way ANOVA (6 levels: 1 control and 5 treatments) and 2) a test for a *post-hoc* *t*-test (two-tailed, independent means). We used the library *pwr* of the R software v. 3.2.3 (R Core Team, 2015) and G Power v. 3.1.9.2 (© Copyright 2010–2016 Heinrich-Heine-Universität Düsseldorf).

3. Results

3.1. Survival

The percentage of nauplii surviving to a copepodid stage did not fulfil the validity criterion in 4 out of 16 treatments (Table 2). The analyses indicated that none of the three factors, nor their interactions, had a significant effect on the survival of this stage (Table 2).

The percentage of copepodids surviving to the adult stage did

Table 2

Mean and standard deviation of the percentages of *Eucyclops serrulatus* surviving to different stages (NC: from nauplius to copepodid; CA: from copepodid to adult; NA: from nauplius to adult) in each treatment. Mean percentages not fulfilling the test validity criterion (at least 70%) are indicated in bold. Treatment abbreviations as in Table 1.

Treatments	NC		CA		NA	
	Mean	SD	Mean	SD	Mean	SD
MIX18V	0.92	0.10	0.89	0.07	0.89	0.07
MIX25V	1.00	0.00	0.80	0.06	0.78	0.08
SCE18V	0.92	0.03	1.00	0.00	0.92	0.03
SCE25V	1.00	0.00	0.90	0.06	0.76	0.02
CHL18V	0.98	0.03	0.85	0.12	0.81	0.16
CHL25V	0.78	0.07	0.89	0.05	0.64	0.02
BW18V	1.00	0.00	0.67	0.00	0.58	0.00
BW25V	1.00	0.00	0.50	0.00	0.50	0.00
MIX18MW	0.93	0.10	0.78	0.05	0.70	0.06
MIX25MW	0.93	0.10	0.50	0.08	0.65	0.01
SCE18MW	0.60	0.26	0.64	0.11	0.48	0.07
SCE25MW	0.60	0.26	0.63	0.03	0.63	0.07
CHL18MW	0.84	0.08	0.43	0.10	0.45	0.13
CHL25MW	0.84	0.08	0.63	0.02	0.51	0.08
BW18MW	0.60	0.29	0.35	0.00	0.20	0.00
BW25MW	0.60	0.29	0.35	0.00	0.30	0.00

not fulfil the validity criterion in 9 out of 16 treatments (Table 2). None of the treatments with the microwells reached the validity criterion, except for the treatment MIX18MW (Table 2). Accordingly, the analyses indicated that the factor volume had a significant effect on the survival of this stage (Pseudo- $F_{1,60} = 48.63$, $p = 0.0001$), as showed in Fig. 2.

The percentages of nauplii surviving to the adult stage did not fulfil the survival validity criterion in 10 out of 16 treatments (Table 2). Except for MIX18MW, neither the treatments with the microwells nor those with bore water in 2 mL-glass vials fulfilled the criterion. The factor volume had a significant effect on the survival of this stage (Pseudo- $F_{1,56} = 96.02$, $p = 0.0001$) as well as the factor diet (Pseudo- $F_{3,56} = 36.26$, $p = 0.0001$). The interaction of the two factors was not significant (Pseudo- $F_{3,56} = 0.94$, $p = 0.4255$). The survivals in the treatments with the bore water were significantly lower than those with the other microalgal diets (*post-hoc t*-tests: BW – CHL, $t = 3.41$, $p = 0.0014$; BW – SCE, $t = 4.99$, $p = 0.0002$; BW – MIX, $t = 7.03$, $p = 0.0001$). The survivals in the treatments with CHL were significantly lower than those with MIX (*post-hoc t*-test: $t = 2.56$, $p = 0.0187$).

3.2. Development

The development of nauplii to copepodids was significantly influenced by the interaction of the three factors (volume \times temperature \times diet: Pseudo- $F_{3,48} = 20.81$, $p = 0.0001$). The developmental rates of nauplii in the treatments at 25 °C were significantly faster than those at 18 °C (Pseudo- $F_{1,48} = 486$, $p = 0.0001$; Table 3). The developmental rates of nauplii in vials were significantly faster than those in microwells (Pseudo- $F_{1,48} = 96$, $p = 0.0001$; Table 3). Ultimately, the nauplii in the treatments with the bore water took longer time to develop with respect to those in the treatments with the algal diets (Table 3).

The development of copepodids to adults was significantly influenced by temperature (Friedman *chi-squared* = 32, $df = 1$, $p = 1.542 \times 10^{-8}$), with the developmental rates at 25 °C being overall faster than those at 18 °C (Table 3). Neither the volume factor (Friedman *chi-squared* = 2.28, $df = 1$, $p = 0.1306$), nor the diet (Friedman *chi-squared* = 3.80, $df = 3$, $p = 0.2834$) influenced the development of this stage.

The development of nauplii to adults was significantly influenced by temperature (Friedman *chi-squared* = 32, $df = 1$, p -value = 1.542×10^{-8}), with the developmental rates at 25 °C being faster than those at 18 °C (Table 3). Neither the volume factor

Table 3

Mean and standard deviation of the development (in days) of *Eucyclops serrulatus* for different stages (NC: from nauplius to copepodid; CA: from copepodid to adult; NA: from nauplius to adult) in each treatment. Treatment abbreviations as in Table 1.

Treatment	NC		CA		NA	
	Mean	SD	Mean	SD	Mean	SD
MIX18V	11.0	1.2	19.3	0.5	29.8	1.5
MIX25V	5.5	0.6	16.0	0.0	20.5	0.6
SCE18V	12.0	0.0	19.5	0.6	30.5	0.6
SCE25V	5.5	0.6	16.8	1.0	21.3	1.0
CHL18V	10.5	0.6	22.5	0.6	32.0	1.2
CHL25V	6.5	0.6	15.0	0.0	20.5	0.6
BW18V	12.0	0.0	21.0	0.0	32.0	0.0
BW25V	8.0	0.0	15.5	0.6	22.5	0.6
MIX18MW	13.0	2.4	23.8	2.8	36.0	0.8
MIX25MW	7.5	0.6	15.5	0.6	23.0	0.8
SCE18MW	13.5	1.7	23.5	1.7	36.0	0.0
SCE25MW	6.5	1.3	14.5	0.6	20.0	1.6
CHL18MW	11.0	1.2	26.0	1.2	38.0	0.0
CHL25MW	8.0	0.8	15.5	0.6	22.5	1.0
BW18MW	23.8	2.1	25.5	2.4	48.3	0.5
BW25MW	9.8	0.5	12.5	0.6	21.3	1.0

(Friedman *chi-squared* = 14.28, $df = 1$, $p = 0.1571$) nor the diet (Pseudo- $F_{3,60} = 0.53$, $p = 0.6599$) influenced the development of this stage.

3.3. Reproduction

The virgin females in the treatments with the microwells did not produce clutches within the end of the experiment (21 days after the start of the respective cohort; Table 4). Moreover, the validity criterion (at least 75% of control females producing offspring; OECD, 2014) was only reached in the treatment in glass vials with the MIX diet at 18 °C and 25 °C (Table 4). Diet and temperature affected the percentages of females producing the first clutch (diet \times temperature: Pseudo- $F_{3,24} = 4.26$; $p = 0.0162$), with the percentages in the treatments with CHL and BW being lower at 18 °C than at 25 °C (CHL: $t = 2.63$, $p = 0.0380$; BW: $t = 2.70$, $p = 0.0374$; Table 4).

The diet factor affected the percentage of females producing the second clutch as well (Pseudo- $F_{2,23} = 25.56$; $p = 0.0001$; *post-hoc t*-tests: $p < 0.001$). However, none of the treatments reached the validity criterion (Table 4).

Ultimately, the diet factor affected the percentage of hatchings

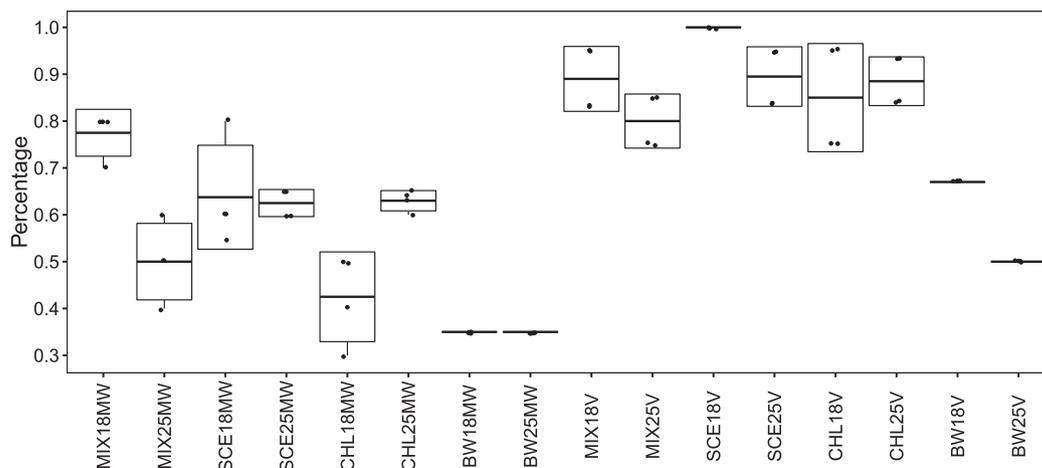


Fig. 2. Boxplots showing mean, SD and min and max percentages of copepodids of *Eucyclops serrulatus* surviving to the adult stage, in each treatments. Treatments in microwells (on the left) significantly differed from those in 2 mL-glass vials (permutational ANOVA: Pseudo- $F_{1,60} = 48.63$, $p = 0.0001$). Treatment abbreviations as in Table 1.

Table 4

Mean and standard deviation of the percentages of hatchings and females producing one (I clutch) and two (II clutch) clutches, in each treatment. Treatment abbreviations as in Table 1.

Treatment	I clutch		II clutch		hatchings	
	mean	SD	mean	SD	mean	SD
MIX18V	0.84	0.01	0.58	0.09	0.52	0.02
MIX25V	0.75	0.17	0.53	0.15	0.45	0.09
SCE18V	0.08	0.09	0.00	0.00	0.39	0.21
SCE25V	0.25	0.17	0.25	0.13	0.39	0.08
CHL18V	0.35	0.12	0.33	0.09	0.38	0.11
CHL25V	0.57	0.11	0.37	0.04	0.38	0.15
BW18V	0.05	0.06	0.05	0.06	0.13	0.14
BW25V	0.16	0.01	0.00	0.00	0.05	0.06
MIX18MW	0	0	0	0	0	0
MIX25MW	0	0	0	0	0	0
SCE18MW	0	0	0	0	0	0
SCE25MW	0	0	0	0	0	0
CHL18MW	0	0	0	0	0	0
CHL25MW	0	0	0	0	0	0
BW18MW	0	0	0	0	0	0
BW25MW	0	0	0	0	0	0

(Pseudo- $F_{3,24} = 15.63$; $p = 0.0001$). The percentages in the treatments with the bore water were significantly lower than those with the microalgal diets (MIX-BW: $t = 8.69$, $p = 0.0001$; CHL-BW: $t = 4.84$, $p = 0.0004$; SCE-BW: $t = 4.36$, $p = 0.0009$). The hatching success ranged from 52% in the treatment with the MIX to 5% in the treatment with BW (Table 4).

3.4. Population growth rates

Lambda values were <1 in the treatments with the bore water, indicating that the populations will go extinct within the 16th step (Table 5). The mean λ of the other treatments were >1 indicating significant increasing of the populations in each treatment. However, the λ CIs overlapped, indicating no significant differences in the population growth rates among these treatments (Table 5).

3.5. Sensitivity power tests

We obtained an effect size (EF) of 0.16SD for the ANOVA sensitivity test and an EF of 0.31SD for the t -test sensitivity (SD = standard deviation of the control). The minimum detectable effects (MDE) in future tests with the two controls that obtained the best performances, that is MIX18V and MIX25V, are showed in the Supplementary File 2. The MDEs are indicated for the following endpoints: survival, development and reproduction. The results highlighted that the sample size applied in this study, that is the number of individuals in each treatment, is large enough to catch small effects ($<1.5\%$) of the treatments with a chance of 80%.

Table 5

Mean population growth rates (lambda) and 95% confidence limits (CLs) calculated from 1000 stochastic Lefkovich matrices generated with the Monte Carlo. Treatments abbreviations as in Table 1.

Treatment	Mean λ	Lower CL	Upper CL
MIX18V	2.20	1.35	3.35
MIX25V	2.23	1.32	3.53
SCE18V	2.10	1.13	4.10
SCE25V	2.20	1.34	3.39
CHL18V	2.00	1.10	4.37
CHL25V	2.15	1.24	3.62
BW18V	0.96	0.37	1.19
BW25V	0.96	0.35	1.19

4. Discussion

The standard full life-cycle test with *A. tenuiremis* has proven to be fully valuable as it is with other marine harpacticoids, such *Nitokra spinipes* and *Tisbe battagliai*, and also with the marine calanoid *Acartia tonsa* (Hutchinson et al., 1999; Breitholtz et al., 2003). However, according to our results, the test needs to be modified to be applicable for the freshwater copepod *E. serrulatus*. The 300- μ L microwells used for *A. tenuiremis* are suitable for nauplii but not for copepodids and adults of *E. serrulatus*. Nauplii spend a larger proportion of their life in a stationary position (Suárez-Morales, 2015 and personal observations) than adults and copepodids, requiring less space. Larger volumes (2 mL) proved to serve better for rearing this species, as was also observed by Reiss and Schmid-Araya, 2011. The better performance of copepodids and adults in 2 mL-glass vials may also be due to a more accurate test medium renewal, that likely reduced the accumulation of metabolic wastes and faecal pellets. Copepod faecal pellets consume oxygen in the test vessels because they are nutrient-enriched microenvironments that act as hotspots for microbial colonization (Tang et al., 2010). In the treatments with the microwells, medium renewal occurred by aspiration with a Hamilton syringe, as indicated in the *A. tenuiremis* method (OECD, 2014). This procedure did not allow to see what was actually aspirated. Accordingly, not all faecal pellets were likely removed from the test microwells. In addition, two accidental aspirations of test specimens occurred during this procedure.

A mixture (1:1) of microalgal species performed better than the monospecific microalgal diets with *E. serrulatus* as it had been observed for *A. tenuiremis*. The good performance may be due to the combined nutrient contents, which meet the nutritional requirements of *E. serrulatus* more closely, as it was observed for other freshwater (Vidhya et al., 2014) and marine copepods (Knuckey et al., 2005). The performances of *C. sorokiniana* and *S. quadricauda* were not significantly different. The non-algal diet performed significantly worse than the others. Although *E. serrulatus* is considered a weak predator (Dos Santos and De Andrade, 1997), it does not significantly benefit from the presence of rotifers in its diet, as it was also observed by Nandini and Sarma (2007), although the species had proven to feed on ciliates (Reiss and Schmid-Araya, 2011) and especially on paramecia (Reiss, 2007).

Copepod developmental time and life spans are much longer at lower temperature and may vary considerably with species (Suárez-Morales, 2015). Accordingly, we observed significantly faster developmental rates of *E. serrulatus* at 25 °C than at 18 °C. Since the short duration of a full life-cycle test is a prerequisite that should be fulfilled to reduce the cost of the trials (Van Leeuwen and Vermeire, 2007), 25 °C could have been considered the most suitable temperature for a developmental test with *E. serrulatus*. However, the best results in terms of survival from copepodid to adult and from nauplius to adult, as well as of reproduction, were obtained at 18 °C, albeit with significantly longer test duration. The treatment with the microalgal mixture in 2 mL-vials at 25 °C met the reproduction criterion with a higher degree of variability respect to the treatment at 18 °C. This result does not provide a strong level of confidence that the test at 25 °C would be able to meet the acceptability criterion on a consistent basis. In addition, 25 °C is a temperature widely reached in ponds and lentic habitats (Kern et al., 2014) but is uncommon in benthic surface water habitats, in hyporheic zone, in springs and in groundwater habitats of the temperate region (Gunawardhana and Kazama, 2012). High temperatures are known to cause physiologic alterations in invertebrate species, such as doubled locomotion activity, altered phenology and increased oxygen consumption rates (Issartel et al., 2005; Colson-Proch et al., 2010; Stoks et al., 2014). Accordingly, the sensitivity of *E. serrulatus* to ionized ammonia and the herbicide

Imazamox is significantly higher at 18 °C than at lower temperatures such as 15 °C (Di Lorenzo et al., 2015b). Finally, tests at 18 °C represent a valid tool to investigate the sensitivity of specific stage of *E. serrulatus* at temperatures that are nearer to those that this species experiences in most of the temperate freshwater habitats.

Ultimately, we chose to pool adult males and females of *E. serrulatus* in cohorts rather than making individual male:female mating pairs as it was suggested in the standard protocol with *A. tenuiremis*. The bias in identifying the sex of alive individuals, which was required for the mating pair set-up, was considered too high to be managed. *E. serrulatus* has sexually dimorphic antennules like most copepods (Boxshall and Huys, 1998). The antennules of the adult male are typically modified for grasping the female during mating. However, the antennule geniculation of the male of this species is clearly visible when the male is dead but it is hardly discernible when the male is alive and freely moving. Moreover, since a single cyclopoid male may successfully fertilize more than one female (Titelman et al., 2007; Kiørboe, 2007), even though it is not always true that any male may fertilize at least one female during its life time (personal observations), the set-up of cohorts was considered to reflect the natural conditions of the populations in the field better than arbitrary mating pairs. In addition, by using cohorts the accidental death of one individual did not require the disposal of its mate. Finally, the data suggest that incorporation of a second clutch into the protocol does not provide any additional useful information.

5. Summary of test conditions and procedures

Our results have highlighted that the best performance of the control in terms of survival, development, reproducibility and population growth rates with this species is reached under the following test conditions and procedures: (i) 80 less than 48 h old nauplii are used in each treatment; (ii) 4 replicates are used for each treatment, with 20 nauplii randomly loaded in twenty 2 mL-glass vials for each replicate; (iii) 1×10^3 cells of *C. sorokiniana* and 1×10^3 cells of *S. quadricauda* are delivered into each vial every 3 days; (iv) 90% of the test medium is renewed by a glass pipette, every 3 days; (v) test temperature is either 25 °C or 18 °C; (vi) development (number of days spent by 50% of the specimens in a treatment to pass to the next stage) and survival (percentage) are observed under a stereomicroscope daily and analysed over time per each treatment; (vii) when >50% of the animals are sexually mature, a mating cohort is set up for each replicate; (viii) sexually mature males and females are removed from the individual vials and pooled into a Petri dish (5 cm in diameter; one vessel per cohort), containing 15 mL of the test medium; (ix) 2×10^3 cells/individual of *C. sorokiniana* and 2×10^3 cells/individual of *S. quadricauda* are loaded into each Petri dish every 3 days and the test medium is renewed as often; (x) the cohorts are checked daily for ovigerous females, that are then picked up and loaded in individual 2 mL-glass vials and fed 1×10^3 cells of *C. sorokiniana* and 1×10^3 cells of *S. quadricauda* every 3 days; (xi) the percentage of females extruding one viable clutch and the percentage of hatched nauplii/female are recorded over time; (xii) the reproduction experiments end at 21 days from the cohort starting at both temperatures; (xiii) cohorts sex ratios are examined at the end of the test; (xiv) cohorts with sex ratios not falling between 35:65 = % females:%males or %males:%females should be discarded; (xv) test duration in the control is 42 days at 25 °C and 51 day at 18 °C.

6. Conclusion

The present study has aimed at describing test procedures for measuring the (sub)chronic effects of chemicals on the freshwater

cyclopoid *Eucyclops serrulatus*. Our results showed that the best test performance was obtained at 18 °C, albeit with significantly longer test duration. Running the test at 25 °C decreases the overall exposure duration, although with greater risk of not meeting the reproductive acceptability criterion.

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 20 days at 18 °C and in 16 days at 25 °C.

Respect to other recently published methodologies for performing tests with freshwater copepods, the full life-cycle test procedure described in this study has the advantage of: i) being an adaptation of a standard protocol widely used for marine copepods and being statistically robust; ii) using a freshwater species that is ubiquitous in freshwater habitats, including groundwater, and has an almost worldwide distribution; iii) providing procedures for reproduction tests not influenced by cannibalism; iv) providing procedures for subchronic tests.

The test protocol that we have obtained in this study is suitable for most chemicals. However, its utmost importance lies on its use in the risk assessment of compounds that are known to affect the hormonal system in crustaceans and that, in turn, regulates their development and reproduction.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.chemosphere.2016.12.151>.

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