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Application of growth-related sublethal endpoints in ecotoxicological assessments using a harpacticoid copepod

Ulrika Dahl [a,*], Elena Gorokhova [b], Magnus Breitholtz [a]

[a] *Department of Applied Environmental Science (ITM), Stockholm University, S-106 91 Stockholm, Sweden*
[b] *Department of Systems Ecology, Stockholm University, S-106 91 Stockholm, Sweden*

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Abstract

In ecotoxicology, there is an increasing demand for sensitive sublethal endpoints. The primary aim of the present study was therefore to evaluate the relative sensitivity and usefulness of four sublethal endpoints – development time, body length, RNA content and growth rate – in the harpacticoid copepod *Nitocra spinipes*, using the reference molecule Simvastatin. Development time decreased significantly at low sublethal concentrations of Simvastatin ($p < 0.001$; $F = 13.249$; 0.16–1.6 $\mu g\,L^{-1}$), while RNA content and body length increased significantly at 0.16 $\mu g\,L^{-1}$ ($p < 0.001$; $F = 6.13$) and 1.6 $\mu g\,L^{-1}$ ($p < 0.01$; $F = 2.365$), respectively. The growth rate increased significantly at 0.16–5 $\mu g\,L^{-1}$ ($p < 0.01$–0.001). Hence, significant responses of growth-related traits were observed already at 0.16 $\mu g\,L^{-1}$, which is about 5000 times lower than the acute toxicity (96 h-LC_{50}: 810 $\mu g\,L^{-1}$). These results show that all assayed endpoints are very sensitive and indicate that current ecotoxicity testing used for environmental protection activities may underestimate the risk for harpacticoid copepods and most likely for other small invertebrates, when relying exclusively on acute toxicity measurements.

Keywords: Development time; RNA content; Body length; Growth rate; ERA; Crustacea

1. Introduction

Environmental risk assessment (ERA) of chemicals is one of the main elements of environmental protection activities. Endpoints are characteristics of an ecological component that may be affected by exposure to a stressor (Suter, 2000). Assessment endpoints are explicit expressions of the actual environmental values that are to be protected (e.g. change in abundance and functioning of aquatic populations), while stressor endpoints are the measurable responses related to an assessment endpoint (e.g. change in survival, growth rate, reproductive potential, etc.). Important characteristics of a stressor endpoint are feasibility and reliability of determination. The stressor endpoint selection is perhaps the most critical aspect of ERA, as it sets the limits for the remainder of the process. Any component from virtually any level of biological organization or structural form can be used as stressor endpoint; hence, the selection of endpoints and organisms to be used for effect characterisation is a tremendous challenge. Current ERA relies on a number of standard test organisms, such as small crustaceans. Among those, copepods are continuously used in growth models and life-cycle tests to predict the impact of chemicals on early development and reproduction (e.g. Hutchinson et al., 1999; Breitholtz and Bengtsson, 2001; Breitholtz et al., 2003; Breitholtz and Wollenberger, 2003; Brown et al., 2003; Chandler et al., 2004).

Most commonly used stressor endpoints are variables related to growth performance (Sibly and Hone, 2002). Growth is defined as a net-difference between the catabolic and anabolic metabolism (Becker et al., 2000) and is usually expressed as a change in body size or reproduction output (Winberg, 1971). The body size correlates with many ecological as well as life-history traits and may thus influence the abundance of species as well as population structure and dynamics (Gaston et al., 2001), and it is an important stressor endpoint that is commonly used for growth assessment (e.g. Winberg, 1971). Identification of biochemical changes, such as RNA contents, which are related to growth and metabolism (Dahlhoff, 2004), can be used to determine if an organism has been exposed to a stress, including contaminants (Yang et al., 2002). The rationale is based on the fact that the RNA content of tissues or whole organisms consists primarily of

* Corresponding author. Fax: +46 8 674 76 36.
E-mail address: ulrika.dahl@itm.su.se (U. Dahl).

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ribosomal RNA (rRNA). Consequently, concentration of rRNA, at any given time is directly related to the protein synthesis of a cell (Elser et al., 2000). The quantity of RNA is directly linked to the growth of the individual (Saiz et al., 1998). For example, in small metazoans, which have high metabolic rates of biosynthesis, much RNA is required (Brown et al., 2004). DNA exists in a quasi-constant quantity in a somatic cell and therefore might be used as an index of the number of cells (Buckley et al., 1999). Based on this, it is becoming increasingly common to assess growth rates in a variety of aquatic animals by using the RNA:DNA ratio (fish: Buckley et al., 1999; lobsters: Rosa and Nunes, 2003; krill: Cullen et al., 2003; copepods: Saiz et al., 1998; Campbell et al., 2001; Gorokhova, 2003; daphniids: Vrede et al., 2002; cirripeds: Desai and Anil, 2002; corals: Meesters et al., 2002). In toxicological tests, these indices were also found to be useful in nematodes (Ibiam and Grant, 2005) and algae (Yang et al., 2002). However, in small crustaceans, such as daphniids, copepods and decapod larvae, RNA content alone may be a more sensitive endpoint than the RNA:DNA ratio due to the growth- and ontogeny-related fluctuations in DNA content (Gorokhova and Kyle, 2002; Rosa and Nunes, 2003; Gorokhova, 2003).

The primary aim of the present study was to evaluate the sensitivity and usefulness of four stressor endpoints; development time (DT), body length (BL), RNA levels and growth rate (GR), all related to individual growth in the harpacticoid copepod *Nitocra spinipes*, a species which has earlier been used to study effects of single substances (e.g. brominated flame-retardants and synthetic musk fragrances) on development, reproduction and population growth rate (Breitholtz and Bengtsson, 2001; Breitholtz et al., 2003; Breitholtz and Wollenberger, 2003). To evaluate the sensitivity and usefulness of the four endpoints it was important to use a reference molecule with a known mode of action. Statins have been shown to inhibit the crustacean juvenile hormone analogue methyl farnesoate (MF) (Li et al., 2003), which could result in developmental alterations (Borst et al., 1987). We therefore chose the drug Simvastatin as our reference molecule in the present study.

2. Material and methods

2.1. Test organisms

N. spinipes is a harpacticoid copepod that is widely distributed around the world (Lang, 1948). Detailed information on the *N. spinipes* strain used in the present study, including culturing conditions, has been published elsewhere (e.g. Bengtsson, 1978; Bengtsson and Tarkpea, 1995; Breitholtz and Bengtsson, 2001).

2.2. Test substance

Simvastatin was obtained from Aldrich Chem, Stockholm, Sweden (CAS 79902-63-9). Acetone (analytical grade) stock solutions were prepared at the beginning of the experiment and stored at +4 °C. Working test solutions were prepared each time the medium was changed, by diluting the acetone stock solutions in 0.03 mm filtrated, pre-heated (80 °C) and GF/C-filtrated brackish water. Acetone was used as a solvent carrier of Simvastatin. A solvent control was therefore used in addition to a dilution water control. The acetone concentration in the test wells did not exceed 0.1‰, which is well below the NOEC for copepod naupliar development (Andersen et al., 2001).

2.3. Acute toxicity test

The acute toxicity of Simvastatin was performed according to Swedish standard procedures (SIS, 1991). Briefly, adult copepods (3–4 weeks old) were exposed to six different concentrations of Simvastatin for 96 h at 19 ± 1 °C in the dark under static conditions without feeding. For each concentration, including the control, three replicates were used, each containing ten animals. Mortality was recorded after 96 h under a stereo microscope with strong illumination.

2.4. Subchronic tests

Approximately 100 females with well-developed egg sacs were isolated from a continuous culture. Nauplii (NI) released within 24 h were collected and randomly allocated into separate wells of transparent 96-well NUNC microplates (VWR International, Norway) together with 295 μL of test solution and 5 μL of feed suspension (i.e. commercial salmon feed; see Breitholtz and Bengtsson, 2001). For each treatment including the control and solvent control, 20 replicates were used. The copepods were held in darkness at 19 ± 1 °C, at high humidity. The wells were covered with loose lids to allow oxygen exchange. Every second day, test medium was renewed (80%), another 5 μL of feed suspension were added and animals were observed in an inverted microscope and their developmental stage and mortality were recorded. When the 3rd copepodite stage (CIII) was reached, the experiment was terminated. In a pilot study (unpublished), we found this stage (CIII) to be the most suitable to use for RNA content analysis in *Nitocra*, because higher variability of RNA concentration at more advanced developmental stages, most probably due to sexual maturation and gonad development (Biegala et al., 1999), hampered inter-treatment comparison. For RNA measurements, 4–5 CIII animals were placed in eppendorf tubes with 100 μL RNAlater and kept at 4 °C until analysis (Gorokhova, 2005); the remaining animals were preserved in glutaraldehyde for BL measurements. The total BL of copepodites (μm) defined as the distance between the anterior part of prosome to the posterior part of the last urosomite, was estimated from the allometric regression:

$$\mathrm{BL} = 10.03 \times \mathrm{FP},$$

where FP is the length of the 2nd free prosomite (μm). The coefficient 10.03 (±0.39) was obtained by dividing the BL with FP of live CIII individuals ($n = 23$). The nauplii to CIII mean development times (DT) were calculated as the mean time in days elapsed from NI to CIII. The GR (day^{-1}), a direct measure of growth, was calculated according to Winberg (1971):

$$\mathrm{GR} = \ln\left(\frac{\mathrm{BL}_t}{\mathrm{BL}_0}\right) \times \left(\frac{1}{\mathrm{DT}}\right),$$