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Intercalibration of ecotoxicity testing protocols with *Artemia franciscana*

L. Manfra[a,b,*], F. Savorelli[c], B. Di Lorenzo[a], G. Libralato[d,e], S. Comin[f], D. Conti[a], B. Floris[g], M. Francese[h], M.L. Gallo[i], I. Gartner[j], M. Guida[k], T. Leoni[l], G. Marino[m], F. Martelli[n], D. Palazzi[c], E. Prato[b], P. Righini[o], E. Rossi[p], A. Volpi Ghirardini[d,e], L. Migliore[q]

[a] *Italian Institute for Environmental Protection and Research, Via Vitaliano Brancati, 60, 00144 Rome, Italy*
[b] *Institute for the Coastal Marine Environment, National Research Council (CNR IAMC), Via Roma 3, 74100 Taranto, Italy*
[c] *Regional Agency for Environmental Protection, Via Bologna 534, 44124 Ferrara, Italy*
[d] *Department of Environmental Sciences, Informatics and Statistics, University Ca' Foscari Venice, Campo della Celestia, 2737/b, I-30122 Venice, Italy*
[e] *Institute of Marine Sciences, National Research Council (CNR ISMAR), Arsenale Tesa 104, Castello 2737/F, I-30122 Venice, Italy*
[f] *Regional Agency for Environmental Protection, Via Lissa, 6, 30174 Venice, Italy*
[g] *Regional Agency for Environmental Protection, Viale Ciusa 6, Cagliari, Italy*
[h] *Shoreline, Area Science Park Padriciano 99, 34149 Trieste, Italy*
[i] *Regional Agency for Environmental Protection, Via Don Bosco 4/F, 80141 Naples, Italy*
[j] *Regional Agency for Environmental Protection, Via Adige 12, 57025 Piombino, Italy*
[k] *Department of Biology, University of Naples Federico II, via Mezzocannone 16, 80134 Naples, Italy*
[l] *Regional Agency for Environmental Protection, Via Federico II 41 – loc. Villa Potenza, 62010 Macerata, Italy*
[m] *Regional Agency for Environmental Protection, Via Nairobi 4, 90100 Palermo, Italy*
[n] *Regional Agency for Environmental Protection, Via Fiume 35, 58100 Grosseto, Italy*
[o] *Regional Agency for Environmental Protection, Via Marradi 114, 57126 Livorno, Italy*
[p] *Regional Agency for Environmental Protection, via Fontevivo 21, 19125 La Spezia, Italy*
[q] *Department of Biology, University Tor Vergata, Via della Ricerca Scientifica, 00133 Rome, Italy*

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ABSTRACT

The brine shrimp, *Artemia* spp., is widely used in ecotoxicology as a target biological model. Although several protocols were available in the early 1980s, only the 24-h acute mortality toxicity test was evaluated in a European intercalibration exercise during that period. Nevertheless, documentation of standard methods serving to provide specifications, guidelines or detailed characteristics of the 24-h protocol is still unavailable. This paper present the results of an intercalibration study of three toxicity-testing protocols using *Artemia franciscana*: (a) the 24-h static acute mortality test, (b) the 48-h static hatching test and (c) the 14-d static-renewal long-term mortality test. A first tier of experiments was conducted by a reference laboratory, which investigated the repeatability of the three methods. The feasibility and reproducibility of these protocols were then investigated by an intercomparison exercise involving 11 participants for the acute mortality test, seven for the acute hatching test and nine for the long-term mortality test. Protocols were tested on reference toxicants (copper sulphate pentahydrate and sodium dodecyl sulphate). The coefficients of variation were <20% and <50% for intra- and interlaboratory activities, respectively. These results encourage the standardization of the proposed methods and their use as regulatory procedures.

1. Introduction

In recent years, many countries' environmental legislation has introduced the use of bioassays as additionally recommended analyses assessing the status of aquatic environments (*i.e.*, water and sediment) and characterizing the ecotoxicity of chemicals (Chapman, 2007). Toxicity tests using a wide variety of taxa have been developed and new methodologies are in progress (Gorbi

* Corresponding author at: ISPRA – Istituto Superiore per la Protezione e la Ricerca Ambientale, Italian Institute for Environmental Protection and Research, Via Vitaliano Brancati, 60 Roma, Italy. Tel.: +39 0650074787/0810663914.
E-mail address: loredana.manfra@isprambiente.it (L. Manfra).

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et al., 2012). In 2014, three national standard toxicity test methods were published: (a) two protocols (acute and chronic mortality) with the copepod *Acartia tonsa* Dana (Gorbi et al., 2012); and (b) one protocol (acute swimming behavior) with the cirripede *Balanus amphitrite* L. (Piazza et al., 2012).

Artemia spp. (Crustacea, Anostraca) is among the most commonly used live food sources in aquaculture; it has a key role in the food chain energy flow in the marine environment and is frequently utilized as a saltwater biological model in ecotoxicology (Migliore et al., 1997). Its use is well documented in the scientific literature of the past 30 years (Nunes et al., 2006; Libralato et al., 2007; Libralato, 2014). The main advantage of the species in this context is that nauplii can be hatched from commercial available durable cysts (eggs), allowing homogeneity of the population and its continuous year-round use, without animal breeding in the laboratory, as required for almost all the species used in ecotoxicity tests (Manfra et al., 2012). Other advantages are: (a) good knowledge of its biology and ecology; (b) easy manipulation and maintenance under laboratory conditions; (c) small body size that allows accommodation in small beakers or plates; (d) adaptability to a wide range of salinities and temperatures (USEPA, 2002).

Some criticisms about *Artemia* sensitivity have been presented in the context of a learning-by-doing approach (Libralato et al., 2010; Libralato, 2014). For example, the cysts' production may reflect the occurrence of genetic variation caused by their geographical origin that is rarely known (Migliore et al., 1997), although certified cysts are usually utilized in toxicity testing.

Various toxicity test protocols are available in the literature and are easily applicable in a minimally equipped laboratory by personnel with little experience. Short-term toxicity test protocols (≤96-h) are the most common. They include various endpoints: (a) survival (Persoone et al., 1993; Guzzella, 1997; Artoxkit, 2014); (b) hatching and growth (Migliore et al., 1997; Sarabia et al., 2008); and (c) behavioral (*i.e.*, swimming) (Garaventa et al., 2010; Gambardella et al., 2014). Some long-term protocols have also been studied in the last 10 years, showing that survival is the most sensitive endpoint compared to growth and reproduction (Brix et al., 2003, 2004; Savorelli et al., 2007; Manfra et al., 2012; UNICHIM, 2012). These long-term procedures employ the larval developmental stages of *Artemia* spp. (Instar II–III), which are the most sensitive stages in the entire life cycle (Gorbi et al., 2012).

The existence of several approaches but the absence of standardized methods (ISO, ASTM or OECD) with *Artemia* spp. is a crucial gap that should be filled as soon as possible to make *Artemia* spp. an official standard biological model in ecotoxicology and nanoecotoxicology (Libralato, 2014). The availability of standardized protocols is a great concern for all test species because the procedures should be reproducible in different laboratories and from different operators. In particular, *Artemia* spp. is widely used in several countries as a result of the above-described advantages and the rich storehouse of information existing in the literature for this species.

Despite the frequent and widespread use of *Artemia* spp. in toxicity testing, the harmonization of protocols followed by standardization activities is still lacking, and round-robins are urgently necessary (Libralato, 2014). To date, only the results from the 24-h toxicity test intercalibration exercises have been published, providing data on copper sulfate as a reference toxicant (Persoone et al., 1993).

To remedy this lack of information, the aim of this paper is to present the Italian intercalibration outcomes of three toxicity-testing protocols with *Artemia franciscana*: (a) the static acute mortality test (24-h); (b) the static acute hatching test (48-h); and (c) the static-renewal long-term test (14-d).

Attention was mainly focused on these methods because: (a) the 24-h mortality test is routinely used for toxicity screening, such as in the case of industrial pollution monitoring or within specific national regulatory requirements; (b) the 48-h hatching procedure is easy to perform, sensitive in a short-time-lag framework and performed on a huge number of individuals (Migliore et al., 1997); (c) the 14-d mortality test presents the same starting conditions (hatched nauplii as testing organisms) of the 24-h protocol, being already recognized by UNICHIM (UNI associated agency, the Italian organization devoted to standardization and unification of methods) and illustrated in a peer reviewed scientific video journal (Manfra et al., 2012). As a preliminary step, a reference laboratory assessed the repeatability of the suggested protocols. Afterwards, seventeen laboratories (three Institutes from the National Research Center, three Universities, one private laboratory and 10 Regional Environmental Protection Agencies) were involved in evaluating the reproducibility of protocols; these laboratories conducted intercomparison exercises on two reference toxicants.

2. Materials and methods

2.1. Intercalibration design and set-up

National intercomparison exercises took place between 2006 and 2009 with 11 participating laboratories. These laboratories were designated Lab 1, Lab 2, Lab 3, Lab 4, Lab 5, Lab 6, Lab 7, Lab 8, Lab 9, Lab 10 and Lab 11.

All participating laboratories were supplied with: (a) certified cysts; (b) reference toxicants; (c) algal cultures (as brine shrimp feeding only for the 14-d test); (d) detailed protocols (also for microalgae culturing) and (e) specific training courses organized by the reference laboratory.

The reference laboratory performed each assay five times (*i.e.*, the static acute mortality test (24-h), the static acute hatching test (48-h) and the static-renewal long-term mortality test (14-d)) to evaluate the intra-laboratory repeatability of the methods. The participating laboratories iterated the acute mortality test three times, the long-term mortality test three times and the acute hatching test one time to evaluate the inter-laboratory reproducibility of the methods.

2.2. Organisms and chemicals

Certified cysts (AF/N2000) of *A. franciscana* were obtained from the Laboratory for Biological Research in Aquatic Pollution of Ghent University (Belgium). The declared sensitivity of cysts for the positive control ($K_2Cr_2O_7$), expressed as median lethal concentration at 24 h (24 h-LC_{50}), was 30.9 mg/L (26.7–35.6 mg/L).

Artificial seawater with a salinity equal to 35 PSU (Practical Salinity Unit) was prepared with Instant Ocean® and ultra-pure deionized water. Prior to use, it was aerated for 48 h and filtered through a 0.45 μm cellulose acetate filter. Artificial seawater was used both as a negative control and as a diluent to prepare toxicity testing solutions. The salinity was maintained at 35 PSU. Oxygen saturation was maintained at a level >60%.

Copper sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$, Sigma Aldrich, ≥98%, CAS# 7758-99-8) was used as the reference toxicant for the static toxicity tests (24-h and 48-h), whereas sodium dodecyl sulphate (SDS, Sigma Aldrich, ≥99%, CAS# 151-21-3) was used for the static-renewal (14-d) bioassays. Actually, copper could kill the algae used as the food for the brine shrimp. The copper could adhere to the algae or clog the algal complexes or the feeding apparatus of the brine shrimp, thus increasing their mortality. These toxicants were employed because are among the reference toxicants (SDS, $CuSO_4$ and $K_2Cr_2O_7$) reported in the *Artemia* spp. protocols (Guzzella, 1997; Libralato et al., 2007; Manfra et al., 2012). A stock solution (1 g/L) of each reference toxicant was prepared by dissolving the toxicant in deionized water and stirring the stock solution