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Intercalibration of ecotoxicity testing protocols with *Artemia franciscana*

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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

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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

until the toxicant is completely dissolved. The stock solutions were diluted with filtered artificial seawater to prepare the testing exposure media. The reference toxicants ranged as follows: (a) 5.0–7.1–10.1–14.4 mg $CuSO_4 \cdot 5H_2O$/L for the acute mortality test; (b) 5.0–7.1–10.1–14.4 mg $CuSO_4 \cdot 5H_2O$/L for the acute hatching test; (c) 1.6–3.1–6.2–12.5–25.0 mg SDS/L for the long-term mortality test. The reference laboratory established the testing ranges on the basis of specific range-finding toxicity tests and previous literature data (Guzzella, 1997; Manfra et al., 2012). The toxicity tests were conducted at least in triplicate. The experiments were based on solutions with nominal concentrations. The solutions were prepared with analytical balances and class A higher-standard glassware. Nominal concentrations of reference toxicants were used to calculate the median lethal or effect concentrations (LC_{50}-EC_{50}) and their 95% confidence intervals.

2.3. Hatching phase for acute and long-term mortality methods

Approximately 20 mg of cysts placed into a Petri dish with 12 mL of artificial seawater were exposed to a light source (1000–4000 lux) for 1 h and then incubated in the dark (25 ± 1 °C). After 24-h, under a stereomicroscope and using a cold light source, migrated phototropic larvae were easily transferred with a glass Pasteur pipette to new Petri dishes containing fresh artificial seawater and incubated for additional 24-h at the same conditions, allowing the larvae to grow till the second–third instar stage. Only the hatched larvae were transferred. Cysts or larvae still within the membranes were not transferred. Finally, second–third instar stage larvae were exposed to the reference toxicants and negative controls (12 mL) minimizing their dilution as much as possible during the organism transfer process.

2.4. Acute mortality procedure

The second–third instar stage larvae were transferred to 24 multi-well plates (10 nauplii per well). These plates contained 1 mL of the negative control or the toxicity testing solutions. The plates were placed for 24 h in the dark at 25 ± 1 °C. After mechanical stimulation (*e.g.*, touching the larvae with the tip of a glass Pasteur pipette) and observation for approximately 10 s, mortality was defined operationally as the total absence of movement (*i.e.*, swimming activity or movement of appendices). The procedure is detailed in Guzzella (1997) and Artoxkit (2014). The experimental conditions are specified in Table 1.

2.5. Acute hatching procedure

The hatching test was performed using a commercial brine shrimp hatchery dish (HYCRO-Encia) that allows the nauplii to separate from their shells. The hatchery was completely covered, except for a hole in the center that allowed only light to pass through it. Cysts (1 g) were added to the outermost ring of the hatchery, which contained 750 mL of the negative control or treatment solutions. The newly hatched brine shrimp were attracted to the light and slowly migrated from the outermost ring to the center of the hatchery dish. Before reaching the center, they were forced to navigate through a series of baffles; once in the center, where they aggregated, the nauplii could be easily harvested using a removable small sieve (included in the hatchery dish); the cyst shells and unhatched cysts remained in the outermost ring. At a time point 24 h from hatching in clean artificial seawater and 48 h of exposure to treatments, free-swimming nauplii were harvested on pre-weighed Millipore 45 μm cellulose filters. Filters were dried at 60 °C for 24-h to determine the brine shrimp biomass on a dry weight basis. The hatching test method was described in Migliore et al. (1997) and Canepa (2015).

2.6. Long-term mortality procedure

The second–third instar stage larvae were transferred to 100 mL glass testing chambers (10 nauplii per chamber), containing 50 mL of the artificial seawater and the testing solutions. Test chambers were incubated at 25 ± 1 °C at 1000 lux with a 14:10 h photoperiod. The renewal of SDS treatment solutions and larvae feeding was performed two days after the beginning of the test and then after 5, 7, 9, and 12 days. A stabilized culture of the marine alga *Dunaliella tertiolecta* Butcher ($1.3–2.0 \times 10^6$ cells/mL) was obtained from the Plant Physiology Institute of Gottingen University (Germany) and was supplied to feed the crustaceans using a final algal density of 10^5 cell/mL in each test chamber (for details, see Supplementary materials).

The mortality was recorded as described for the acute tests. Manfra et al. (2012) and UNICHIM (2012) have provided the details of the procedure. The experimental conditions are documented in Table 1.

2.7. Statistical analysis

The acceptability of toxicity tests was considered on the basis of the criteria reported in Table 1 and established in the protocols.

Table 1
A. franciscana toxicity test conditions within the three selected testing methods; TSK = Trimmed Spearman-Karber.

	24-h acute mortality test	48-h acute hatching test	14-d long-term mortality test
Starting larval stage	Nauplii II–III stage	Dry cysts	Nauplii II–III stage
Test type	Static	Static	Renewal
Temperature (°C)	25 ± 1	25 ± 1	25 ± 1
Photoperiod (h of light per day) and light intensity (lux)	0	24 (3000)	14 (1000)
Salinity (PSU)	35 ± 1	35 ± 1	35 ± 1
Oxygen saturation (%)	>60	>60	>60
Dilution water	Artificial seawater	Artificial seawater	Artificial seawater
Testing volume (mL)	1	750	50
No. of exposure concentrations	4	4	5
Testing vessel	24-well plates	Hatchery dish	Glass beaker
No. of exposed larvae	10	≈200,000	10
No. of replicates per treatment	3	3	3
No. of testing repetitions	5 for intra-lab. tests 3 for inter-lab. tests	5 for intra-lab. tests 1 for inter-lab. tests	5 for intra-lab. tests 3 for inter-lab. tests
Endpoint	Mortality	Hatching rate inhibition	Mortality
Statistical analysis	LC_{50} TSK method	EC_{50} regression analysis	LC_{50} TSK method
Test acceptability criteria	Control mortality ≤10%	Hatched nauplii weight ≥60 mg	Control mortality ≤15%

Table 2
Intralaboratory variability of the acute mortality test, the acute hatching test and the long-term mortality test with *A. franciscana*.

No. test repetitions	Exposure time	Toxicant	Mean E(L)C_{50} (mg/L)	SD	CV (%)	*r*
5	24-h	$CuSO_4{\cdot}5H_2O$	6.60	1.06	16.11	2.97
5	48-h	$CuSO_4{\cdot}5H_2O$	4.07	0.53	13.13	1.48
5	14-d	SDS	8.03	1.11	13.88	3.11

EC_{50} = effective concentration in relationship to 50% of the population tested; SD = standard deviation; CV = coefficient of variation; *r* = repeatability value.

The LC_{50} or EC_{50} values of the acute/long-term mortality test and the hatching tests were calculated using the Trimmed Spearman–Karber method (Finney, 1978) and a nonlinear regression model (four-parameter logit model) (ISO/TS, 2006), respectively. The Benchmark Dose Software 2.4.0 (USEPA, 2012) calculated LC_{50}/EC_{50} values by selecting a benchmark response equal to 50% of the control hatching rate.

The coefficient of variation (CV), calculated as the ratio between the standard deviation (SD) and the overall mean, was considered acceptable if it was between 14 and 30% for intra-laboratory exercises (USEPA, 2000) and ≤50% for interlaboratory variability (ISO 6341, 1996).

The repeatability factor (*r*) was defined as the 95% confidence limit for the absolute value of the difference between two measurements in the same laboratory. The reproducibility factor (*R*) was considered to be the 95% confidence limit for the absolute value of the difference between two measurements, not necessarily produced by the same laboratory. The difference between two results obtained in the same laboratory or from different laboratories can be considered significant if greater than *r* or *R*, respectively (European Commission, 2000/60/EC; Mandel, 1991). The *Z*-score index, used for the identification of potential outliers, was calculated according to the formula Z score = $(X - m)$/SD, where X is the median of the replicate results of each laboratory and m and SD are the median and standard deviation, respectively, of replicated results of all laboratories ($|z| \leq 2$, acceptable; $2 < |z| \leq 3$, questionable; and $|z| > 3$, unsatisfactory) (Thompson et al., 2006). The Cochran test is a homogeneity test based on variance outliers that verifies the precision of each laboratory by calculating the value of the statistic $C = \frac{s^2_{MAX}}{\sum_{i=1}^{2} s_i^2}$, where s^2_{MAX} is the greatest laboratory variance and $\sum_{i=1}^{2} s_i^2$ is the sum of all variances (Hibbert, 2007).

3. Results

3.1. Acute mortality method

All LC_{50} values generated by participants were considered valid because the mortalities of control tests were <10% (Guzzella, 1997; Artoxkit, 2014). The values of mean LC_{50}, standard deviation (SD), coefficient of variation (CV) and repeatability (*r*) of the reference laboratory are reported in Table 2. The CV value and the differences between results obtained in two different repetitions (not greater than *r* value) indicated acceptable intra-laboratory test–reliability. In Fig. 1A, the mean LC_{50} value (calculated from three testing repetitions) for each of the eleven laboratories was reported. The LC_{50} values ranged between 5.63 and 23.31 mg/L indicating a good precision (CV < 40%) for all participants except for Lab 4. Lab 4 generated a CV = 51% and did not prove accurate on the basis of a Cochran test ($\alpha = 0.05$). All average LC_{50} values fell in the interval of the overall mean ± SD except for Lab 2 and Lab 9. Applying the *Z*-score index, only Lab 2 did not obtain satisfactory values (α level = 0.05) (Fig. 2A). In Table 3, average LC_{50}, SD, CV and reproducibility (*R*) values were reported showing explicitly the role of Lab 2 data. CV values are <40%, also including Lab 2. Excluding Lab 2, the differences between the results obtained in two different repetitions were not greater than the *R*-value.

3.2. Acute hatching method

The weight of the hatched nauplii in the control batches complied with the acceptability criteria (hatched nauplii weight ≥60 mg) (Migliore et al., 1997; Canepa, 2015). Therefore, the results from all laboratories were considered valid. The intralaboratory variability (within the reference lab) showed CV = 13.13% and *r* = 1.48%. As each laboratory conducted only one test, the CV and the Cochran test were applied on the average weight of hatched larvae obtained from each replicated weight. All seven participants showed a CV < 50% except for Lab 6 (CV = 82%), which also proved imprecise based on a Cochran test (α level = 0.05). The EC_{50} values ranged between 2.21 and 11.60 mg/L; the Lab 6 value was not among the mean range values (Fig. 1B). The *Z*-score index found that only Lab 6 was an outlier (Fig. 2B). The parameters for reproducibility were also satisfied without the Lab 6 data (Table 3).

3.3. Long-term mortality method

The negative control mortality was always <15%, as required by the protocol (Manfra et al., 2012; UNICHIM, 2012). The intralaboratory variability results are shown in Table 2 and represent reliable values. The LC_{50} values ranged between 3.24 and 15.39 mg/L (Fig. 1C). A Cochran test recorded lower precision for Lab 9 (CV = 31%) compared with the others (CV < 30%). All average LC_{50} values fell within the overall mean ± SD except for Lab 4, Lab 6 and Lab 8. All *Z*-score values were in the range −2 and +2, suggesting that the participating members obtained acceptable results except for Lab 8 (Fig. 2C). The CVs were <40% with or without Lab 8, but the differences between the participating member results were considered significant only if the Lab 8 values were excluded (Table 3).

Table 3
Interlaboratory variability of the acute mortality test, the acute hatching test and the long-term mortality test with *A. franciscana*.

No. labs used	Exposure time	Toxicant	No. results used	Mean E(L)C_{50} (mg/L)	SD	CV (%)	*R*
11[a]; 10	24-h	$CuSO_4$ $5H_2O$	33[a]; 30	11.66; 10.71	4.32; 3.13	37.03; 29.23	12.10; 8.76
7[b]; 6	48-h	$CuSO_4$ $5H_2O$	7[b]; 6	5.90; 4.95	3.25; 2.26	55.18; 45.75	9.10; 6.33
9[c]; 8	14-d	SDS	27[c]; 24	8.49; 8.50	2.39; 3.34	28.15; 39.26	6.69; 9.35

EC_{50} = effective concentration in relationship to 50% of the population tested; SD = standard deviation; CV = coefficient of variation; *R* = reproducibility value.
[a] Including Lab 2.
[b] Including Lab 6.
[c] Including Lab 8.

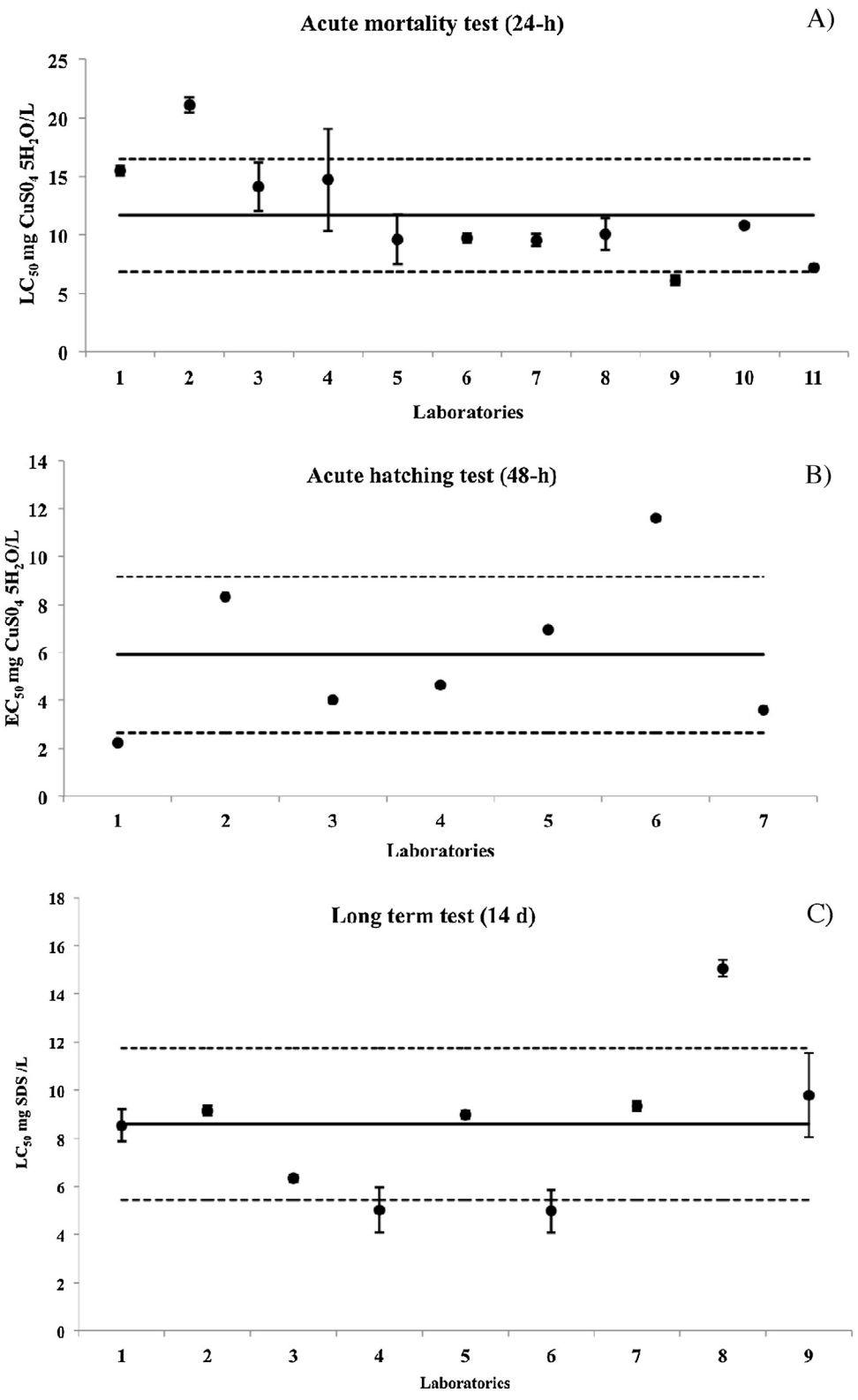

Fig. 1. Mean L/EC_{50} values (mg/L) of participating laboratories (including the reference laboratory) for the 24-h acute mortality test (A), 48-h acute hatching test (B) and 14-d long-term mortality test (C) with *A. franciscana*. The error bars correspond to the standard error, and the horizontal dotted lines indicate the range given by the overall mean ± standard deviation (SD).

4. Discussion

The intercomparison exercises with *A. franciscana* involved eleven, seven and nine participants for the acute mortality test, the acute hatching test and the long-term mortality test, respectively. Most of the participants in the intercalibration activities already knew the acute mortality test protocol. This method did not require specific technical expertise but simply required that the operator pay attention when transferring the hatched larvae from the Petri dish to the multi-well plate. The hatching test is feasible, but some

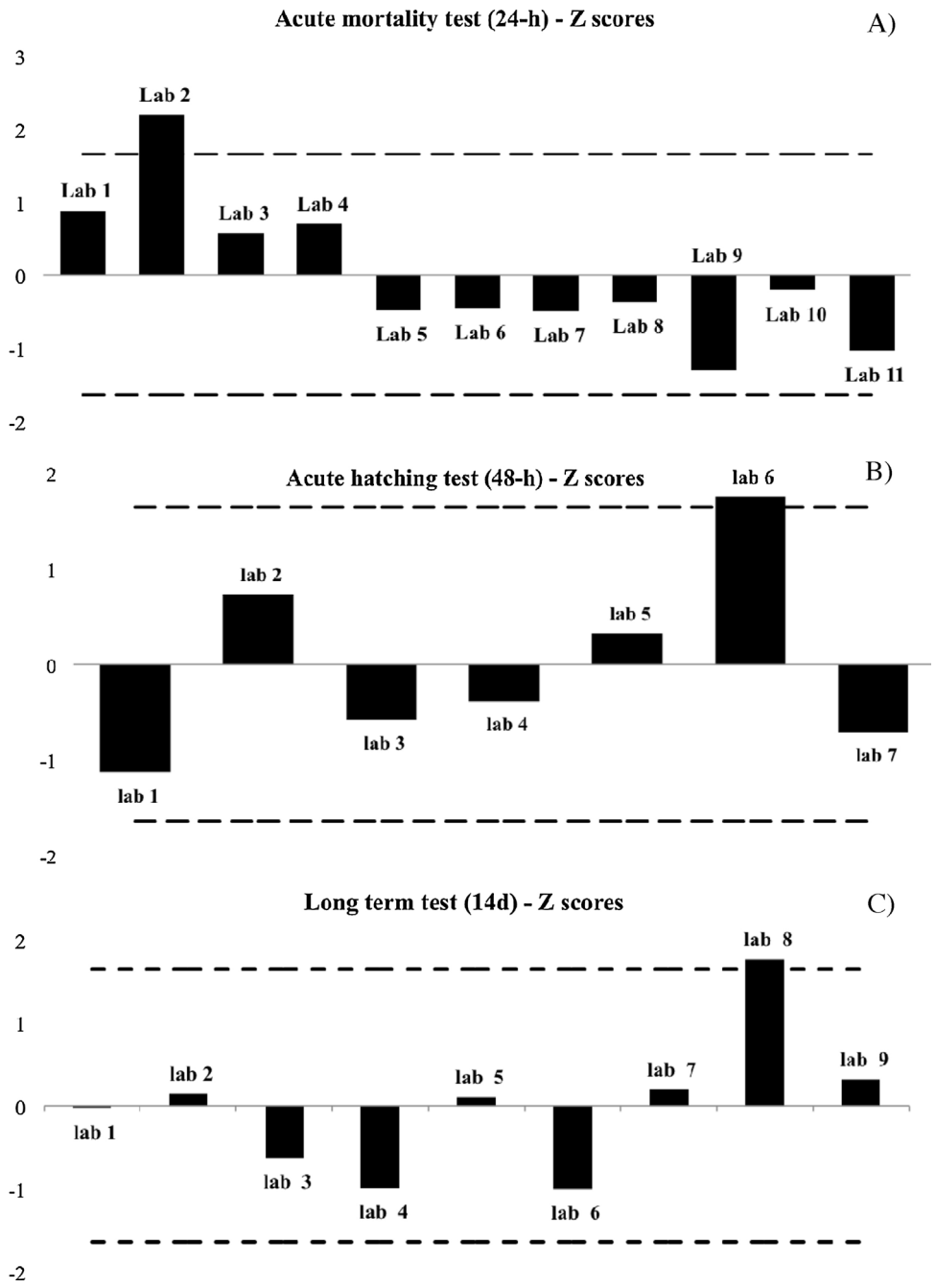

Fig. 2. *Z*-score and confidence limits ($\alpha = 0.05$, dashed lines) of participating laboratories (including the reference laboratory) for the 24-h acute mortality test (A), 48-h acute hatching test (B), and 14-d long-term mortality test (C) with *A. franciscana*.

attention was required to harvesting the free nauplii aggregated at the center of the hatchery. The 14-d assay posed some problems: participants experienced some difficulties in microalgae culturing, in manipulating larvae during the renewal of testing solutions and in feeding the organisms. To overcome these difficulties, a short video presenting an experiment and visually explaining how to manage this procedure was published (Manfra et al., 2012).

The comparison between the acute mortality test and the hatching test at $CuSO_4{\cdot}5H_2O$ exposures showed that the hatching test was the most sensitive assay. Canepa (2015) applied all the methods proposed in this paper to SDS and diethylene glycol (DEG), showing that the hatching test was more sensitive than the acute mortality test and less sensitive than the long-term assay (*i.e.*, EC_{50} values approximately one-half of the acute test values, and approximately twice those of the long-term test).

In addition, the mean EC_{50} values from the hatching test for $CuSO_4{\cdot}5H_2O$ (5 mg/L, this study), SDS (14 mg/L) and DEG (50 g/L) (Canepa, 2015) were comparable to those of other species belonging to different trophic levels: fish (17 mg $CuSO_4{\cdot}5H_2O$/L) (Savorelli et al., *personal communication*); algae, crustaceans, urchins, fish and bacteria (3–7 mg SDS/L) (Mariani et al., 2006); bacteria, algae, rotifers and fish (40–60 g DEG/L) (Tornambè et al., 2012).

The hatching assay, like a short-term test, is able to yield toxicity data with a short time lag (48-h) and, like a long-term mortality test, provides a highly sensitive response incorporating several larval stages.

5. Conclusions

Intercalibration exercises are highly essential steps in standardization processes. This study focused on a series of round-robins to validate and standardize the acute mortality test, the acute hatching test and the long-term mortality test. The outcomes from the inter-laboratory exercises suggested a good repeatability and reproducibility of all protocols. This favorable result was demonstrated by intralaboratory coefficients of variation <30% and interlaboratory coefficients of variation <50% for all toxicity tests. These outcomes can encourage and stimulate the standardization of the proposed methods and their use as regulatory procedures. Since 2010, the long-term mortality assay has been officially implemented by UNICHIM along with other acute toxicity tests on crustaceans (*i.e., A. tonsa, B. amphitrite, Corophium orientale* and *Tigriopus fulvus*). Hopefully, the short-term protocols on *Artemia* (24-h mortality test and 48-h hatching test) may follow the same process of recognition for the harmonization and standardization of these commonly used procedures.

The results of this paper serve to increase the breadth, depth and availability of background information on *A. franciscana* as a target biological model in ecotoxicology from the perspective of the standardization of methods at the international level.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ecolind.2015.04.021

References

Artoxkit®, 2014. http://www.microbiotests.be/toxkits/artoxkit.pdf (MicroBioTest, 28.04.14).

Brix, K.V., Cardwell, R.D., Adams, W.J., 2003. Chronic toxicity of arsenic to the Great Salt Lake brine shrimp, *Artemia franciscana*. Ecotoxicol. Environ. Saf. 54, 169–175.

Brix, K.V., Deforest, D.K., Cardwell, R.D., Adams, W.J., 2004. Derivation of a chronic site-specific water quality standard for selenium in the Great Salt Lake, Utah, USA. Environ. Toxicol. Chem. 23, 606–612.

Canepa, S., (PhD Dissertation in Environmental Sciences) 2015. Application of Innovative Methods to Evaluate the Toxicity of a Compound of Environmental Interest: Diethylene Glicol (DEG). University of Genova, pp. 202.

Chapman, P.M., 2007. Determining when contamination is pollution – weight of evidence determinations for sediments and effluents. Environ. Int. 33, 492–501.

European Commission, 2000. Directive of the European Parliament and of the Council Concerning Establishing a Framework for Community Action in the Field of Water Policy (2000/60/EC), 23 October 2000.

Finney, D.J., 1978. Statistical Methods in Biological Assay, 3rd ed. Griffin, London, pp. 394–401.

Gambardella, C., Mesaric, T., Milivojevic, T., Sepcic, K., Gallus, L., Carbone, S., Ferrando, S., Faimali, M., 2014. Effects of selected metal oxide nanoparticles on *Artemia salina* larvae: evaluation of mortality and behavioural and biochemical responses. Environ. Monit. Assessess. 186, 4249–4259.

Garaventa, F., Gambardella, C., Di Fino, A., Pittore, M., Faimali, M., 2010. Swimming speed alteration of *Artemia* sp. and *Brachionus plicatilis* as a sub-lethal behavioural end-point for ecotoxicological surveys. Ecotoxicology 19, 512–519.

Gorbi, G., Invidia, M., Savorelli, F., Faraponova, O., Giacco, E., Cigar, M., Buttino, I., Leoni, T., Prato, E., Lacchetti, I., Sei, S., 2012. Standardized methods for acute and semi-chronic toxicity tests with the copepod *Acartia tonsa*. Environ. Toxicol. Chem. 31, 2023–2028.

Guzzella, L., 1997. Saggio di tossicità acuta con *Artemia* spp (Toxicity test on *Artemia* spp.). Biol. Ambient. 1, 4–9.

Hibbert, D.B., 2007. Systematic errors in analytical measurement results. J. Chromatogr. 1158 (1–2), 25–32.

ISO, 1996. Water Quality Determination of the Inhibition of the Mobility of *Daphnia magna* Straus (Cladocera, Crustacea). ISO 6341, International Standardization Organization, Geneva, Switzerland.

ISO/TS, 2006. Water Quality – Guidance Document on the Statistical Analysis of Ecotoxicity Data. Ref. n. ISO/TS 20281., pp. 1–252.

Libralato, G., Losso, C., Volpi Ghirardini, A., 2007. Toxicity of untreated wood leachates towards two saltwater organisms (*Crassostrea gigas* and *Artemia franciscana*). J. Hazard. Mater. 144, 590–593.

Libralato, G., Volpi Ghirardini, A., Avezzù, F., 2010. How toxic is toxic? A proposal for wastewater toxicity hazard assessment. Ecotoxicol. Environ. Saf. 73, 1602–1611.

Libralato, G., 2014. The case of *Artemia* spp. in nanoecotoxicology. Mar. Environ. Res. 101, 38–43.

Mandel, J., 1991. Evaluation and Control of Measurements. Marcel Dekker, New York, USA.

Manfra, L., Savorelli, F., Pisapia, M., Magaletti, E., Cicero, A.M., 2012. Long term mortality lethal toxicity test with the crustacean *Artemia franciscana*. J. Vis. Exp. 62, 2182–2185, http://dx.doi.org/10.3791/3790

Mariani, L., Pascale, D., Faraponova, O., Tornambè, A., Sarni, A., Giuliani, S., Ruggiero, G., Onorati, F., Magaletti, E., 2006. The use of a test battery in marine ecotoxicology: the acute toxicity of sodium dodecyl sulphate. Environ. Toxicol. 21, 373–379.

Migliore, L., Civitareale, C., Brambilla, G., Dojmi di Delupis, G., 1997. Toxicity of several important antibiotics to *Artemia*. Water Res. 31, 1801–1806.

Nunes, B.S., Carvalho, F.D., Guilhermino, L.M., Van Stappen, G., 2006. Use of the genus *Artemia* in ecotoxicity testing. Environ. Pollut. 144, 453–462.

Persoone, G., Blaise, Chr., Snell, T., Janssen, C., van Steertegem, M., 1993. Cyst-based toxicity test: II-Report on an international intercalibration exercise with three cost-effective Toxkits. Angew. Zool. 1, 17–34.

Piazza, V., Ferioli, A., Giacco, E., Melchiorre, N., Valenti, A., Del Prete, F., Biandolino, F., Dentone, L., Frisenda, P., Faimali, M., 2012. A standardization of *Amphibalanus (Balanus) amphitrite* (Crustacea, Cirripedia) larval bioassay for ecotoxicological studies. Ecotoxicol. Environ. Saf. 79, 134–138.

Sarabia, R., Del Ramo, J., Varó, I., et al., 2008. Sublethal zinc exposure has a detrimental effect on reproductive performance but not on the cyst hatching success of *Artemia parthenogenetica*. Sci. Total Environ. 398, 48–52.

Savorelli, F., Palazzi, D., Gorbi, G., Invidia, M., Sei, S., Magaletti, E., Manfra, L., Gelli, F., 2007. Set up of a standard methodology for 14-day bioassay on *Artemia franciscana* and *A. parthenogenetica*. Biol. Ambient. 21, 27–36.

Thompson, M., Ellison, S.L.R., Wood, R., 2006. The international harmonized protocol for proficiency testing of analytical chemistry laboratories (IUPAC Technical Report). Pure Appl. Chem. 78, 145–196.

Tornambè, A., Manfra, L., Mariani, L., Faraponova, O., Onorati, F., Savorelli, F., Cicero, A.M., Virno Lamberti, C., Magaletti, E., 2012. Toxicity evaluation of diethylene glycol and its combined effects with produced waters of off-shore gas platforms in the Adriatic Sea (Italy): bioassays with marine/estuarine species. Mar. Environ. Res. 77, 141–149.

UNICHIM MU, 2012. 2244 – 2012, Qualità dell'acqua – Determinazione della tossicità letale a 14 giorni con il crostaceo marino anostraco *Artemia franciscana* (Determination of the Lethal Toxicity at 14 days with the Marine Anostracan *Artemia franciscana*).

USEPA, 2000. Understanding and accounting for method variability in whole effluent toxicity applications under the national pollutant discharge elimination system program. In: Denton, D.L., Fox, J., Fulk, F.A., Greenwald, K., Narvaez, M., Norberg-King, T.J., Phillips, L. (Eds.), EPA/833/R-00-003. Office of Water, Washington, DC.

USEPA, 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, Fifth edition, EPA-821-R-02-012, pp. 266.

USEPA, 2012. Benchmark Dose Software (BMDS), http://www.epa.gov/ncea/bmds