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. EC50 and LC50 values after 24–48 h exposures were calculated for two reference toxicants, copper sulphate pentahydrate (CuSO4·5H2O) 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.

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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 (Tahedi 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; Xuerreb 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 (CuSO4·5H2O) 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
term of sensitivity, between swimming speed, mortality (data in this study) and hatching capability (Rotini et al., in press; Manfra et al., 2015).

(CuSO$_4$·5H$_2$O) 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/l, 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 above-mentioned 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 Sarrebourg, France) to deionized water. Solutions for treatments were prepared by diluting CuSO$_4$·5H$_2$O (Sigma Aldrich, ≥98%, CAS# 7758-99-8), SDS (Sigma Aldrich, ≥99%, CAS# 151-21-3) and DEG (Carl Roth GmbH, ≥99%, CAS# 111-46-6) in the SSW to obtain the following concentrations: 2–4–8–16–32 mg/l CuSO$_4$·5H$_2$O, 3–6–12–24–48 mg/l SDS and 10–20–40–80–160 g/IDEG. 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).

The results obtained selecting these sublethal endpoints are comparable to those in literature. We compared our swimming speed alteration and mortality percentages of A. franciscana after 24 and 48 h of exposure to CuSO$_4$·5H$_2$O, 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$_4$·5H$_2$O 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 endpoints 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$_{50}$ (7.49 ± 1.33) appeared comparable to 14 d LC$_{50}$ (8.50 ± 3.34 mg/l) (Manfra et al., 2015).

Studies on the 48 h hatching rate inhibition of Artemia exposed to CuSO$_4$·5H$_2$O (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$_4$·5H$_2$O, 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$_4$·5H$_2$O, [2.36–7.42] mg/l for CuSO$_4$·5H$_2$O.
SDS, [5.90–90.40] g/l for DEG (Nipper et al., 1993; Ribelles et al., 1995a, 1995b; Rosety et al., 2001; Verbruggen et al., 2005; Blanchard and Grosell, 2006; Mariani et al., 2006; Tornambè et al., 2012).

Several locomotor responses (distance travelled, travel velocity, frequency of direction change, time spent active, response to light, and tail-flip escape response) have been assessed on various test organisms. Swimming speed is perhaps the most frequently used behavioural measurement of the physiological status of an aquatic organism (Faimali et al., 2006). Behaviour is a determinant that results from molecular, physiological and ecological aspects of toxicology (Little et al. 1990). Therefore, behaviour can provide insights into various levels of biological organisation (Scott and Sloman, 2004). How might the contaminants interact on Artemia to produce the reduction in swimming velocities (compared to controls)?

Davenport and Healy (2006) assessed the relationship between physical parameters (medium salinity, body density, buoyancy) and swimming in *Artemia franciscana* larvae. They found that the horizontal swimming speed was unaffected by salinity and viscosity (over the range 8.5–100 ‰) but the observed differences in vertical swimming rates are solely due to relatively constant body density. Larsen et al. (2008) also suggested that changes in swimming velocity of *Artemia* are due to change in kinematic viscosity. Previously, Williams (1994a, 1994b) published an analysis of locomotion in *Artemia*, showing that larvae have a single pair of antennae and exhibit jerky, discontinuous swimming in which the animal’s body moves to and fro with each rowing stroke. No information seems to be available in literature on the swimming speed alteration of *Artemia* exposed to CuSO4·5H2O, SDS and DEG, except a mobility study of Kokkali et al. (2011). They quantified the Cu2⁺ EC50 value (7.6 mg/l), which resulted to be 2.5 times lower than the LC50 (19.5 mg/l) but did not explain the

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**Table 1**

EC50/LC50 mean values (s.d.) after 24–48 h of exposure to CuSO4·5H2O (mg/l), SDS (mg/l) and DEG (g/l).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>24 h EC50</th>
<th>48 h EC50</th>
<th>24 h LC50</th>
<th>48 h LC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuSO4·5H2O (mg/l)</td>
<td>5.03 (0.58)</td>
<td>2.51 (0.37)</td>
<td>14.21 (10.63)</td>
<td>2.51 (0.22)</td>
</tr>
<tr>
<td>SDS (mg/l)</td>
<td>16.15 (0.32)</td>
<td>7.49 (1.33)</td>
<td>19.41 (1.00)</td>
<td>15.60 (0.27)</td>
</tr>
<tr>
<td>DEG (g/l)</td>
<td>80.37 (3.95)</td>
<td>64.56 (3.42)</td>
<td>80–160</td>
<td>80–160</td>
</tr>
</tbody>
</table>

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toxic effect mechanisms. Moreover, some authors studied the effects of copper and SDS on aquatic organisms, proving to explain the toxicity mechanisms. Blanchard and Grosell (2006) studied to copper effects on fish: gills became frayed and lose their ability to regulate transport of salts such as sodium chloride and potassium chloride into and out of fish. These salts are important for the normal functioning of the cardiovascular and nervous systems and their uncorrected transport may cause fish death. In addition, copper can affect the activation of the olfactory receptor neurons or intracellular signalling in the neurons. Sublethal copper exposure alter a number of behaviours in invertebrates. It reduces the ability of decapods to detect and orient to food odours in a water plume. It produces adverse effects on locomotory and sensory systems and these can also be correlated with adversely affected predator avoidance behaviours. It induces a slowing of heart rate in mussels and it adversely affects the ability of worms to produce body reversal or helical swimming behaviours following tactile stimulation (O’Gara et al., 2004).

Barbieri et al. (1998) revealed that increasing concentrations of SDS affect fish metabolism (the oxygen consumption increases) and locomotor capacity (swimming capacity decreases). These authors observed that at the highest concentration (10 mg/l), swimming capacity was reduced 5 times and oxygen consumption increased 2.8 times in comparison to the control. SDS is characterized by high amphility and adsorption ability. The basis of its toxicity seems to be mainly related to the alteration of the cellular ionic balance caused by cellular membrane permeability alterations and to the induction of oxidative stress (which, in turn, can generate other physiological and biochemical stresses (Messina et al., 2014).

Hymela et al. (2002) observed variation in fish swimming performance after exposure to glycols (ethylene glycol). The buildup of metabolic products and increased metabolic cost of removing ethylene glycol from the body may have caused or contributed to the decrease in swimming performance of exposed fish.

Earlier reports indicated that the decrease in velocity in other organisms could be caused by food deprivation, anoxic conditions or inhibition of acetylcholine (ACh) enzyme (Westertep, 1977; Nilsson et al., 1993). In short-term tests (24–48 h) Artemia is not facing either anoxic conditions or deficiency of food (Guzzella, 1997). In the past, Artemia species have been referred as organisms that accumulate toxic compounds with no effect on their life cycle (Barabina et al., 2002). After that, Rao et al. (2007) observed that the (ACh) enzyme activity may be inhibited by the toxicant (i.e., pesticides), resulting in accumulation of acetylcholine at neuromuscular junctions. The accumulation of this neurotransmitter (ACh) may alter the locomotion behaviour of the organism, interrupting the coordination between the nervous and muscular junctions. Toxicity testing of copper-contaminated sediments to amphipods (Hyalella azteca) and daphnids (Daphnia magna) using techniques of enzyme inhibition and growth rate showed that these variables are more sensitive in accurately predicting copper sensitivity than LC_{50} (48 h) values (Eisler, 1998). Recently, this neurotoxic effect has been investigated in Artemia salina nauplii exposed to organic compounds (i.e., Eserine) (Garaventa et al., 2010) but there are not clearly evidences for copper, SDS and DEG exposures.

For these toxic mechanisms, the swimming speed alteration may potentially deliver more sensitive indicator of stress than conventional mortality assay for Artemia, as already observed for behavioural responses compared to endpoints such as survival, growth, and reproduction (Gerhardt et al. 2005; Dell’Orno, 2002).

4. Conclusion

To promote alterative endpoints for Artemia, the sensitivity of the swimming speed and the mortality have been evaluated by using two well-known reference toxicants and a potentially environmental hazardous compound (Manfra et al., 2015).

This study has proved that an high sensitivity was detected for Artemia by tracking naupliar swimming and estimating their average speed using digital image processing. This endpoint seems to be sensitive, ecologically relevant and comparable to another end-point: the hatching rate. Both of them showed to be preferable to mortality in order to point out a toxic effect at low concentrations. The swimming speed alteration is of great ecological importance as the alteration of behaviour represents an integrated whole-organism response that can link the physiology and ecology of an organism to its environment (Little and Brewer, 2001). In addition, changes in swimming behaviour of invertebrates may have important indirect effects on marine and freshwater communities, such as alteration in predation pressure, zooplankton prey pressure, and phytoplankton abundance (Charoy and Janssen, 1999).

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

Informed consent was obtained from all individual participants included in the study.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2015.08.017.

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