Designation: E 1203 – 98 (Reapproved 2004)

Standard Practice for Using Brine Shrimp Nauplii as Food for Test Animals in Aquatic Toxicology[1]

This standard is issued under the fixed designation E 1203; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This practice describes procedures for hatching, harvesting, and testing the acceptability of brine shrimp nauplii for use as a food for certain fish and invertebrate species that are used in aquatic toxicity tests. The term "brine shrimp" refers to all species in the genus *Artemia* **(1)**[2] although this practice specifically deals only with those species for which cysts (encysted embryos) are commercially available.

1.2 These procedures are applicable to all brine shrimp nauplii that are obtained by incubating commercially available cysts. With appropriate processing, cysts collected by noncommercial harvesters can be subjected to these same procedures.

1.3 Modification of these procedures might be justified by special needs or circumstances.

1.4 This practice is organized as follows:

1.5 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* Specific hazard statements are given in Section 6.

2. Referenced Documents

2.1 *ASTM Standards:* [3]

E 1191 Guide for Conducting Life-Cycle Toxicity Tests with Saltwater Mysids

E 1241 Guide for Conducting Early Life-Stage Toxicity Tests with Fishes

3. Terminology

3.1 The words "must," "should," "may," "can," and "might" have very specific meanings in this practice. "Must" is used to express an absolute requirement, that is, to state that the test ought to be designed to satisfy the specified condition, unless the purpose of the test requires a different design. "Must" is only used in connection with factors that directly relate to the acceptability of the test. "Should" is used to state that the specified condition is recommended and ought to be met if possible. Although violation of one "should" is rarely a serious matter, violation of several will often render the results questionable. Terms such as "is desirable," "is often desirable," "might be desirable" are used in connection with less important factors. "May" is used to mean "is (are) allowed to," "can" is used to mean "is (are) able to," and "might" is used to mean "could possibly." Thus the classic distinction between "may" and "can" is preserved, and "might" is never used as a synonym for either "may" or "can."

3.2 *Descriptions of Terms Specific to This Standard:*

3.2.1 *brine shrimp cyst*—a gastrula-stage embryo that is enclosed in an envelope and cuticle for resistance to desiccation. Dried brine shrimp cysts are often incorrectly referred to as eggs. Upon hydration, embryonic development proceeds until a nauplius emerges from the encysting shell.

3.2.2 *brine shrimp nauplius*—a newly hatched, freely swimming, instar I stage larva. Nauplii are incapable of exogenous feeding until the instar II stage that occurs approximately 24 h after hatch at 25°C. The reddish-brown color of the nauplii is due to the presence of yolk on which they rely for endogenous food.

[1] This practice is under the jurisdiction of ASTM Committee E47 on Biological Effects and Environmental Fate and is the direct responsibility of Subcommittee E47.01 on Aquatic Assessment and Toxicology.

Current edition approved April 1, 2004. Published April 2004. Originally approved in 1987. Last previous edition approved in 1998 as E 1203 – 98.

[2] The boldface numbers in parentheses refer to the list of references at the end of this practice.

[3] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

3.2.3 *feeding assay*—a test in which one or more life stages of an aquatic animal species is raised on a diet to determine the adequacy of the diet for the life stage(s) of the species.

3.2.4 *Reference Artemia cysts*—a homogeneous collection of cysts that has been tested and declared a Reference sample. Formerly two repositories of reference cysts existed,[4] but both supplies have been exhausted without replacement.

3.2.5 *strain*—a geographical population of brine shrimp that is genetically distinct from other brine shrimp populations of the same species. Genetic distinction is determined electrophoretically **(2)**.

3.2.6 *time to 90 % hatch*—the amount of time that expires between the immersion of dried brine shrimp cysts in water and the hatching of 90 % of the hatchable cysts. Time to 90 % hatch is usually abbreviated T_{90}.

4. Summary of Practice

4.1 One can of cysts is purchased. Samples are hatched in aerated salt water and nauplii are collected on a small-mesh screen and rinsed. Nauplii are fed to a species of aquatic animal to determine if the nauplii are an acceptable complete food for that life stage of that species. Chemical and physical measurements might also be performed on the cysts or on the nauplii, or on both. If the nauplii are acceptable, a large number of cans from the same lot are purchased, appropriately stored, hatched, and fed to that life stage of that species. An alternative is to feed algae in addition to the brine shrimp to the test organisms.

5. Significance and Use

5.1 In certain toxicity tests during which small aquatic animals must be fed, the food of choice is often live brine shrimp nauplii because of their availability, ease of use, and presumed good nutritional quality. In addition, many test species that are cultured or held in the laboratory must be fed prior to toxicity testing, even if they are not fed during the test.

5.2 Brine shrimp nauplii can be readily hatched in the laboratory from cysts that are obtained naturally from many geographical areas around the world. Cysts from a few of those areas are available commercially, but might represent two or more species of brine shrimp **(1)**.

5.3 Nauplii of different strains of the same brine shrimp species from different geographical areas can differ substantially in their nutritional value, contaminants, and acceptability as a complete food for the life stage of the species to which they are fed **(3-8)**.

5.4 The results of a toxicity test can depend on the brine shrimp nauplii used as food **(9)**. It is desirable to determine prior to use whether a particular batch of brine shrimp is of adequate quality for the test organisms.

5.5 The primary requirements of acceptable brine shrimp are that they are an appropriate size for the test organisms, have adequate nutritional value, and do not contain excessive concentrations of contaminants. Whether a given lot of brine shrimp meets these requirements for a given species can only be determined by a feeding assay.

5.6 Standardization of the hatching, harvesting, testing, and using of brine shrimp will probably increase the reproducibility of results of toxicity tests with some test species by decreasing the use of unhealthy organisms caused by feeding them poor quality brine shrimp.

6. Hazards

6.1 Because water is a good conductor of electricity, use of ground fault systems and leak detectors should be considered to help avoid electrical shocks. Salt water is such a good conductor that protective devices are strongly recommended.

7. Obtaining Cysts

7.1 *Selecting a Source*—Selection of a given lot of cysts for a feeding assay can be based on information concerning the geographical origin of the cysts, the way the cysts were dried and stored, and possibly the size of the cysts and resulting nauplii. However, commercial suppliers might not divulge this information, and information that is provided might not be correct. The preferred processes for drying and storing brine shrimp cysts are, respectively, fluidized bed drying and storage under vacuum or nitrogen **(10)**. Cysts processed and stored in other ways might be acceptable, but hatchability will probably be reduced. Size of nauplii at hatch varies from strain to strain but is relatively constant within a strain **(11)**. Mortality of some larval fish increases with increasing naupliar size **(12)** because some larvae simply cannot ingest the larger nauplii. Nutritional value and levels of contaminants vary both among and within strains, and depend mostly on the quality of the water and the algae in the water from which the cysts were harvested **(13)**. The only way to determine the acceptability of cysts is to perform a feeding assay. Therefore, it is desirable to purchase and test a small sample (for example, one can) of cysts from one lot before purchasing a large quantity.

7.2 *Storage*—Properly sealed cans of cysts can be stored for several years at room temperature, under refrigeration, or frozen. If they are frozen, they should be removed from the freezer at least 48 h, and preferably about 1 week, prior to use to ensure adequate hatching. After a can has been opened, the optimal conditions for long term storage are, of course, lost. If the contents will be used up in 1 to 2 months, it is only necessary to close the can tightly (for example, with a plastic lid) after each use and store it in a cool (about 4°C), dry place, such as in a refrigerator. If the contents will not be used up within 2 months, it is best to distribute the cysts among smaller containers that hold about a 1 to 2 month supply of cysts and seal the containers under nitrogen or vacuum or freeze them. One simple method is to place the cysts in a sealable plastic freezer bag and introduce nitrogen via a pipet inserted through a small hole. After about 1 min, the pipet is removed and the hole is sealed.

8. Hatching Cysts

8.1 Brine shrimp cysts may be hatched in either reusable or disposable containers, such as conical plastic bags. Separatory funnels are probably the most convenient reusable containers,

[4] Artemia Reference Center, State University of Ghent, Ghent, Belgium, or Quality Assurance Research Division, U.S. Environmental Protection Agency, Cincinnati, OH 45268.

because the stopcock allows harvesting of newly hatched nauplii from the bottom of the container. Substances used to clean a reusable container should be nontoxic and the container must be rinsed completely after cleaning so that the brine shrimp are not exposed to substances such as detergents.

8.2 Salt water used for hatching should have a salinity of 25 to 35 g/kg salinity.

8.3 Aeration is necessary for hatching and survival of brine shrimp nauplii. Introduction of air into the bottom of a funnel shaped container provides best continuous circulation of cysts. Containers can be aerated using a glass tube or pipet attached to a flexible tube leading to an air supply. Air used for aeration should be free of fumes, oil, and water; filters to remove oil and water are desirable. Although brine shrimp cysts will hatch under a wide range of temperatures, standard hatching tests are conducted at 25°C **(14)**, and all discussion of hatching in this practice assumes a temperature of 25°C.

8.4 Brine shrimp cysts should be subjected to light intensity of at least 1000 lx for the first 3 h after immersion in salt water, because they need a light trigger to initiate hatching **(15-17)**.

8.5 Brine shrimp cysts should be added to the water in the proportion of 5 cm^3 of cysts/L.

8.6 Cysts can be decapsulated by soaking in water for 2 h, dissolving the chorion with a hypochlorite solution with appropriate control of temperature, soaking in thiosulfate, and rinsing before incubation **(18, 19)**. Although decapsulation disinfects the cysts and increases hatchability and the energy content of the nauplii, it is not recommended because of the labor involved and because of the possibility of contamination by hypochlorite, thiosulfate, and chlorinated materials.

9. Harvesting Nauplii

9.1 Brine shrimp hatch as instar I nauplii, which is a nonfeeding stage. They diminish substantially in weight, caloric value **(20)**, and biochemical composition **(21)** until they begin feeding, and these changes might reduce their value as food for test organisms **(22)**. Therefore it is important to harvest nauplii shortly after they hatch. However, because they do not all hatch at once, they should be harvested about 2 to 6 h after T_{90}, that is, about 2 to 6 h after 90 % have hatched. If T_{90} is not known, it can be determined by periodically sampling the aerated hatching solution and counting the hatched nauplii, unhatched cysts, and empty shells **(10)**. At 25°C, T_{90} for most brine shrimp is between 20 and 32 h (see Table 1) **(14)**. Therefore, brine shrimp should be hatched in a constant temperature room or water bath so T_{90} is as similar as possible from day to day and from season to season. After the feeding schedule for the test organisms has been selected, hatching chambers should be set up at appropriate times so nauplii can be harvested when needed. The setup schedule can either be planned on the basis of the T_{90} of the cysts and temperature that are available or one can try to select cysts and a temperature that fit into a desirable setup and harvesting schedule.

9.2 At the end of the incubation period, aeration is stopped. Hatched nauplii and unhatched cysts will sink to the bottom, whereas empty shells will float to the top. There might also be some heavy debris that sinks very rapidly and can be drawn off immediately, before the nauplii and unhatched cysts reach the bottom. Because nauplii are phototactic, shading the top of the container with black plastic and shining a light on the bottom of the container can hasten the settling of nauplii. To avoid extreme packing of nauplii at the bottom of the container, it is best to harvest nauplii 5 min after removal of aeration, wait 5 min more and harvest again, repeating the procedure as often as necessary. After the nauplii have been harvested, they should be washed onto a 150-µm screen with clean salt water to remove small extraneous matter and substances such as glycerol that are present in the hatching water. If the nauplii are mixed with excessive amounts of debris, phototaxis can again be used for separation, for example, in a separator box **(23)**. Nauplii can also be separated by reimmersing them in clean salt water, shining a light on one side of the container, and collecting nauplii with a siphon or pipet as they move toward the light. In certain circumstances, some brine shrimp emerge from the cysts but do not become freely swimming. These immobile brine shrimp sink to the bottom of any chamber and are unavailable to larval predators in the water column **(24)**. The above separation procedure is therefore especially important to ensure use of only freely swimming nauplii.

TABLE 1 Hatching Characteristics of Geographical Sources of *Artemia* and Reference *Artemia* (14)

NOTE—Times to 90 % hatch (T_{90}) are given in hours at 25°C. Individual naupliar size is expressed as dry weight, as is hatching output (total naupliar biomass produced from 1 g of cysts).

Source	T_{90} at 25°C	Individual nauplius dry weight, µg	Hatching output, mg
San Francisco Bay, California	21	1.63	440
San Pablo Bay, California	20	1.92	544
Great Salt Lake, Utah	22	2.42	257
Macau, Brazil	24	1.74	564
Barotac Nuevo, Philippines	22	1.68	401
Shark Bay, Australia	28	2.47	563
Chaplin Lake, Canada	32	2.04	400
Buenos Aires, Argentina	23	1.72	424
Lavalduc, France	31	3.08	567
Tientsin, Peoples' Republic of China	27	3.09	406
Margherita di Savoia, Italy	26	3.33	463
Reference Artemia	32	1.78	516

10. Testing Nauplii

10.1 *Feeding Assay*:

10.1.1 Before being routinely fed to test organisms, brine shrimp should be tested for their ability to support good survival, growth, and (for life-cycle tests only) reproduction of the test species.

10.1.2 The organisms used to test the quality of a brine shrimp sample should be of the same species, life stage, and geographical origin, as those that are to be used in the toxicity tests.

10.1.3 When this standard was first established, it was based on a comparison of a batch of unknown quality with a reference standard batch of *Artemia* cysts. That original experimental design is provided in 10.1.3.1 for use should a reference standard be re-established. Two additional designs are provided as feasible and useful alternatives in 10.1.3.2 and 10.1.3.3.

10.1.3.1 If a supply of reference cysts is available, the experimental design for the feeding assay requires two treatments: Treatment A contains test organisms that are fed the experimental brine shrimp and Treatment B contains test organisms that are fed a known, good-quality food such as a Reference brine shrimp **(25, 26)**. There should be at least three replicates within each treatment so that statistically significant differences in survival can be distinguished. If the organisms in Treatment A survive, grow, or reproduce significantly less than those in Treatment B, or less than desired, then the experimental brine shrimp nauplii are likely to be unsuitable for use as a food for the test organisms.

10.1.3.2 If a supply of reference cysts is not available, a laboratory may designate a laboratory-specific supply of reference cysts, based on results of feeding assays with one or more species used by that laboratory. The feeding assays determine the biological performance of the test species in one or more of the following categories: survival, growth, reproduction. The performance category(-ies) chosen should match those used in toxicity tests with each test species. The performance criteria are species-specific and should be based on established standards for the life stage(s) being tested (for example, acceptable performance of control organisms in a toxicity test). Some investigators have also used "stress tests" as sensitive indicators of performance in feeding assays **(27)**. The duration of the feeding assay for a species should be at least that of the longest duration toxicity test performed in that laboratory with that species.

10.1.3.3 An alternative to the feeding assay approach to designating a batch of laboratory-specific reference cysts is the conduct of acute toxicity tests with a reference chemical. In this case, one or more species used by the laboratory is (are) fed nauplii from the specific batch and exposed to the chemical. The LC50 derived from the toxicity test is compared with either (*a*) literature values for the acute toxicity, or (*b*) laboratory-specific performance tests, for example, those used in control charts or other performance-based records. If the LC50 falls within an acceptable range, often defined as the upper and lower 95 % confidence limits of the data, then the batch of *Artemia* can be designated as reference cysts.

10.1.3.4 Once an acceptable laboratory-specific reference batch of cysts has been identified, a substantial amount of the tested batch is then stored frozen or in a nitrogen atmosphere in small bags, each sufficient to perform a single reference comparison with new batches of cysts of unknown quality.

10.1.4 The chambers in which the feeding assay is conducted will depend on the test species used. The chambers should be the same as those that will be used in the subsequent toxicity test. Water exchange between chambers containing different treatments should be prohibited so that brine shrimp nauplii fed to one treatment cannot swim into the other treatment. Organisms in each treatment should be fed the same as in the toxicity test. The duration of the experiment should be the same as (*a*) the duration of the chronic toxicity test in which organisms of the test species will be used, or (*b*) the duration of the laboratory culture period for organisms of the generation of that species that are used in acute toxicity tests, depending on the investigator's intended use of the batch of brine shrimp being assayed. The biological data collected from a brine shrimp feeding assay are survival, growth, and (in some cases) reproduction. Recommendations for the collection of these data for early life-stages of fish and for mysids are given in Guide E 1241 and Guide E 1191, respectively.

10.1.5 The results of a brine shrimp feeding assay, when there are only two treatments, can be statistically analyzed using a t-test. If there are more than two treatments, an analysis of variance can be used **(28)**.

10.2 *Chemical and Physical Measurements*:

10.2.1 Although the feeding assay is the primary test of the acceptability of the nauplii, chemical and physical measurements might provide useful information, particularly if it is eventually possible to correlate results of chemical and physical measurements with results of feeding assays.

10.2.2 It might be desirable to determine the diameter of the hydrated cysts or the length of the nauplii, or both, using published methods **(11, 29)** in case the size of the brine shrimp makes them unacceptable to the test species.

10.2.3 The nauplii should be chemically analyzed for pesticides, metals, and fatty acids. Freshwater fish require one essential fatty acid (18:3ω3) whereas saltwater species require others (20:5ω3 or 22:6ω3) in their respective diets **(30-33)**. If the sample cannot be analyzed immediately, it should be rinsed with deionized or distilled water, sealed under nitrogen, and frozen.

10.2.4 Chemical data should be obtained using appropriate ASTM standards whenever possible. For those measurements for which an ASTM standard does not exist or is not sensitive enough, methods should be obtained from other reliable sources **(34)**.

10.3 *Enrichment of Brine Shrimp*—The nutritional value of brine shrimp nauplii can be improved by feeding them on algae or some inert enrichment product **(35-38)**. However, the newly hatched instar I stage is a nonfeeding stage and uptake of enrichment foods does not begin until instar II, approximately 16 to 24 h after hatching. Growth and development of nauplii during these stages means that enriched instar II nauplii are substantially larger and faster swimming and therefore less easily captured by the test organisms. In some cases, an alternative to testing brine shrimp nutritional quality is to feed the test organisms with either *Selenastrum capricornutum* (cultured axenically in revised Bold's medium) or *Skeletonema costatum* (cultured axenically in revised Provasoli's medium) **(39)** in addition to the *Artemia* nauplii, depending on whether the species being fed is freshwater or saltwater, respectively.

11. Using Nauplii

11.1 If the nauplii are of acceptable quality, a large number of cans of the same lot of cysts should be purchased to provide good quality brine shrimp nauplii over an extended period of time. These cysts should be stored, hatched, and harvested as described previously.

11.2 Procedures for feeding the nauplii to test organisms are given in Guides E 1191 and E 1241. It is usually desirable that nauplii be constantly available to the test organisms so they can feed ad libitum. However, in a test, it is also important that the nauplii not be present in the test solution long enough to (*a*) accumulate substantial amounts of the test material, (*b*) suffer

mortality and thereby reduce food availability in a concentration-dependent manner, or (*c*) deplete oxygen levels or increase metabolites in the test chamber. Therefore, during a test, the organisms should be fed small quantities of brine shrimp nauplii two or more times per day, rather than a large quantity only once per day.

12. Report

12.1 Whenever brine shrimp nauplii are used to feed test organisms before or during a toxicity test, the record of the results of the test should contain the following information either directly or by reference to available documents:

12.1.1 The commercial source of the brine shrimp cysts, geographical source, and lot number;

12.1.2 How the cysts were stored and incubated;

12.1.3 How the nauplii were harvested, rinsed, and fed to the test organisms;

12.1.4 Incubation temperature and time to 90 % hatch; and

12.1.5 Results of all feeding assays and chemical and physical measurements made on the nauplii.

12.2 If a batch of cysts is prepared as described in Sections 10.1.3.2 or 10.1.3.3, the data for feeding tests and reference chemical toxicity tests should be included by reference in all reports of test results using cysts compared to that batch. This information is in addition to that required under Section 12.1.

13. Keywords

13.1 aquatic toxicology; *Artemia*; brine shrimp; feeding; nutrition

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