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A review of toxicity testing protocols and endpoints with *Artemia* spp.

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ABSTRACT

Artemia spp. is an historically popular biological model still requiring an official internationally based standardization. Several endpoints are currently available. Short-term acute endpoints include biomarker (acetylcholinesterase; heat stress proteins; lipid peroxidation; thiobarbituric acid reactive substances; thioredoxin reductase; glutathione-peroxidase; glutathione *S*-transferase; glutathione reductase; aldehyde dehydrogenase; and adenylpyrophosphatase and Fluotox), hatching (dry biomass, morphological disorders and size), behavioral (swimming speed and path length), teratogenicity (growth), and immobilization (meaning mortality after 5–30 s observation). Long-term chronic tests focus on growth, reproduction and survival or mortality after 7–28 d exposure from larval to adulthood stage. We analyzed each test looking at its endpoint, toxicant and experimental design including replicates, exposure time, number of exposed cysts or organisms and their relative life stage, exposure conditions during hatching and testing (salinity, pH, light intensity, aeration dilution media, and food supply), type of testing chambers, and quality assurance and quality control criteria. Similarities and differences between the identified approaches were highlighted. Results evidenced that hatching 24 h short-term and 14 d long-term mortality are the most promising *Artemia* spp. protocols that should go forward with international standardization.

1. Introduction

The adoption and implementation of the European legislation about the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) (EC, 2006) required several additional ecotoxicity data promoting the decrease of vertebrates used in toxicity testing encouraging alternative strategies with invertebrates, plants as well as organ, tissue, and cell cultures (Dvorak et al., 2012). During the last 50 years, various invertebrates were assessed to investigate their sensitivity to many physical and chemical agents for their possible use as pre-screening or screening models. Internationally, *Artemia* spp. brine shrimps (*Crustacea*, *Branchiopoda*, *Anostraca*), commonly known also as sea monkeys, are one of the most frequently used species for toxicity testing (Van Steertegem and Persoone, 1993a,b).

Artemia spp. is a major taxon in many hypersaline biotypes throughout the world feeding primarily on phytoplankton and being an important primary consumer (Persoone and Sorgeloos, 1980; Vanhaecke et al., 1987; Triantaphyllidis et al., 1998). They are of economical importance being used in aquaculture and in aquariology. They also act as an efficiency and productivity stimulator for salt production in solar salt works (Jones et al., 1981; Migliore et al., 1997; Treece, 2000).

The main advantages of using brine shrimps in toxicity testing are: (i) rapidity (i.e. 28–72 h from hatching to the first endpoint); (ii) cost-effectiveness;(iii) the availability of nauplii hatched from commercial durable cysts (eggs) (i.e. homogeneity of the population, availability all year-round without the necessity of culturing) (Nunes et al., 2006a; Manfra et al., 2012). Other advantages are: (i) good knowledge of its biology and ecology; (ii) easy manipulation and maintenance under laboratory conditions; (iii) small body size allowing accommodation in small beakers or microplates; (iv) high adaptability to various testing conditions (Nunes et al., 2006a; Kokkali et al., 2011). Conversely, several criticisms about *Artemia* spp. sensitivity were presented by a learning-by-doing approach (Libralato et al., 2010a,b; Libralato, 2014). For example, the cysts' production can reflect the occurrence of genetic variation caused by crustaceans' geographical origin that is rarely known (Migliore

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et al., 1997), although certified cysts are usually utilized in toxicity testing. Their origin can have consequences on the growth, survival and reproduction of *Artemia* spp. specimens considering especially salinity and temperature (Vanhaecke and Sorgeloos, 1989; Triantaphyllidis et al., 1995).

Artemia spp. nauplii were used to test the toxicity of a wide range of chemicals such as arsenic (As) (Brix et al., 2003), cadmium (Cd) (Kissa et al., 1984; Hadjispyrou et al., 2001; Sarabia et al., 1998a, 2002, 2006; Brix et al., 2006; Leis et al., 2014), chromium (Cr) (Hadjispyrou et al., 2001; Leis et al., 2014), cobalt (Kissa et al., 1984), copper (Cu) (Browne, 1980; Jorgensen and Jensen, 1977; Brix et al., 2006), mercury (Hg) (Sarabia et al., 1998b; Leis et al., 2014), nickel (Kissa et al., 1984), tin (Sn) (Hadjispyrou et al., 2001), zinc (Zn) (Brix et al., 2006; Garaventa et al., 2010), potassium permanganate, potassium dichromate, and silver nitrate (Boone and Baas-Becking, 1931; Vanhaecke et al., 1980), antibiotic drugs (Migliore et al., 1993a,b, 1997), engineered nanomaterials (Libralato, 2014; Minetto et al., 2014; Corsi et al., 2014; Callegaro et al., 2015), nano-sized polystyrene (Bergami et al., 2016), asbestos (Stewart and Schurr, 1980), phenolic compounds (Guerra, 2001), ethanolamines (Libralato et al., 2010a) and trace elements (Petrucci et al., 1995), triazine herbicides, insecticides, pesticides (Kuwabara et al., 1980; Varó et al., 1997, 2002), acrylonitrile (Tong et al., 1996), carbammates (Barahona and Sánchez-Fortún, 1999), phthalates, antifouling agents (Grosch, 1980; Persoone and Castritsi-Catharios, 1989a,b; Okamura et al., 2000; Castritsi-Catharios et al., 2007, 2013, 2014; Koutsaftis and Aoyama, 2007), pharmaceuticals (Xu et al., 2015), anticorrosive agents (Tornambè et al., 2012; Manfra et al., 2015a, 2016), oil (Trieff, 1980) and oil dispersants (Zillioux et al., 1973; Savorelli et al., 2007), various plant extracts (Cáceres et al., 1998), toxins (Granade et al., 1976; Medlyn, 1980; Vezie et al., 1996; Beattie et al., 2003) and environmental matrices such as wood leachates (Libralato et al., 2007), wastewater (Krishnakurmar et al., 2007; Libralato et al., 2010b), seawaters (Manfra et al., 2011) and marine discharges (Manfra et al., 2010).

Currently, various toxicity tests with *Artemia* spp. are available including short-term and long-term methods. Short-term toxicity tests are more frequently used, some long-term protocols have been developed in the last 10 years, but none of them is an internationally standardised method like International Standard Organization (ISO), American Society for Testing and Materials (ASTM) or Organization for Economic Co-operation and Development (OECD). Methods for testing immobilization/mortality were standardised only in Italy by the Italian Agency for Environmental Protection and Italian Institute for Water Research (APAT IRSA-CNR) and Italian Agency for Standardization in the Chemical sector (Unichim). Despite the frequent and widespread use of *Artemia* spp. in toxicity testing, the harmonization of protocols followed by international standardization activities is still lacking, and intercalibration exercises are urgently necessary (Libralato, 2014).

The aim of this review paper is to collect, organize, select and discuss the existing knowledge about *Artemia* spp. methods for toxicity testing including both short- and long-term bioassays and organism hatching and maintenance conditions providing tips for protocols definition, implementation and standardization.

2. Hatching of cysts

Artemia spp. cyst hatching conditions can vary greatly as reported in Table S1 ($n = 42$). This can result in a different evaluation of cysts/nauplii sensitivity, although the first factor that can affect organism sensitivity is the geographical origin of cysts. Other species are commercially available, but their sensitivity must be evaluated on Vanhaecke et al. (1980) a case-by-case basis if no certification or traceability is available (Guzzella, 1997).

The second key point is to start the toxicity tests with nauplii belonging to the same class of age because some stages are more sensitive than others (i.e. Instar I stage is less sensitive than Instar II–III stage) (Sorgeloos et al., 1978). Cyst hatching occurred 24 h before starting the toxicity test (Instar I stage) in 10 papers (Vanhaecke et al., 1981; Barahona et al., 1994; Brix et al., 2003, 2004; Caldwell et al., 2003; Venkateswara Rao et al., 2007; Garaventa et al., 2010; Bustos-Obregon and Vargas, 2010) and 30 h in 3 papers (Persoone et al., 1993; Guerra, 2001; Koutsaftis and Aoyama, 2007). Most authors used 48 h old larvae hatched at 25 ± 2 °C involving during the exposure larvae at Instar II–III (Guzzella, 1997; Hadjispyrou et al., 2001; APAT and IRSA-CNR, 2003; Favilla et al., 2006; Libralato et al., 2007; Savorelli et al., 2007; Pimentel et al., 2009; Manfra et al., 2011, 2010; Kokkali et al., 2011; Prato et al., 2011; Manfra et al., 2012; Tornambè et al., 2012; Leis et al., 2014; Veni and Pushpanathan, 2014; Manfra et al., 2015a,b, 2016; Rotini et al., 2015).

Either artificial or natural seawaters are used as hatching media. Artificial media are more frequently used because salt blends to create the ideal saltwater are commercially available (i.e. *Crystal Sea® Marinemix, Forty Fathoms®, Coral Reef Red Sea Salt®, Instant Ocean®*) or are provided along with toxkits (Artoxkit, 2014). The required seawater can be obtained dissolving these salts in distilled and deionized water.

Cysts are usually incubated at between 18–28 °C and largely 35‰ salinity. The values of pH vary between 7.5–9.0 and pH should not be lower than 7 to obtain good hatching (Vanhaecke et al., 1980). During the hatching process, seawater is sometimes aerated by an air pump being the medium adequate aeration a prerequisite to obtain a successful hatching (Vanhaecke et al., 1980). Manfra et al. (2016) proposed an oxygen saturation level >60%. The hatching phase occurred in presence of light (1000–4000 lux) (Varó et al., 2002; Artoxkit, 2014) or partly in darkness like just 1 h of light during 48 h exposure (Guzzella, 1997; Unichim, 2012) or 12–16 h of light during 24 h exposure (Garaventa et al., 2010; Gambardella et al., 2014).

The hatching efficiency needs to be considered, but only Guzzella (1997) proposed a threshold evaluating cysts hatching efficiency that should be >90% in ≤32 h.

Two main cyst-hatching procedures were identified on the basis of Artoxkit (2014) or Unichim (2012). Artoxkit (2014) suggests hatching cysts 30 h before toxicity testing. Cysts are transferred into a Petri dish with 9 mL of seawater prepared with the toxkit salts and gently swirled to distribute them evenly. The Petri dish is exposed to a light source (1000–4000 lux) for 30 h. According to Unichim (2012), cyst hatching starts 48 h before the toxicity test. Seawater is used as hatching medium. *Artemia* cysts (100 mg) are transferred into a Petri dish with 12 mL of seawater exposed at 1000–4000 lux for 1 h and for 23 h in darkness at 25 ± 1 °C. After 24 h, hatched nauplii are pipetted to a new Petri dish for moulting containing new fresh seawater that is incubated for 24 h at 25 ± 1 °C in the dark allowing larvae to reach II or III Instar stage.

Sometimes, *Artemia* embryos were sterilized and dechorionated before use, as in the protocols for aquaculture purposes (Sorgeloos et al., 1977). Dechorionated cysts are *Artemia* embryos enveloped only by the embryonic cuticle and the outer cuticular membrane (Léger et al., 1986). The technique was set up by Nakanishi et al. (1962), which used a chilled diluted antiformin solution to dissolve the chorion. Morris and Afzelius (1967) improved this technique, which allows removing the outer part of the shell without affecting embryo viability. The temperature of the medium must be kept below 40 °C to maintain the maximal hatching efficiency (Sorgeloos et al., 1977). The use of antibiotics (i.e. penicillin (50 units/mL), streptomycin (50 μg/mL) and sodium tetraborate (0.2% w/v)) was highlighted by Bagshaw et al. (1986) and Brix et al. (2006). Embryos