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Lethal and sublethal endpoints observed for *Artemia* exposed to two reference toxicants and an ecotoxicological concern organic compound

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ABSTRACT

Swimming speed alteration and mortality assays with the marine crustacean *Artemia franciscana* were carried out. EC_{50} and LC_{50} values after 24–48 h exposures were calculated for two reference toxicants, copper sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$) and Sodium Dodecyl Sulphate (SDS), and an ecotoxicological concern organic compound, Diethylene Glycol (DEG).

Different end-points have been evaluated, in order to point out their sensitivity levels. The swimming speed alteration (SSA) was compared to mortality values and also to the hatching rate inhibition (literature data). SSA resulted to be more sensitive than the mortality and with a sensitivity comparable to (or even higher than) the hatching rate endpoint.

1. Introduction

Aquatic invertebrates are widely used in ecotoxicological studies, because they are relatively easy to maintain under test conditions, their use is not (as yet) restricted by ethical concerns, and they are generally more sensitive to a range of pollutants than vertebrates or plants (Piazza et al., 2012).

Several bioassays using larval stages of marine crustaceans have been proposed to investigate the biological effects of contaminants and environmental matrices on primary consumers, i.e. with the cirriped *Amphibalanus amphitrite* (Greco et al., 2006), with the copepods *Tigriopus fulvus* (Faraponova et al., 2003) and *Acartia tonsa* (Gorbi et al., 2012), with the brine shrimp *Artemia franciscana* (Manfra et al., 2015).

Among the endpoints mainly observed on larval stages, behavioural parameters are accurate, sensitive and reliable indicators of stress. The behaviour of an organism is the endpoint of a sequence of complex neurophysiological events (stimulation of neurons via the release of chemical messages and muscular contractions) (Thiéry et al., 2012).

The swimming of aquatic organisms is a behavioural response well defined and practical to measure, sensitive to a wide range of contaminants and adaptable to different species, ecologically relevant and has representation across species. Moreover, it is simple to automate in order to be useful for a wide range of applications (Rand, 1985). Changes in locomotor behaviour can therefore be used as a stress indicator in ecotoxicological studies, thus obtaining a realistic picture of the effects of contaminants at the ecosystem level (Tahedl and Häder, 2001).

Mostly in the last years, changes in motion behaviour as a response to exposure to organic or inorganic pollutants, have been observed in a range of aquatic invertebrates such as *Artemia salina, A. Amphitrite, Brachionus calyciflorus, Corophium volutator, Gammarus fossarum* and other copepods (Charoy and Janssen, 1999; Faimali et al.; 2006, Rao et al., 2007; Kienle and Gerhardt, 2008; Xuereb et al., 2009; Seuront, 2011).

In particular, Faimali et al. (2006) developed a Swimming Speed Alteration (SSA) recording system, by applying a video-camera tracking system to detect linear swimming speed as behavioural end-point. This system has been already used on larvae of *A. amphitrite* (Crustacea Cirripedia), on the brine shrimp *Artemia* sp. and the rotifer *Brachionus plicatilis* (Garaventa et al., 2010), demonstrating that alterations in swimming speed can be detected.

Taking into account these previous information, aims of this study were: (a) to measure the swimming speed alteration and mortality of *A. franciscana* exposed to two reference toxicants i.e. copper sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$) and Sodium Dodecyl Sulphate (SDS); (b) to record the swimming speed alteration and mortality of *Artemia* exposed to an ecotoxicological concern additive i.e. Diethylene Glycol (DEG); (c) to compare the response, in

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term of sensitivity, between swimming speed, mortality (data in this study) and hatching capability (Rotini et al., in press; Manfra et al., 2015).

($CuSO_4 \cdot 5H_2O$) and SDS were selected because commonly used in *Artemia* biotests (Persoone et al., 1993; Guzzella, 1997; Manfra et al., 2012). Regards to DEG, it is used to prevent hydrate formation during the gas production process and since it may be discharged into the sea from the gas platforms, its ecotoxicological characterisation is required (M.D. 28.07.1994). The most previous results showed lethal and sub-lethal (i.e. bioluminescence inhibition for bacteria, growth rate inhibition for algae, development inhibition for mussels) toxic effects at DEG concentration higher than 9 g/L (Tornambè et al., 2012). Fish biomarker outcomes also did not show significant toxic effects up to 5 g/1, with the only exception of a slight genotoxic damage (Gorbi et al., 2009). The hazard assessments on aquatic organisms indicated Predicted No Effect Concentrations for marine waters of 1–10 mg/L (European Chemicals Agency Database) and 5.9–59 mg/L (Manfra et al., 2015), for constant or intermitted release of DEG. These abovementioned concentrations are higher than quantities really measured in the Adriatic Sea (<2 mg/L) (Cianelli et al., 2008).

2. Materials and methods

A control sample (Synthetic Sea Water SSW, 0.22 μm filtered, at 35‰ salinity) was obtained by adding the marine salt mixture *Instant Ocean* (Aquarium Systems Mentor, Ohio, USA and Sarreborg, France) to deionized water. Solutions for treatments were prepared by diluting $CuSO_4 \cdot 5H_2O$ (Sigma Aldrich, $\geq$98%, CAS# 7758-99-8), SDS (Sigma Aldrich, $\geq$99%, CAS# 151-21-3) and DEG (Carl Roth GmbH, $\geq$99%, CAS# 111-46-6) in the SSW to obtain the following concentrations: 2–4–8–16–32 mg/l $CuSO_4 \cdot 5H_2O$, 3–6–12–24–48 mg/l SDS and 10–20–40–80–160 g/lDEG. These intervals have been based on previous results obtained in hatching rate and mortality tests (Guzzella, 1997; Manfra et al., 2015; Rotini et al., in press). Preliminarily, the stability of the compounds (24 h measured concentrations) was evaluated under the toxicity test conditions. The copper was measured according to Clescerl et al. (1999); for SDS and DEG the ISO no. 7875 method (ISO, 1984) and the UNICHIM no. 1367 method (UNICHIM, 1999) were applied, respectively. The 80–90% of the measured initial concentration at 24 h was maintained (Table 2, Supplemental Data).

Dehydrated cysts of *A. franciscana* (certified from the Laboratory for Biological Research in Aquatic Pollution, University of Ghent, Belgium) were used for the experiments. To obtain Instar II–III stage larvae (less than 48 h old), the hatching was performed as described in Manfra et al. (2015). Larvae were added into the multiwell-plates with 24 compartments (Barloworld Scientific Ltd.). Ten individuals were put in each well/replicate containing 1 ml of test solution; the control was also analyzed. The plates were kept at 25 °C in the dark.

The test solutions and the control were analyzed in three replicates and three test repetitions were performed for each toxic compound. The average of the linear speed of 10 test organisms and the dead individual number were observed for each replica. The swimming speed and mortality were recorded after 24 and 48 h of exposure, according to Garaventa et al. (2010) and Guzzella (1997), respectively. The swimming speed alteration was recorded using the SSA recorder described in Faimali et al. (2006), consisting of a video camera with a macroobjective for recording the larval swimming paths. The larvae were dark-adapted for 2 min before the video recording (time fixed by preliminary tests, to reach steady speeds and a uniform spatial distribution) and the images were analyzed using advanced image processing software to reconstruct the individual paths/tracks (Gambardella et al., 2014).

Larvae that were completely motionless were counted as dead organisms, and the percentage of mortality was calculated compared to the control. The term "motionless" means organisms that do not change their own barycentre position and do not move any appendages in 5 s.

The data referred to the swimming alteration have been normalised to the mean swimming speed (S) of the control, where: alteration (%)=[(Streated- Scontrol/Scontrol)]*100.

Median effective concentration on swimming alteration (24 h EC_{50} and 48 h EC_{50}) and mortality (24 h LC_{50} and 48 h LC_{50}) were calculated using Trimmed Spearman-Karber analysis (Finney, 1978).

The tests were considered acceptable when the control mortality was $\leq$10% (Guzzella, 1997, Garaventa et al., 2010).

Significant differences between the exposure times (24 h EC_{50s} vs. 48 h EC_{50s} and 24 h LC_{50s} vs. 48 h LC_{50s}) and also between lethal and sublethal endpoints (24 h EC_{50s} vs. 24 h LC_{50s} and 48 h EC_{50s} vs. 48 h LC_{50s}) were calculated by statistical analysis (*t*-test).

3. Results and discussion

The tests resulted acceptable because the death rate of the controls was $<$10%, according the methods used in the experiments.

The swimming speed alteration and mortality percentages of *A. franciscana* after 24 and 48 h of exposure to $CuSO_4 \cdot 5H_2O$, SDS and DEG are shown in Fig. 1. These were slightly higher after 48 h than 24 h of exposure. The EC_{50} and LC_{50} values are summarised in Table 1.

The results indicated the lowest toxicity of DEG compared to the toxic effects of the reference toxicants. DEG LC_{50} values were within 80–160 g/l concentration range. For $CuSO_4 \cdot 5H_2O$ and SDS, 48 h EC_{50} values were lower (about half) than 24 h results. The difference was less marked for DEG. These differences between the two exposure times were always statistically significant ($p<0.05$) for SDS and DEG but not for $CuSO_4$ LC_{50} values (see Table 3, Supplemental Data).

The differences between lethal and sub-lethal end-points were significant ($p<0.05$) for SDS but not for $CuSO_4$ (see Table 4, Supplemental Data).

Our LC_{50} values were comparable to literature results: 12.57 and 10.71 ± 3.13 mg/l for $CuSO_4$ (Persoone et al., 1993; Manfra et al., 2015), 25.60 ± 5.50 mg/l for SDS (Guzzella, 1997).

The swimming test is a short-chronic bioassay and it may give results similar to long-term exposures. In fact, our SDS 48 h EC_{50s} (7.49 ± 1.33) appeared comparable to 14 d LC_{50s} (8.50 ± 3.34 mg/l) (Manfra et al., 2015).

Studies on the 48 h hatching rate inhibition of *Artemia* exposed to $CuSO_4 \cdot 5H_2O$ (Manfra et al., 2015), SDS and DEG (Rotini et al., in press) are available in literature. We compared our swimming speed alteration results (48 h EC_{50}) to these data. For $CuSO_4 \cdot 5H_2O$, we observed a mean value (2.51 ± 0.37 mg/l) 1.5–2 times lower than 48 h hatching EC_{50} (4.95 ± 2.26 mg/l). For SDS, the mean value (7.49 ± 1.33 mg/l) was about 2 times lower than hatching value (>15 g/l). Similar results were measured for DEG (swimming: 64.56 ± 3.42 g/l; hatching: >50 g/l). Generally, the swimming speed alteration and the hatching rate inhibition resulted more sensitive than the acute (24–48 h) mortality. In addition, our results showed a swimming test sensitivity comparable or higher than hatching test.

The results obtained selecting these sublethal endpoints are comparable to the response of other marine organisms (as algae, rotifers, amphipods, crustaceans, urchin and fish) that give an EC_{50} range of [1.30–17.40] mg/l for $CuSO_4 \cdot 5H_2O$, [2.36–7.42] mg/l for