Video Article

Long-term Lethal Toxicity Test with the Crustacean *Artemia franciscana*

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Abstract

Our research activities target the use of biological methods for the evaluation of environmental quality, with particular reference to saltwater/ brackish water and sediment. The choice of biological indicators must be based on reliable scientific knowledge and, possibly, on the availability of standardized procedures. In this article, we present a standardized protocol that used the marine crustacean *Artemia* to evaluate the toxicity of chemicals and/or of marine environmental matrices. Scientists propose that the brine shrimp (*Artemia*) is a suitable candidate for the development of a standard bioassay for worldwide utilization. A number of papers have been published on the toxic effects of various chemicals and toxicants on brine shrimp (*Artemia*). The major advantage of this crustacean for toxicity studies is the overall availability of the dry cysts; these can be immediately used in testing and difficult cultivation is not demanded[1,2]. Cyst-based toxicity assays are cheap, continuously available, simple and reliable and are thus an important answer to routine needs of toxicity screening, for industrial monitoring requirements or for regulatory purposes[3]. The proposed method involves the mortality as an endpoint. The numbers of survivors were counted and percentage of deaths were calculated. Larvae were considered dead if they did not exhibit any internal or external movement during several seconds of observation[4]. This procedure was standardized testing a reference substance (Sodium Dodecyl Sulfate); some results are reported in this work. This article accompanies a video that describes the performance of procedural toxicity testing, showing all the steps related to the protocol.

Video Link

The video component of this article can be found at https://www.jove.com/video/3790/

Protocol

1. Method Standard

Our testing involves exposing *Artemia* larvae to an interval of substance concentrations or a test sample for the purpose of determining the concentration or dilution levels that would cause the death of 50% of the organisms (LC_{50}) exposed over 14 days and meeting the conditions defined by this method. If necessary and possible, the following may also be determined: a) the concentration level that causes the death of 20 % of organisms exposed (LC_{20}); b) the highest analyzed concentration level that does not determine a higher mortality rate than that of the negative control (NOEC); the lowest tested concentration level that determines, after 14 days, a higher mortality rate than that of the negative control (LOEC).

2. Testing Materials

2.1 *Artemia* eggs

To perform testing, *Artemia franciscana* larvae of less than 48 hours old (nauplii) are required, at stage II or III, obtained from newly hatched eggs[5]. Certified eggs or cysts utilized to support testing are commercially available, e.g. from the Quality Assurance Research Division, U.S. Environmental Protection Agency, Cincinnati OH 45268, U.S.A. or from the Laboratory for Biological Research in Aquatic Pollution, University of Ghent, Belgium.

2.2 Water dilution

Synthetic saltwater may be used as dilution water, prepared by dissolving recognized analytical quality reagents or a commercially available formulation in distilled or de-ionized water. Salt blends for creating the ideal saltwater are commercially available, e.g. the *Instant Ocean* mixtures. It is recommended a dilution water with a salinity equal to 35 (±2) PSU as well as a dissolved oxygen concentration of more than 80% of the value of saturation and a temperature of 25 (±2) °C. After aeration for 48 hours and filtration in filters with a porosity of at least 0.45 µm, the dilution may be stored for a maximum of 30 days in the dark at a temperature of 0-4 °C.

2.3 Algal culture

It is recommended that test organisms are feed on *Dunaliella tertiolecta* microalgae, which grow exponentially and reach densities of $1.3x10^6$ - $2.0x10^6$ cells/ml. Testing on environmental samples requires cultures with densities higher than $2.0x10^6$ cells/ml[6]. It is suggested that *Dunaliella tertiolecta* grows in a culture media containing seawater (30 (±2) PSU and 20 (±1) °C) with addition of essential nutrients, minerals and vitamins. Some indications on the algal media composition and preparation are reported in the Appendix. The initial cell density is suggested to be approximately 10^5 cells/ml. The algal culture is maintained in a room thermostat at 20 (± 1) °C, illuminated with fluorescent lamps 3000 (± 300) lx, with a photoperiod ratio of 16 hours of light to 8 hours of darkness.

Other phytoplankton species could also be used for feeding but it would be necessary to make comparative studies with these phytoplankton species to find the "equivalent" species to *Dunaliella tertiolecta*, because the experimental conditions (e.g. initial cell densities, light illumination, temperature, composition of culture media and exposure time) varies among phytoplankton species. Besides the tolerance of the microalgae to different pollutants needs to be considered because the pollutant effects on the growth and development of phytoplankton impact also to the *Artemia*.

2.4 Reference substance

The reference substance in this test is Sodium Dodecyl Sulfate.

2.5 Laboratory glassware

In addition to the usual laboratory equipment, the following material is needed:

1. Testing containers including 100 ml borosilicate glass beakers (18 to perform testing and 18 for transferring solutions).
2. A flask (e.g. 500 ml flask) to prepare the standard solution.
3. Six flasks (e.g. 200 ml flasks) to prepare the testing solutions.
4. Glass or polystyrene 5 cm diameter Petri dishes with removable covers to be used for activating the cysts and transferring *Artemia* from the hatching dish to the testing containers.
5. Glass Pasteur pipettes (with tips rounded by flame) for transferring the nauplii.
6. Glass cannulae or Pasteur 3 ml disposable plastic pipettes (to be cut) for transferring *Artemia*.
7. Micropipettes and graduated pipettes to prepare the testing solutions.
8. Six graduated cylinders (e.g. 50 ml cylinders) for transferring testing solutions from the flasks to the testing containers.

3. Cyst Activation

3.1. Petri dishes containing cysts + pouring water

To generate test organisms, 20 mg of cysts are placed into a Petri dish containing 12 ml of dilution water 48 hours before testing.

3.2. Incubation in the light and in the dark

The Petri dish is maintained at 25 (±2) °C and at a 4000 (±1000) lux (lumens per square meter) light intensity for one hour.

3.3. Replacing the medium using the microscope

After 24 hours, the hatching larvae are transferred into a new Petri dish filled with artificial water. The transfer is performed using the microscope, i.e. using a light source so that the hatching, phototropic larvae migrate towards the light beam. A glass Pasteur pipette is used to make the transfer, ensuring that only the nascent larvae are transferred and not the cysts or the larvae still in membranes.

3.4. Incubation in the dark

The dish containing the larvae is placed in a dark thermostatic chamber for 24 hours at 25 (±2) °C.

4. Preparation of Testing Solutions

4.1. Weighing the substance + transfer to the flask

It is recommended that the standard solution for the testing substance is prepared by dissolving 0.5 g of the substance in a 500 ml flask. The flask is filled up with de-ionized or distilled water and the solution is stirred until the testing substance is completely dissolved. Solutions have to be prepared at the time of use unless it is not known whether the substance is stable in solution. In that case, the standard solution may be prepared up to two days before testing.

4.2. Preparing the negative control, adding dilution water and algae with the 10 ml pipette

The negative control is prepared in a flask (e.g. 200 ml volume) by adding an aliquot of the algal suspension to the dilution water so that a density of 10^5 cells/ml is obtained.

4.3. Preparing the testing solutions: adding dilution water, algae and standard solution

It is suggested that testing solutions are prepared in five 200 ml flasks by adding the standard solution to the dilution water in the quantities specified so that the desired concentrations for testing is obtained. To feed the organisms, aliquots of a *Dunaliella tertiolecta* microalgae suspension may be used and added to reach a density of 10^5 cells/ml when testing solutions are prepared. The following order of addition is

recommended: dilution water, algal suspension and standard solution. After the preparation of testing solutions, they have to be used in the assay as soon as possible.

4.4. Transferring solutions from the flask to the testing containers, then to the Petri dishes

Equal quantities (50 ml) of the testing solutions are introduced into the testing containers utilizing the graduated cylinder. Three replicas for each concentration are made. For each series of tests, a control container is prepared with an amount of dilution water equal to the volume of the test solutions. Equal volumes (about 12 ml) of the test solutions are introduced into the Petri dishes. For each series of tests, a control Petri dish is used.

5. Preparation for Testing

5.1. Nauplii at the II-III stage

Forty-eight hours after cyst activation, the nauplii reach the II-III larval stage and are then usable for testing.

5.2. Nauplii transfer into the Petri dishes using the microscope

The Petri dish used for cyst activation is taken out of the thermostatic chamber. A small quantity of larvae are transferred into the Petri dishes containing the control and the testing solutions. This task is to be performed with the microscope utilizing a pipette of sufficiently wide diameter so that the organisms do not get damaged. In this phase of the test, the transfer may be performed with a glass Pasteur pipette (with tip rounded by flame) and a laterally positioned light source that encourages the *Artemia* to aggregate.

5.3. Nauplii transfer from the Petri dishes into the test containers with the microscope

Ten larvae are transferred from the testing solutions Petri dishes to the testing containers. Also this operation has to be performed by utilizing the Pasteur pipette and microscope. It is recommended that a volume not exceeding 1 mL is transferred during the passage of larvae not to affect the overall volume of test system.

5.4. Full containers covered with parafilm

The testing beakers are covered with parafilm (leaving a gap for air passage), and maintained at a temperature of 25 (±2) °C for the whole duration of the test and at an illumination of 900 (±100) lx with a photoperiod ratio of 14 hours of light to 10 hours of darkness.

6. Replacing the Medium and Food Supplement

6.1. Survival rate control

Two days after the test has started, and then after 5, 7, 9, and 12 days, the *Artemia* is observed under the microscope to verify the survival rate and to replace the medium and the food supplement.

Number of live organisms is counted in each test container. After observation under the microscope and slight mechanical stimulation (e.g. touching the larvae with a glass Pasteur pipette) organisms that do not show some movement for about 10 seconds should be considered dead.

6.2. Preparation of a test solution showing algae sampling with a 10 ml pipette and sampling of the substance to be tested with a micropipette. Transferring solutions from the flask to the test containers

While testing, the test solutions must be periodically replaced, and prepared in the same day as they are to be used. Test solutions are made from the standard solution previously prepared.

6.3. *Artemia* transfer, utilizing a cut Pasteur pipette, from the old test containers to the three new containers

The transfer of *Artemia* is performed by using a pipette with a sufficiently wide diameter so as not to damage the organisms. During this phase, a 3 ml plastic Pasteur pipette (to be cut) may be used.

6.4. Larvae in a beaker containing either live or dead *Artemia*

After 14 days of exposure at the end of testing, the number of surviving larvae is counted and recorded on the worksheet. LC_{50} and/or LC_{20}, NOEC and LOEC at 14 days, are calculated and recorded, as well as confidence limits at 95 % where appropriate, and calculation methods.

7. Representative Results

At the end of the test (14 days), calculate the percentage of mortality at each concentration, according to the following formula:

$(M_s/N) * 100$

Where:

M_s is average number of died individuals in the sample analysed

N is total number of exposed individuals

Table 1 reports an example of data of a test (14d) with *Artemia* exposed to Sodium Dodecyl Sulfate.

Concentrations (mg/l)	Number of died individuals in each replica			Mortality (%)
	I	II	III	
Negative control	0	2	1	10
1.56	1	2	0	10
3.12	2	0	2	13
6.25	3	2	3	27
12.5	8	9	10	90
25.0	10	10	10	100
50.0	10	10	10	100

Moreover, determine the LC_{50} value by a graphical Gaussian logarithmic plot or using appropriate statistical methods (for example Spearman-Karber or Probit Method). The calculation of LC_{50} for the data of table 1 is 8.18 (7.15-9.37) mg/l. If the maximum concentration tested caused a lower mortality rate of 50%, you should not proceed to the calculation of the LC_{50} value which would then be unreliable or even non-computable. In this case, however, would be appropriate to repeat the test by extending the range of concentrations tested. Alternatively, the LC_{50} value is more correctly expressed as greater than the highest concentration tested, possibly indicating the percentage of mortality at the highest concentration tested and the highest concentration corresponding to a no effect.

Results are considered valid if at the end of testing the following conditions have been met:

a. the control's average mortality rate is ≤ 20 %;
b. where using Sodium Dodecyl Sulfate, the LC_{50} at 14 days is included in the 8.0 (±5) mg/l interval.

If the above conditions have not been met, all data obtained with the same batch of organisms should be considered invalid and testing repeated.

Discussion

Artemia is one of the most valuable test organisms available for ecotoxicity testing and research done so far allows us to state that it is possible to sustain several options related to *Artemia* use in Toxicology and Ecotoxicology. The characteristics that turn this organism into a suitable species for bioassays are: a wide geographic distribution, high adaptability to adverse environmental conditions and varied nutrients, relatively simple laboratory culture and maintenance, resistance to manipulation, short life-cycle, large offspring production and the existence of a considerable amount of information about some species. A criticism against the use of *Artemia* is referred to its sensitivity to chemical exposures: earlier studies refer *Artemia* (test to 24 hours exposure) as a less sensitive species for ecotoxicity studies, when compared to other test organisms under the same experimental conditions[3]. The choice of method for ecotoxicity testing is crucial in this respect. We wanted to standardize a method that had the same starting conditions (nauplii as a test organism, obtained from the use of cysts) of the protocol to 24 hours of exposure[4], but with relatively long exposure times (14 days) could provide a more sensitive response. This protocol certainly allows a more sensitive response than the acute tests but it cannot be used as a substitute for a chronic test because a exposure of 14 days is not relevant if we consider the estimate lifespan of *Artemia*. The endpoint selection for this method has been widely discussed. Initially both mortality and growth (i.e. carapace length after 14-day exposure) were selected, because we also wanted to ensure the observation of a sublethal effect for a long-term test. However, the sublethal endpoint was found to be less sensitive compared to mortality[6]. For this reason mortality is the only endpoint mentioned in the description of the protocol and in the video. This finding is consistent with studies of other researchers[7,8] who observed that survival is the most sensitive endpoint among those considered by them (survival, growth and reproduction).

The proposed method is useful for evaluating the toxicity of chemicals, effluents and environmental matrices[9]. In the feature it might be interesting to test other sub-lethal chronic toxic effects, such as biomarkers (i.e. enzymatic activity)[10].

Disclosures

No conflicts of interest declared.

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