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A short-term swimming speed alteration test with nauplii of *Artemia franciscana*

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ABSTRACT

The presence of toxicant needs to be assessed within short time in order to effectively protect the aquatic environment from serious threat. Based on the observation that at high temperatures aquatic organisms become more vulnerable to stressors than those maintained at room temperature, a new test was developed.

The proposed bioassay consisted in the evaluation of the swimming speed alteration (SSA) of nauplii of *Artemia franciscana* incubated at 39 °C (± 1) for 6 h, using a Swimming Behavior Recorder system (SBR). A comparative ecotoxicological study between the 6 h SSA test and the 24 h mortality test was carried out in order to validate the new method in terms of sensitivity by means of EC_{50} values.

The bioassay was applied to screen different toxicants: $K_2Cr_2O_7$, $Cu(SO_4)_2$, NaClO, SDS and Sertraline hydrochloride. The EC_{50s} calculated for the short-term SSA test and those of the mortality test showed comparable values. For all toxicants, the 6 h SSA test was proved to be as sensitive as the 24 h mortality test.

The method developed in this study is the first temperature-based toxicity test with nauplii of *Artemia franciscana* and it represents an attractive assay in ecotoxicology because of its convenience in terms of time and costs, feasibility and sensitivity.

1. Introduction

Ecotoxicological bioassays represent important tools for evaluating water quality status, together with the assessment of physical, chemical and microbiological parameters. The need of simple and rapid methods to detect contaminants is necessary in order to manage effectively the environmental preservation (Sadik et al., 2004). Numerous efforts are underway to develop fast-response and sensitive tools for ecotoxicological surveys, filling the gap for the needed “early warning” signals (Hellou, 2011).

The use of the genus *Artemia* spp. (Arthropoda, class Crustacea) is widely spread in ecotoxicology (Nunes et al., 2006). The life cycle of the common brine shrimp begins by the hatching of dormant cysts, small spherical-like structures of high physical and chemical resistance. The cysts can remain dormant for extended period, until they are activated with salt water. In form of cysts, *Artemia* spp. can resist for a long period, as long as cysts are kept dry (Pelka et al., 2000).

The high adaptability to different testing conditions, the wide geographical distribution, the simple laboratory culture and maintenance, the short life-cycle, the large offspring production and the low cost of the tests make this species a suitable model organism for ecotoxicological tests (Manfra et al., 2015; Nunes et al., 2006; Persoone and Wells, 1987).

The reliability, replicability and validity of toxicity bioassay with nauplii of *Artemia* spp. has been confirmed by several ecotoxicological studies performed with different stressors, including metals (Brix et al., 2003; Garaventa et al., 2010; Leis et al., 2014), antibiotic drugs (Migliore et al., 1993a, 1993b, 1997), nanomaterials (Corsi et al., 2014; Libralato, 2014; Minetto et al., 2014), microplastics (Gambardella et al., 2017), pesticides (Varó et al., 1997, 2002), ionizing radiations (Easter and Hutchinson, 1961; Grosch and Erdman, 1955), radionuclides (Boroughs et al., 1958) and UV light (Dattilo et al., 2005).

The mortality test with nauplii of *Artemia* spp. is widely employed for cost-effective routine screening of chemicals in aquatic environment (Libralato et al., 2016). It has been standardized basing on the results of a large intercalibration exercise in Europe, U.S. and Canada (Persoone et al., 1992;Van Steertegem and Persoone, 1992). Lethality test can be generally performed for 24 h up to no more than 48 h: after that time, mortality of *Artemia* nauplii showed high variability in both treatments and controls as possible consequence of starvation (Sundh et al., 2012). Besides mortality, several sub-lethal endpoints can be investigated, e.g. hatchability of cysts, biomarkers, teratogenicity test, behavioral test

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(Carballo et al., 2002; Gambardella et al., 2014; Huang et al., 2016; Rotini et al., 2015; Varó et al., 2002). Among sub-lethal behavioral assays, alterations in locomotion have been found to be a sensitive endpoint frequently investigated. In a recent review, Faimali et al. (2017) identified *Artemia* spp. as the most used model organism in the swimming alteration studies as response to contaminants. The swimming speed alteration (SSA) test with nauplii of *Artemia* spp. proved to be a suitable tool to assess the impact of contaminants at concentrations far below the lethal effects (Alyuruk et al., 2013; Anufriieva and Shadrin, 2014; Costa et al., 2016; Gambardella et al., 2014; Garaventa et al., 2010; Huang et al., 2016; Kokkali et al., 2011; Manfra et al., 2016; Mesarič et al., 2015; Venkateswara Rao et al., 2007). In addition to alterations in speed, Alyuruk (Alyuruk et al., 2013) and Venkateswara Rao (Venkateswara Rao et al., 2007) considered swimming path alteration of *Artemia* exposed to contaminants, whereas Di Delupis and Rotondo (1988) evaluated the phototactic response of nauplii to environmental stressors as an additional sub-lethal endpoint.

Several aspects in the locomotor behavior of *Artemia* nauplii have been analyzed, including swimming alterations as response to different wavelengths of light, predators, food availability and changes in water temperature (Ali et al., 2011; Anufrieva and Shradin, 2014; Beck and Turingan, 2007; Larsen et al., 2008). Temperature is an important factor that can significantly affect the physiological state of aquatic organisms (Heugens et al., 2003), directly (e.g. changes in the rates of biochemical reactions and fluidity of membranes) and indirectly (e.g. changes in metabolic fluxes and cellular signalling), which could, in turn, modify the response to environmental stressors (Bao et al., 2008).

To evaluate the relevance of an ecotoxicological bioassay, it is of main importance to take account of the influence of temperature variations on the endpoint investigated. For example, Piazza et al. (2016) assessed temperature effects on cadmium toxicity on lethal and sub-lethal response of *Amphibalanus amphitrite* nauplii. The obtained results showed an increase in cadmium toxicity at higher temperatures for all the endpoints investigated, thus highlighting how incubation parameters can actually change organism responses, affecting final test results.

Taking advantage of the natural tendency of aquatic organisms to respond to an increase in water temperature by accelerating their metabolism (Varó et al., 1993), becoming therefore more sensitive to stressors, we developed a short-term test able to detect a fast-response for ecotoxicological surveys. Indeed, once exposed to chemicals, at high temperatures *Artemia* nauplii resulted to be affected by toxicants prior than nauplii maintained at room temperature (Cairns et al., 1975; Grasset et al., 2016; Oetken, et al., 2009). Despite the high number of studies focused on the synergic action of water temperature and toxicant on aquatic organisms, nobody have so far exploited this combined impact to develop a rapid-response test. To our knowledge, only Kim and colleagues (Kim et al., 2012) proposed a 1-h toxicity test using *Daphnia magna* incubated at 36.5 °C, while, according to the OECD Guideline for testing of chemicals, the test temperature should be between 18 and 22 °C (OECD, 2004). This study highlighted similar results between the 1 h and the standard 24 h acute toxicity test.

In the present work, i) the swimming speed of *A. franciscana* nauplii incubated in Filtered Natural Seawater at different temperatures (25, 33 and 39 °C) for few hours (1, 2, 4 and 6 h) was assessed, together with organisms survival; ii) preliminary SSA tests were carried out evaluating different temperatures and times of incubation with a reference chemical ($K_2Cr_2O_7$) in order to identify the most suitable parameters for the new test; iii) then, the definitive short-term SSA test was applied with different stressors and, for each toxicant, a comparative 24 h mortality test was performed to confirm the sensitivity of the new SSA test by means of comparison with EC_{50} and LC_{50}.

2. Materials and methods

2.1. Model organism

Commercially available dehydrated cysts of *Artemia franciscana* were used for the experiments. Instar I stage nauplii (24 h post hatching) were obtained as described by Garaventa et al. (2010). The hatched nauplii were separated from non-hatched cysts based on their positive phototaxis and then transferred by a Pasteur pipette into beakers containing the Filtered Natural Seawater (FNSW).

2.2. Swimming speed and survival assessment at different temperatures and incubation times

Swimming speed of *A. franciscana* nauplii incubated at different temperatures (25, 33 and 39 °C) was recorded using an automatic recording system (Swimming Behavioral Recorder- SBR). In parallel, the survival percentage was checked at the stereomicroscope (Stereo Discovery V.8, Zeiss, Germany).

The SBR system, developed at ISMAR-CNR, is a video camera-based system (Kenko, Japan) with a macro-objective, which records the paths of a sample of swimming larvae as described in detail in Faimali et al. (2006). Each test was performed by adding 15 nauplii into 25-well polystyrene plates that contained 1 mL of FNSW. The plates were kept at 25, 33 and 39 °C. Each test has been performed in 6 replicates; swimming speed and mortality percentage were evaluated after 1, 2, 4 and 6 h.

2.3. Preliminary SSA tests

In order to identify the most suitable parameters for the new test, preliminary SSA tests were performed, testing different temperatures (25, 33 and 39 °C) and times of incubation (1, 2, 4 and 6 h) in the presence of a reference toxicant. Each test was carried out by adding 15 nauplii to each well of a 25-well polystyrene plate that contained 1 mL of FNSW with solutions of different concentrations (2.5–5–10–25–50–75 mg/L) of the reference chemical $K_2Cr_2O_7$ (Huang et al., 2016). Once larvae were added to the well, the plates were stored in dark condition for 1, 2, 4 and 6 h at 25, 33 and 39 °C, respectively. All tests were performed in triplicate. For each test, the swimming speed alteration percentage of *A. franciscana* nauplii was recorded through the SBR system after 1, 2, 4 and 6 h. Swimming speed data (the average of all instantaneous speed values ± SE) were referred to as swimming speed alteration, normalized to the average swimming speed of the control.

2.4. Definitive short-term SSA test

The definitive short-term SSA test was applied to screen different compounds. Stock solutions were made in MilliQ water (Millipore Corp., Bedford, MA, USA). The tested compounds, $K_2Cr_2O_7$, $Cu(SO_4)_2$, Sodium dodecyl sulfate (SDS), NaClO and Sertraline hydrochloride (≥ 98%; HPLC) were purchased from Sigma–Aldrich Co. (St. Louis, USA). A wide range of nominal concentrations was used (2.5–5–10–25–50–75 mg/L for $K_2Cr_2O_7$; 2.5–5–10–25–50 mg/L for Cu $(SO_4)_2$; 2.5–5–10–25–50 mg/L for SDS; 0.1–0.5–1–5–10 mg/L for NaClO; 0.1–0.5–1–5–10 mg/L for Sertraline hydrochloride) in order to expose organisms to a significant chemical stress gradient, allowing to calculate the Effective Median Concentrations (EC_{50} values).

Each test was carried out as previously described (Subsection 2.3), selecting 39 °C and 6 h as temperature and time of incubation, respectively.

2.5. Comparative 24 h mortality test

A comparative 24 h mortality test was run, exposing *Artemia* nauplii