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**Review of *Artemia* *salina* L. hatching methods and lessons for the Nigerian Ecotoxicologist**

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**Abstract**

*Artemia* *salina* also known as the brine shrimp has been used for *in-vivo* toxicological investigations for quite some time and different methods of hatching the cysts have been developed by researchers worldwide. However, the nigerian ecotoxicologist is faced with the challenge of availability of the cyst in small packs, epilleptic power supply and dense population of scientists in the hinterland who may not have access to natural seawater, therefore there is need to develop a kit that can work for nigerian scientists. This review thus highlights some of the existing methods used in hatching the cyst, comprehensive details of the methods were given, prospects and constraints were listed and the design of NigArtoxKit is proposed. Hatching the brine shrimp requires continous aeration and illumination in a saline medium so the design of the kit should consider identification of artemia strain most suitable for nigerian environment with diverse regional weather variations. Alternative medium to seawater must be explored while source of power for continous aeration and illumination can be incorporated into the design.

**Key words:** Brine shrimp, *in-vivo*, toxicological investigations, cyst

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**Bioassay**

 Bioassay is a term used properly only to describe the use of a living organism to quantitate the amount of a particular toxicant present. It is frequently used to describe any *in vivo* toxicity test (Hodgson, 2004). In addition, it signifies a test in which a living tissue, organism or group of organisms is used as a reagent for the determination of the potency of any physiologically active substance of unknown activity (FAO, 1977). Bioassays are carried out with the assumption that the test organisms are surrogates for the large body of organisms comprising natural environments (Cairns and Pratt, 1989).

 Laboratory mice have been used in several bioassays (toxicological tests) taking into account such factors as age, sex, weight, species, diet and environmental conditions (Cáceres, 1996). Presently, there is a tendency to limit the use of laboratory animals in toxicological tests (Yajes, 1997). The alternative methods include the 3Rs procedures that could replace
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experiments carried out with animals, reduce the number of animals used in every test, or refine the existing methodology in order to reduce pain and stress on the animals (Johnston and Rusche, 1997; Yajes, 1997). This has lead many researchers to investigate the use of single cell organisms in toxicity tests known not to form significant aggregates with established culture methods. Some of them include: freshwater green algae species (*Ankistrodesmus falcatus*, *Chlorella vulgaris*, *Monoraphidium arcuatum*, *Scenedesmus quadricauda*, *Scenedesmus subspicatus*, *Pseudokirchneriella subspicata* (previously *Selenastrum capricornutum*), marine green algae (*Dunaliella tertiolecta*), diatoms (*Phaeodactylum tricornutum, Cyclotella* sp*, Synedra* sp, *Nitzschia closterium, Skeletonema costatum, Thalassiosira pseudonana*) (Reish and Oshida,1986; Lewis, 1993; Nasha *et al*., 2005) Zooplanktons (*Daphnia magna, Acartla tonsa, Tisbe sp. Tigriopus sp. Acanthomcysis sp, Metamysidopsis sp. and Mysidopesis bahia*) (Reish and Oshida,1986) and Brine shrimp (*Artemia salina*) (Meyer *et al.*, 1982).

 A variety of test methods exist for determining the toxic effects of chemicals, effluents, hazardous chemical wastes, contaminated sediments and bioremediation products to algae. These are being improved to increase their utility to the scientific community (Lewis, 1993). The traditional toxicity tests with algae have been considered ‘simple’, ‘reliable’, ‘inexpensive’, and ‘sensitive’ by those familiar with the methodology (Elnabarawy and Welter, 1984; Nyholm and Kallqvist, 1989), nevertheless some have had difficulty using the published test methods (Sirois, 1990).

 The objective of this review is to highlight some of the available methods for hatching the brine shrimp, list out the optimal conditions necessary for the shrimps to hatch and conclude by proposing the design and development of a Nigerian based Artemia toxicology kit.

***Artemia salina* LEACH (Artemiidae)**

 *Artemia salina* L. (the brine shrimp), is an invertebrate component of the fauna in marine ecosystems (Parra *et al.*, 2001) with more than 50 geographical strains (Treece, 2000). It is a crustacean whose larvae are sensitive to a variety of substances (Cáceres, 1996). It plays an important role in the energy flow of the food chain (Sanchez-Fortun *et al.*, 1995) and it can be used in a laboratory bioassay in order to determine toxicity through the estimation of the medium lethal concentration (LC50 values) (Lewan *et al*., 1992), which have been reported for a series of toxins and plant extracts (Meyer *et al*., 1982). This method, which determines the LC50 value of the active compounds and extracts in saline medium in μg/ml (Massele *et al*., 1995) has been used in research on medicinal plants carried out in different countries in order to evaluate toxicity, gastro-protective action and other biological actions, which in some cases have been related to pharmacological studies carried out for different chemical compounds (Matthews, 1995; Fumaral and Garchitorena, 1996) and as a screening method mainly for products of plant origin (Parra *et al.*, 2001) since activities of a broad range of known plant extracts are manifested as toxicity to the shrimp (Taha & Alsayed, 2000; Parra *et al.,* 2001; Coe *et al*., 2010; Nondo *et al*., 2011). It has also found occasional use in behavioural (Richter and Goldstein, 1970; Schott *et al.,* 1980) and structural–activity relationship studies (Ferrigni *et al.*, 1984; Bottalico *et al*., 1990). It has also been considered a bioindicator of environmental contamination by trace elements such as arsenic, lead, copper, zinc, cadmium, mercury, selenium (Parra *et* *al.*, 2001) and mycotoxin indicator (Harwig and Scott, 1971).

 *Artemia salina* has gained popularity as a test organism because of its ease of culture, short generation time, cosmopolitan distribution and the commercial availability of its dormant eggs (cysts). Since test animals hatching from cysts are of similar age, genotype and physiological condition, test variability is greatly reduced (Barahona and Sanchez-Fortun, 1999). Although, some authors claimed that there is no correlation between this bioassay and the toxicological effects in a whole animal (Sanchez *et al*., 1993), however Parra *et al.*, (2001) toxicologically examined 20 plant extracts using “*in vivo*” and “*in vitro*” methods, their results showed a good correlation (r = 0.85, p < 0.05), suggesting that the brine shrimp bioassay is a useful alternative model to acute toxicity tests in mice. In toxicity evaluation of plant extracts by brine shrimp bioassay, an LC50 value lower than 1000 μg/ml is considered bioactive (Meyer *et al*. 1982).

**ARTEMIA HATCHING METHODS**

 Parra *et al.,* (2001) reported incubation of the cysts with artificial sea water, at temperatures from 20 to 30 °C (Meyer *et al*., 1982). The artificial salt water consisted of 23 g NaCl, 11 g MgCl2·6H2O, 4 g Na2SO4, 1.3 g CaCl2·2H2O or CaCl2·6H2O, 0.7 g KCl in 1000 ml distilled water. The pH was adjusted to 9.0 using Na2CO3 to avoid risk of death to the *Artemia* larvae by decrease of pH during incubation (Lewan *et al*., 1992). After 24 hours, 15 ml of yeast solution 0.06 % was added to the chamber for every litre of salt water in order to feed the larvae.

 Caldwell *et al.*, (2003) reported hatching *A. salina* cysts by vigorously aerating 1 litre of freshwater in a conical flask at 18oC for 24 h while Grzimeks (1974) reported jam jar containing a solution of three to eight percent salt was prepared, to which a small quantity of eggs was added. The developmental stages of hatched metanauplius larva to maturity and reproduction can then be followed. The larvae are fed with a solution of bakers yeast, and the growing and mature animals are fed with dry, powdered fishfood. Abimbade (2010) reported the use of a shallow rectangular plastic container containing 250 ml of natural sea water, about 70 mg of the cyst was then sprinkled on the water. The plastic container was thereafter placed beside a window for light rays and proper ventilation. The shrimps hatched in 48 hrs.

 Park *et* *al*., (2007) reported brine shrimp cysts (Aquafauna Bio-Marine, CA, USA) were placed in a 1 L beaker containing 700 ml of 35psu sterile-filtered seawater with 0.2 mm membrane filter (PALL, MI, USA). The seawater was aerated from the bottom of the beaker using a standard aquarium aerator at 250C to keep the cysts in suspension and allow for hatching. The cysts hatched between 24 and 48 hrs after being placed in the beaker. The seawater in the beaker was exchanged by one third using fresh sterile-filtered seawater every alternate day. Nauplii hatched from cysts were allowed to grow to 1 mm length for 2–3 days, and then used for artemia bioassays.

 Middaugh *et al.*, (1993) reported the use of a separating funnel filled to two-thirds capacity with 20 $μ$m filtered seawater with a salinity of 20 – 30 ppt. Artemia cysts, 20 – 50 ml dry measure are then added to the separating funnel. After adding the cysts, clean air is then vigorously bubbled through the neck of the funnel until the tip rests on the bottom adjacent to the stopcock. Aeration keeps the cyst and newly hatched artemia in suspension. Cysts hatch in 24 – 36 hrs. A light source placed near the bottom of the separating funnel will enhance the settling process, empty cysts will rise to the surface. After approximately 5 minutes, the stopcock/drain is used to collect the nauplii into a 500 ml beaker with a 100$ μ$m mesh screen bottom. Hatching water is discarded and the nauplii are rinsed into a clean 500 ml beaker.

 Treece (2000) reported optimum conditions for hatching *Artemia* cysts to be: 1) temperature above 25oC (77oF), with 28oC (82oF) being optimum; 2) diluted seawater with salinity of 5 ppt (1.030 density); 3) heavy, continuous aeration; 4) constant illumination (example: two 40- watt fluorescent bulbs for a series of four 1-litre hatching cones); and 5) a pH of about 8. Stocking density is set by adding not more than 5 grams of cysts per litre of water. Good circulation is needed to keep the cysts in suspension. A container that is V-shaped or cone-shaped is best (2-litre bottles work well; glue a valve on the bottle cap and invert it). The best container is a separation column, found in any laboratory supply, although it is more expensive. Unhatched cysts, empty shells and hatched nauplii can be easily removed separately. The hatching percentage and density are usually a function of water quality, circulation, and the origin of the cysts. The author also recommendedhydrati onanddecapsulation of the cysts before incubation and advised incubation of 1 to 3 grams of cysts per litre of water. He also discouraged incubating more than 5 grams of cysts per litre of water because a density higher than this could cause foaming. The diluted seawater (as low as 5 ppt) is preferable because less energy is required for the nauplii to emerge from the cysts at the lower salinity. If cysts are allowed to settle, hatch rates may be poor.

 A method used by the authors of this review to hatch artemia is illustrated in Fig 1.



Fig. 1: Hatching of *Artemia* cysts

 Six to eight perforations are made round an empty 50 cl plastic container at about 2 cm height. It is then cut at the base to a height of about 3 cm (A & B), (NB. It is better the nail or any sharp object to be used for the perforation is inserted from outside, do not perforate from inside. Perforation is easier done before cutting). This perforated container is then kept within a transparent disposable lunch box of 65 cl capacity (B & C). The lunch box is filled with natural sea water such that the perforated container floats freely within the lunch box (D). The set up is kept by the window sill (E) (NB. It should be exposed to light and ventilation but not rain) and a pinch of artemia cyst is dropped inside the perforated container (D). This is then left undisturbed and the cysts will hatch between 24 – 36 hrs. When the cysts start hatching, they will swim out through the perforations into the lunch box (F) and can then be harvested clean using a pasteur pipette. This set up is better initiated early in the morning so that harvesting can commence in daylight the following day.

 All the discussed methods showed that for the brine shrimps to hatch, the conditions required include: saline medium, continous agitation of water, illumination and some other physico-chemical parameters of the water must be optimal.

**Conclusion**

 From the aforementioned, hatching the brine shrimp requires continous aeration and illumination in a saline medium. With the peculiar nature of our country without a stable power supply and dense population of the hinterland who may not have access to natural seawater, the nigerian ecotoxicologist therefore needs to develop a kit that can work for nigerian scientists. Persoone and Janseen (1993) reported the research teams that developed the first cyst-based toxicity tests have endeavoured to mould their tests into kits (Toxkits) for rapid and cost effective use. The principle on which these are built is the incorporation into a kit of the materials necessary to perform a full acute toxicity test i.e the cysts, the hatching process, test containers, the media to hatch the cysts and to prepare the toxicant dilution series. To date, the Artoxkit M, Rotoxkit F, Rotoxkit M and Streptoxkit F are available commercially. What then can the Nigerian Ecotoxicologist contribute?

**Recommendation**

 The Artemia strain that is most suitable for our environment must be identified; a source of information on availability, nutritional quality and purity of artemia cysts is the artemia research center, state University of Ghent, Belgium (Middaugh *et al*., 1993). The use of normal saline (physiological solution) that can easily be diluted in order to achieve the desired concentration should be investigated and if not, different salts that can be used to constitute a litre of artificial seawater can be measured and packaged. The various methods for hatching the cyst as highlighted can be investigated in different regions of the country. The design of aerators that are powered by direct current (DC) motors and use of light emitting diodes (LEDs) with spherical lenses to give the desired illumination can all be coupled together in a chamber of specified length, breadth and height that can be easily assembled, portable, accessible and affordable by the Nigerian Ecotoxicologist. All these can be achieved through the collaborative efforts of multidisciplinary research. We can then produce “NigArtoxKit”.

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