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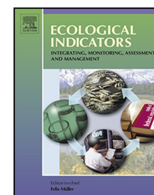

A review of toxicity testing protocols and endpoints with *Artemia* spp.

G. Libralato[a,*], E. Prato[b], L. Migliore[c], A.M. Cicero[d], L. Manfra[d,e]

[a] Department of Environmental Sciences, Informatics and Statistics, University Ca' Foscari Venice, Via Torino 155, 30172 Mestre, Venice, Italy
[b] Institute for the Coastal Marine Environment, National Research Council (CNR IAMC), Via Roma 3, 74100 Taranto, Italy
[c] Department of Biology, University Tor Vergata, Via della Ricerca Scientifica, 00133 Rome, Italy
[d] Italian Institute for Environmental Protection and Research, Via Vitaliano Brancati 60, 00144 Rome, Italy
[e] Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Naples, Italy

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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.

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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

* Corresponding author.
E-mail address: giovanni.libralato@unive.it (G. Libralato).

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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

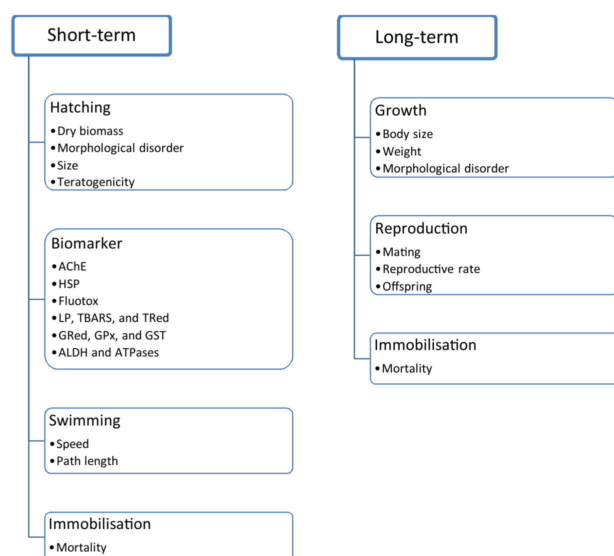

Fig. 1. Summary of *Artemia* short- and long-term toxicity tests. (AChE = AcetylCholinEsterase; HSP = heat stress proteins; LP = lipid peroxidation; TBARS = thiobarbituric acid reactive substances; TRed = thioredoxin reductase; GPx = glutathione-peroxidase; GST = glutathione *S*-transferase; GRed = glutathione reductase; ALDH = aldehyde dehydrogenase; and ATPases = AdenylTriPhosphatase).

were hydrated in sterile seawater containing antibiotics prior to toxicity test in order to prevent potential bacterial infections.

3. Toxicity tests

We decided to cluster toxicity tests with *Artemia* spp. as short-term acute and long-term chronic bioassays (Fig. 1) considering the life span of *Artemia* spp. varying between 2–4 months depending on salinity, temperature and species-specific characteristics (Browne et al., 1991). Acute toxicity tests assess the effects based on relatively high exposure concentrations (i.e. mg/L) for no more than 96 h. Toxicity is generally expressed as lethal concentration causing the death of 50% of the group of test animals (LC_{50}), but also hatching and swimming behavior impairment. The analysis was carried out considering sub-lethal (acute cyst hatching test, biomarkers, teratogenicity test, acute larvae behavioral test) and lethal endpoints.

Chronic toxicity tests investigate the long-term exposure from weeks (*e.g.* 2–4 weeks) up to the whole life cycle of *Artemia* spp. at relatively low concentrations (μg/L) (Pane et al., 2012). Toxicity is expressed as the no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) considering survival, growth and reproduction as endpoints (Brix et al., 2003, 2004; Savorelli et al., 2007; Manfra et al., 2012; Unichim, 2012).

3.1. Short-term toxicity test

Acute endpoints generally investigate the extent of lethality (Persoone et al., 1993; Guzzella, 1997; Artoxkit, 2014) (Table S2) or other significant impacts on *Artemia* spp. such as hatching (Table 1), growth (Migliore et al., 1997; Sarabia et al., 2008), and swimming (Garaventa et al., 2010; Gambardella et al., 2014) (Table 2). In most of the cases, readings are carried out just at the end of the exposure period, keeping to a minimum continuous and/or periodic observations of single animals in a non-endpoint perspective. Generally, long-term toxicity tests require the periodical recording of death animals depicting how contact time may contribute to the final evolution of effects.

Artemia 'death' (i.e. mortality or lethality) presented various definitions generating potential misunderstanding about the observation of moribund or really dead larvae. Generally, mortality evaluation is carried out with a stereomicroscope by eye, but computer based analysis systems are available to decide whether the monitored *Artemia* nauplius is alive or dead (Garaventa et al., 2010; Alyuruk et al., 2013). Widdows (1998) stated that *Artemia*, and other crustaceans, can lie on the bottom of the test container, but still occasionally move an appendage. 'Immobility' may therefore be an easier state to define. Anyhow, differences in how the immobility state is defined exist. Garaventa et al. (2010) considered nauplii as dead when they are completely motionless or do not change their barycenter position, or no appendage movement occurs for 5 s. Zulkifli et al. (2014) reported that nauplii are dead if they do not show any movement after 10 s observation, while Alyuruk et al. (2013) prolonged the observation period up to 30 s.

3.1.1. Acute cyst hatching test

Since 1980s' cysts and their embryonic development were used in ecotoxicology as summarized in Table 1. The hatching toxicity test was developed to assess the effect of toxicants having different composition and mode of action like metals (Bagshaw et al., 1986; Brix et al., 2006; MacRae and Pandey, 1991; Rafiee et al., 1986; Jorgensen and Jensen, 1977; Sarabia et al., 1998a,b, 2003, 2008), organic compounds (Kuwabara et al., 1980; Alyürüc and Çavas, 2013; Rotini et al., 2015; Vismara, 1998), antibiotic drugs (Migliore et al., 1993a,b, 1997) or cell extracts from invertebrates, macro- and microalgae (Caldwell et al., 2003; Carballo et al., 2002) as well as stress events generated by salinity and temperature (Vanhaecke and Sorgeloos, 1989). Hatching toxicity tests are static or semi-static lasting between 24 h and 96 h. They investigate the reduced emergence of nauplii from cysts/eggs when exposed to toxicants compared to negative controls. Results of 'cyst based' toxicity tests with metals, using different procedures, produced apparently inconsistent data. Bagshaw et al. (1986) tested fully hydrated *Artemia* cysts (20–30 organisms per replicate) at 28 °C and 36 Practical Salinity Unit (PSU) under constant illumination and shaking, evidencing that encysted embryos are more sensitive to Cd and Zn than pre-nauplius larvae. Cadmium (0.1 μM) retarded the hatching and development of larvae, while higher concentrations blocked the hatching phase almost completely. Zinc was less toxic than Cd, but it caused similar effects. Bagshaw et al. (1986) protocol was used by Rafiee et al. (1986) and Brix et al. (2006) confirming both the toxicity of Cd and Cu (*Artemia franciscana*), respectively. MacRae and Pandey (1991) showed that *Artemia franciscana* Cu and Pb (0.1 μM) toxicity was similar to Cd reducing the rate and extent of larval development; Zn was less toxic than Cd, Pb and Ni. Sarabia et al. (1998a,b, 2003, 2008), using hydrated cysts of *Artemia parthenogenetica* (10 organisms per replicate) did not highlight any adverse effects of Cd, Hg and Zn to nauplii emergence. The difference in *Artemia* spp. sensitivity to metals was probably due to the differences in testing temperature and interspecific species sensitivities. In 1931, Boon and Baas-Becking observed that temperature affected emergence and hatching. Vanhaecke and Sorgeloos (1989) demonstrated that the incubation temperature between 25 and 37 °C can significantly affect the hatching percentage of *Artemia* cysts even within geographical strains of both *A. franciscana* and *A. partenogenetica*. In *Artemia* spp., temperature is known to profoundly influence chemicals' effects. In particular, low temperature reduced toxicity of organo-metallic compounds in *A. franciscana*: Cu pyrithione and Diuron toxicity at <25 °C were significantly reduced or eliminated, respectively (Koutsaftis and Aoyama, 2008). Thus temperature is a variable of extreme interest to be considered while standardizing from hatching, larvae maintenance up to the end of the exposure to treatments. The use of a full range of temperatures, especially during toxicity testing, may drastically increase the ecological relevance of data.

Table 1
Artemia spp. behavioural toxicity tests.

References	Test	Toxicant	Concentration (mg/L)	Exposure (h)	Cysts (mg)	Endpoint	T (°C)	Salinity (‰)	pH	Test light (lx)	Aeration	Dilution medium	Food (cell/mL)	Organisms per chamber	Replicates	Test vessel (mL)	Test mL	QC	Reference Toxicant	NOEC/LOEC/LC_{50}/EC_{50}
Alyuruk et al. (2013)	S	$K_2Cr_2O_7$, p-coumaric acid	7-30-70-100	24	n.a.	SW velocity and paths covered by nauplii	n.a.	35	n.a.	Low light	n.a.	Saline water	n.a.	5–8/20 μL	n.a.	MP(96) 130 μL	0.2	n.a.	$K_2Cr_2O_7$	% survival and velocities for each concentration and paths
Davenport and Healy (2006)	S	salinity	8.5–250	24	n.a.	SW speed	23	8.5-250	n.a.	n.a.	air-saturated	NSW	No food	n.a.	n.a.	Flat-faced transparent polystyrene canted-neck culture flasks	50	n.a.	n.a.	n.a.
Gambardella et al. (2014)	S	SnO_2, CeO_2 and Fe_3O_4 NPs	10-100-1000	48	500	SW and M	20	37	n.a.	16 h light	n.a.	ASW	n.a.	10-15	9	25 compartment square Petri dish	1	n.a.	n.a.	Nano-SnO_2 M < 20%; nano-CeO_2: LC_{20} = n.c.; % SW alteration < 50%; nano-CeO_2 EC_{20} = 80 mg/L (70–90)
Garaventa et al. (2010)	S	1. zinc pyrithione; 2. Macrotrol® MT-200; 3. Eserine	1. 0,1-1-10-100; 2. 1.5-3-6-9-12; 3. 0.1-1-10-100	24–48	500	SW and M	20	37	n.a.	16 h light	n.a.	ASW	n.a.	10-15	9	Petri dish	1	n.a.	n.a.	1. LC_{50}(48 h) = 35.74 (26.24-48.68); EC_{50}(48 h) = 11.l9 (6.56-19.09); LC_{50}(24 h) > 100; EC_{50}(24 h) = 19.57 (11.31-33.87); 2. LC_{50}(48 h) = 5.31 (4.70-6.00); EC_{50}(48 h) = 4.62 (3.64-5.88); LC_{50}(24 h) = 5.83 (n.c.); EC_{50}(24 h) = 6.39 (5.72-7.15); 3) LC_{50}(48 h) > 100; EC_{50}(48 h) = 6.78 (4.49-10.24); LC_{50}(24 h) > 100; EC_{50}(24 h) = 22.31 (l 2.61-39.46).
Huang et al. (2015)	S	1. $K_2Cr_2O_7$; 2. $Cd(NO_3)_2$	1. 0–100; 2. 0–1000	24	n.a.	speed movement	25	n.a.	n.a.	n.a.	yes	NSW	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	LC_{50}($K_2Cr_2O_7$) = 32 mg/L LC_{50}($Cd(NO_3)_2$) = 626 mg/L (speed movement)
Larsen et al. (2008)	S	Temperature, viscosity	*T* = 10–15–20 °C, viscosity 1.1-1.2-1.4-1.6 (×10–6 m^2 s^{-1})	n.a.	n.a.	SW velocity	n.a.	n.a.	n.a.	n.a.	n.a.	NWS	*Rhodomonas* sp.	n.a.	n.a.	glass flasks	n.a.	n.a.	n.a.	Inhibition of SW velocity for a 10 °C temperature reduction
Manfra et al. (2015b)	S	1. $CuSO_4$; 2. SDS; 3. DEG	1. 2-4-8-16-32; 2. 3-6-12-24-48; 3. 10000-20000-40000-80000-160000	48	20	SW and M	25 ± 1	35 ± 1	n.a.	D	n.a.	ASW	n.a.	10	3	MP(24)	1	control mortality ≤ 10%	1. $CuSO_4$ 2. SDS	1. LC_{50}(24 h) = 14.21 ± 10.63; LC_{50}(48 h) = 2.51 ± 0.22; 1. EC_{50}(24 h) = 5.03 ± 0.58 EC_{50}(48 h) = 2.51 ± 0.37; 2. LC_{50}(24 h) = 19.41 ± 1.00; LC_{50}(48 h) = 15.60 ± 0.27; 2. EC_{50}(24 h) = 16.15 ± 0.32; EC_{50}(48 h) = 7.48 ± 1.33; 3. LC_{50}(24 h) > 160000; LC_{50}(48 h) > 160000; 3. EC_{50}(24 h) = 80370 ± 3950; EC_{50}(48 h) = 64560 ± 3420
Venkateswara Rao et al. (2007)	S	1. CPP; 2. PF; 3. MCP; 4. ACEP	1. 0.385 ± 0.08; 2. 7.71 ± 1.48; 3. 262.68 ± 17.30; 4. 2350.07 ± 131	24	1000 nauplii	SW speed (cm/s) and distance travelled (m/min)	n.a.	n.a.	n.a.	n.a.	n.a.	ASW	n.a.	25/each exposure	n.a.	MP(96)	n.a.	n.a.	n.a.	SW speed inhibition (%): 1. 89.00 ± 3.32CPP 2. 74.68 ± 2.13 PF 3. 62.48 ± 1.87 MCP 4. 49.83 ± 1.53 ACEP
Williams (1994a, 1994b)	S	No toxicant	n.a.	n.a.	n.a.	1. newly hatched larvae (nauplii); 2. larvae with visible limb buds; 3. a series of larval stages identified by the number of actively beating trunk appendages present	21	35–40	n.a.	n.a.	yes	Brine solution	Yeast and green algae	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Initial development of new limbs in *Artemia* larvae is unimportant for propulsion.

ACEP = acephate; ASW = artificial seawater; CPP = chlorpyriphos; D = darkeness; DEG = diethylene glycol; EC50 = effect concentration for 50% population; LC50 = lethal concentration for 50% population; LOEC = lowest observed effect concentration; M = mortality; MCP = monocrotophos; MP = multi-well plate; n.a. = not available; NOEC = no observed effect concentrations; NSW = natural seawater; n.c. = not calculable; NP = nanoparticles; PF = profenofos; QC = quality negative control; S = static; SDS = sodium dodecylsulphate; SW = swimming.

Table 2
Artemia spp. short-term toxicity tests.

References	Test	Toxicant	Concentration (mg/L)	Exposure (h)	Cysts (mg)	Endpoint	T (°C)	Salinity (‰)	pH	Test light (lx)	Aeration	Dilution medium	Food (cell/mL)	Organisms per chamber	Replicates	Test vessel (mL)	Test mL	QC	Reference toxicant	NOEC/LOEC/LC_{50}/EC_{50}
APAT and IRSA-CNR (2003)	S, A	SDS, $K_2Cr_2O_7$, $CuSO_4$	SDS: 5-9-16-28-50; $K_2Cr_2O_7$: 5-9-16-28-50; $CuSO_4$: 1.5-2.7-4.8-8.4-15	24	n.a.	M	25 ± 2	n.a.	6.5-8.5	D	n.a.	ASPM, IO	n.a.	10	3	MP 24 wells	1	CM ≤ 20%	SDS, $K_2Cr_2O_7$, $CuSO_4$	Probit analysis, Chi^2 test: EC_{50}(SDS) = 23.2 ± 6.5 mg/L with ASPM and 25.6 ± 5.5 mg/L with IO. EC_{50}($K_2Cr_2O_7$) = 16 ± 8.4 mg/L with ASPM and 15.1 ± 9.6 mg/L with IO. EC_{50}($CuSO_4$) = 4.5 ± 2.0 mg/L ASPM and 4.5 ± 2.0 mg/L with IO
Artoxkit (2014)	S, A	$K_2Cr_2O_7$	10-18-32-56-100	24	n.a.	M	25	35	n.a.	D	n.a.	ASW	no	10	3	MP 24 wells	1	CM ≤ 10%	$K_2Cr_2O_7$	n.a.
Brix et al. (2004)	S, A	SeO_4^{2-}	42-56-75-100,-133	95	n.a.	M	25 ± 1	80-102	7.9–8.4	n.a.	n.a.	NSW	daily 2 mL of 5×10^5 cell/mL *Platymonas* sp.	n.a.	4	Beakers	400	n.a.	n.a.	LC_{50} = 78 (71–86) mg/L
Guzzella (1997)	S, A	SDS, $K_2Cr_2O_7$, $CuSO_4$	SDS: 5-9-16-28–50 $K_2Cr_2O_7$: 5-9-16-28-50 $CuSO_4$: 1.5-2.7-4.8-8.4-15	24	n.a.	M	25 ± 2	n.a.	6.5–8.5	D	n.a.	ASPM, IO	n.a.	10	3	MP 24 wells	1	CM ≤ 20%	SDS, $K_2Cr_2O_7$, $CuSO_4$	Probit analysis, Chi^2 test: EC_{50}(SDS) = 23.2 ± 6.5 mg/L with ASPM and 25.6 ± 5.5 mg/L with IO. EC_{50}($K_2Cr_2O_7$) = 16 ± 8.4 mg/L with ASPM and 15.1 ± 9.6 mg/L with IO. EC_{50}($CuSO_4$) = 4.5 ± 2.0 mg/L ASPM and 4.5 ± 2.0 mg/L with IO
Kissa et al. (1984)	S, A	1. $CdCl_2$ 2. Na_2CrO_4 3. $Ni(NO_3)_2$ 4. $Co(NO_3)_3$	1,2,3 0-200 4. 0-250	24, 48	n.a.	M	24 ± 0.5	n.a.	n.a.	n.a.	n.a.	NSW	n.a.	20 (3 days old)	n.a.	Glass cuppels	50	n.a.	n.a.	LC_{50}(48 h): 160 mg/L for Cd; 163 mg/L for Ni; 172 mg/L for Co; 8 mg/L for Cr
Libralato et al. (2007)	S, A	Wood leachates	12-25-50-100% v/v	24	n.a.	M	25	35	7.0–8.3	D	n.a.	IO	n.a.	10-15	3	MP 24 wells	2	CM ≤ 10%	$CuSO_4$	Trimmed Spearman–Karber expressed as LC_{50} and transformed into Toxicity Units (TU_{50}): 47.57 (41.65–54.75) for *Quercus* spp. and 78.21 (75.58–84.09) for *Picea abies*

Table 2 (*Continued*)

References	Test	Toxicant	Concentration (mg/L)	Exposure (h)	Cysts (mg)	Endpoint	T (°C)	Salinity (‰)	pH	Test light (lx)	Aeration	Dilution medium	Food (cell/mL)	Organisms per chamber	Replicates	Test vessel (mL)	Test mL	QC	Reference toxicant	NOEC/LOEC/LC_{50}/EC_{50}
Manfra et al. (2015a,b)	S, A	$CuSO_4$	n.a.	24	n.a.	M	25 ± 1	35 ± 1	n.a.	n.a.	n.a.	ASW	n.a.	10	3	MP 24 wells	1	CM ≤ 10%	$CuSO_4$	Trimmed Spearman–Karber: mean EC_{50} between 5.63 and 23.31 mg/L (CV < 40%)
Persoone et al. (1993)	S, A	$CuSO_4$	n.a.	24	n.a.	M	n.a.	n.a.	n.a.	n.a.	n.a.	ASW	n.a.	10	n.a.	MP 24 wells	n.a.	n.a.	$CuSO_4$	Mean LC50 (Cu^{2+} mg/L): from 3.3 to 3.8; CV%: from 32.8 to 50.7
Peteros and Uy (2010)	S, A	crude extracts of four medicinal plants	10-100-1000	24	n.a.	M	n.a.	n.a.	n.a.	L	n.a.	NSW	n.a.	10	3	Vials	5	n.a.	DMSO	Probit analisys, LC_{50} (24 h) in mg/L = *Brucea amarissima:* 37.7; *Intsia bijuga*: 86.5; *Laportea meyeniana*: 89.5; *Pipturus arborescens*: 57.5
Solis et al. (1993)	S, A	21 active agents from plants	n.a.	24	n.a.	M	22–29	n.a.	n.a.	D	n.a.	NSW	n.a.	n.a.	3	MP 96 wells	0.1	n.a.	DMSO	Probit analysis mean LC_{50} value: between $3.8 \times 10^{-4} \pm 1.4 \times 10^{-4}$ and >1000 mg/L
Toğulga (1998)	S, A	SDS, $K_2Cr_2O_7$	n.a.	24	n.a.	M	25 ± 1	n.a.	7.5 ± 0.5	D	yes	NSW	no	n.a.	n.a.	Petri dish	10	n.a.		Litchfield and Wilcoxon' method, LC_{50}: 14.5 SDS mg/L; 34 PDK mg/L. Bliss' method LC_{50} = 13.84 ± 0.005 SDS mg/L; LC_{50} = 32.84 ± 0.007 PDK mg/L
Vanhaecke et al. (1980)	S, A	SDS	n.a.	24	100	M	25 ± 1	35	7.5	D	n.a.	Kinne (1971)	no	10	8	Petri dish	10	n.a.	SDS	Litchfield and Wilcoxon: Instar I mean LC_{50} = 33.9 ± 5.2 mg/L (CV = 7.7%); Instar II–III: mean LC_{50} = 17.8 ± 0.9 mg/L (CV = 5.1%)
Vanhaecke et al. (1981)	S, A	SDS	n.a.	24	100	M	25 ± 1	35 ± 1	8.0 ± 0.5	D	Continuous	IO	n.a.	10	3	Glass Petri dish	10	CM ≤ 10%	SDS	Litchfield and Wilcoxon 13.3 < LC_{50} (24 h-SDS) < 19.9 mg/L

A = acute; ASW = artificial seawater; CM = negative control mortality; D = darkness; DMSO = dimethyl sulfoxide; EC50 = effect concentration for 50% population; LC50 = lethal concentration for 50% population; LOEC = lowest observed effect concentration; IO = Instant Ocean® ASW; L = light; M = mortality; MP = multi-well plate; n.a. = not available; NOEC = no observed effect concentration; NSW = natural seawater; QC = quality control; S = static; SDS = sodium dodecylsulphate.

Methanol and ethanol toxicity was evaluated (Vismara, 1998) by exposing hydrated cysts for 48 h. The hatching test was also successfully used to evaluate the toxicity of two biocides utilized in antifouling paints (Diuron and Irgarol) (Alyürüc and Çavas, 2013) by exposing hydrated cysts for 24 h. The hatching test was also able to evaluate, in a concentration-dependent way, the toxicity of water soluble algal extracts and crude cellular extracts of *Skeletonema costatum* and *Nitzschia commutata*, and the diatom-derived short chain aldehydes decadienal by exposing 20–30 cysts per replicate to fresh seawater (1 mL) at 15 °C in multiplate wells (Caldwell et al., 2003).

A quite different protocol using commercial brine shrimp hatcher was applied to evaluate the toxicity of three antibiotics (Bacitracin, Flumequine and Sulfadimethoxine) (Migliore et al., 1993a, 1997), two organic compounds (diethylene glycol (DEG) and sodium dodecyl sulphate (SDS)) (Rotini et al., 2015), and the bioactivity of extracts of marine invertebrates and macroalgae (isopropanolic extracts from *Hyatella* spp. (sponge), *Dysidea* spp. (sponge), *Pacifigorgia adamsii* (coral) and *Muricea* sp. (coral) already found active against two human cell lines) (Carballo et al., 2002). This method is aimed to evaluate the dry biomass of hatched nauplii from 1 g (~200,000 individuals) of *Artemia* spp. cysts incubated at 25 °C and 35 PSU under constant illumination (3000 lx) and aeration (air bubbling, not compulsory) for 2–4 days. Cysts were intact and not dechorionated. The free nauplii, after the successful emergence from the cyst and hatching, aggregate over a sieve in the center of the hatcher due to their positive phototactic activity easing their collection. This kind of approach seems to be more viable for the evaluation of the reduced emergence and/or hatching than enumerating one-by-one the cysts able to complete their embryonic development when exposed to a toxicant (Kuwabara et al., 1980; Rotini et al., 2015). Phototaxis is a cost-effective and easy way of nauplii grouping for counting or biomass production determination. Anyhow, this method does not allow discriminating the sub-lethal etiology of the treatment exposure. Further investigation would be interesting about *Artemia* spp. light reception, nervous system processing, motor function as well as developmental monitoring.

Another group of 'cyst based tests' evaluated the morphological disorders and size of hatched nauplii (Rafiee et al., 1986; Vismara, 1998; Neumeyer et al., 2014). Bagshaw et al. (1986) found reversible arrest of development of pre-nauplii larvae emerged in Cd spiked waters (10 μM $CdCl_2$) and morphological alteration at higher concentrations, considering the phenotypic alterations an endpoint within the hatching test. Also in the case of Hg, Go et al. (1990) described the presence of morphological abnormalities and delayed development in exposed cysts during emergence and hatching phase.

Rafiee et al. (1986) incubated hydrated cysts up to 100 h in presence of Cd and Zn spiked seawater. Scanning electron micrograph and light microscopy evidenced morphological abnormalities and delayed development. Migliore et al. (1997) according to Rafiee et al. (1986) highlighted severe phenotypic alteration due to Bacitracin and changes in pigmentation due to Flumequine. Neumeyer et al. (2014) investigated the use of fully hydrated and dechorionated embryos (15–28 per replicate) incubated at 21–23 °C and room light condition observing and describing by light microscopy all *Artemia* development phases and relative alterations considering a range of salinity (20, 25, 30 and 35‰) and density (from 1–3 embryo/mL to approximately 350 embryo/mL). They found that (i) aberrant morphologies result from a mismatch between development and emergence; (ii) a small salinity increase or embryo abundance decrease can impair the emergence success having only moderate effects on the development; (iii) aberrant developmental paths produce swimming nauplii and unhatched cysts appearing normal in counts. Neumeyer et al. (2014) improved the design of *Artemia* spp, endpoint assay offering an alternative approach considering the quantitative developmental profile under specified conditions (22 °C, 16:8 or 0:24 light:dark cycle, 20–30‰ artificial seawater), even if it can be a quite time consuming activity.

Some authors compared the sensitivity of hatching and acute/chronic mortality toxicity test. Jorgensen and Jensen (1977) observed that when *Artemia salina* is exposed to Cu ions the hatching rate is much more susceptible than mortality, obtaining EC_{50} values at least 100 times below the analogous LC_{50}. Migliore et al. (1997) showed that in *A. franciscana* both cysts hatching and mortality responded to antibiotics exposure evidencing hatching rate and larvae morphological alterations, and nauplii death, respectively. Carballo et al. (2002) evidenced a similar sensitivity between cysts' hatchability and nauplii mortality exposed to various organic extracts, suggesting a simultaneous application of both assays to test natural marine products' pharmacological activity. Sarabia et al. (2003) conducted experiments by exposing cysts and larvae to Cd. They did not observe toxic effects on cysts (percentage and timing of cyst hatching) and larvae (life span and reproductive parameters). Caldwell et al. (2003) compared hatching success and larval mortality of *A. salina* exposed to diatom extracts and aldehydes, showing that hatchability is a less sensitive endpoint. The most recent paper by Rotini et al. (2015) compared the results of hatching, acute mortality and chronic mortality tests on DEG and SDS ranking the endpoints as follows: acute mortality < hatchability < chronic mortality. The protocol described in Rotini et al. (2015) was intercalibrated at Italian level between 2006–2009 involving eleven laboratories to ring toxicity tests with *A. franciscana* using $CuSO_4$ as reference toxicant (Manfra et al., 2015b).

3.1.2. Biomarkers

Biomarkers are any measurable characteristics of an organism reflecting a specific or general physiological and health state. Biomarkers are often compounds isolated from serum, hemocyanin, or other fluids that can be used as an indicator of the presence or severity of a particular disease state. The brine shrimp *A. salina* was used in few screenings considering biochemical mechanisms (Nunes et al., 2006b). Espiritu et al. (1995) developed a 1 h enzymatic inhibition assay (Fluotox) with *Artemia* nauplii using a fluorigenic enzyme substrate comparing the output with 24 and 48 h mortality test. Addition of 4-methylumbelliferyl-β-D-galactoside (MUF) to the test medium did not influence the toxicity of $CuSO_4$, $CdCl_2$, $K_2Cr_2O_7$, sodium pentachlorophenate, SDS, and phenol. Uptake of MUF and its subsequent hydrolysis form the 4-methylumbelliferone that is strongly fluorescent in alkaline solution using a UV light. The exposure to toxicants may reduce or inhibit this enzymatic pathway that can be used as an early manifestation of toxicity.

Varó et al. (2002) observed the inhibitory potential of chlorpyrifos and dichlorvos on acetylcholinesterase (AChE) activity using *A. salina* and *A. parthenogenetica* provoking the accumulation of acetylcholine (ChE) at neuromuscular junction disrupting the function nerve (Peakall, 1992). Results highlighted the tolerance of *A. salina* to high levels of ChE inhibition (approximately 80%) without lethal effects.

Few data exist on the antioxidant defense mechanisms in *Artemia* spp. Nunes et al. (2006b) investigated the antioxidant skills of *A. parthenogenetica* to pharmaceuticals and personal care products. Benzodiazepine significantly inhibited ChE and glutathione-peroxidase (GPx) activities. Clofibrate and clofibric acid showed significant decreases in Se-dependent GPx. In particular, clofibrate caused a slight increase of lipid peroxidation (LP) (thiobarbituric acid reactive substances, TBARS) (Nunes et al., 2006b). Sodium dodecylsulphate (SDS) widely used in detergents

decreased the activity of both ChE and glutathione reductase (GRed) in *A. parthenogenetica* (Nunes et al., 2006b).

Nauplii of *A. franciscana* were selected as model organisms to assess the induction of acute oxidative stress of *Aloe vera* juice (Sirdaarta and Cock, 2008, 2010) showing 34%, 79% and 90% inhibition of thioredoxin reductase (TRed), GRed and GPx, respectively. Thus, an oxidative stress was induced after acute exposure (Sirdaarta and Cock, 2008, 2010) observing that vitamin E could help to partially reduce the effects of *A. vera* juice exposure (Sirdaarta and Cock, 2010).

Glutathione *S*-transferase (GST) activity was investigated during the early developmental stages of *Artemia* spp. hatching in artificial seawater prepared with municipal wastewater effluent. Alterations of the isoenzyme profile were evidenced with a maximum effect after 48 h from hatching. The isoenzyme profile depended upon the organism age and can be affected by environmental factors such as water quality (Grammou et al., 2011).

Katranitsas et al. (2003) measured adenylpyrophosphatase (ATPase) and aldehyde dehydrogenase (ALDH) in brine shrimps exposed to Cu-based antifouling paints evidencing an alteration of *Artemia* osmoregulation up to death. Also Castritsi-Catharios et al. (2012) suggested that ALDH activity in *Artemia* nauplii could be a valuable biomarker for the evaluation of antifouling paint.

The heat stress proteins (HSPs) in *Artemia* were observed in early developmental stages, in encysted gastrula embryos and nauplii. HSP26 is undetectable in unstressed cells, appearing in presence of heat or salt shock, cell cycle arrest, nitrogen or carbon starvation, oxidative stress and low pH. The protein p26 is a small heat shock/α-crystalline protein exhibiting an in vitro molecular chaperone activity conferring a thermo-tolerance (Thomas, 2003). Its synthesis plays a critical role in embryo encystment, diapause and quiescence (Delinger, 2002; Miah et al., 2010).

Proteomic approaches were considered to compare non-stressed (negative control) and stressed organisms (Migliore et al., 2007) improving the understanding of physiological mechanisms underlying stress response and/or tolerance and highlighting putative biomarkers in the differentially expressed proteins (Rotini et al., 2013). For example, the microtubule proteome encompasses tubulin and a group of proteins associated with tubulin upon microtubule formation determining microtubule organization and functioning. In post-diapause development (0 and 12 h) cell free extracts of *A. franciscana*, tubulin assembly was investigated in presence and absence of taxol (Paclitaxel, 10 μM) (O'Connell et al., 2006) evidencing poorly formed microtubules and abundant low molecular mass proteins (Day et al., 2003). No proteins were present in the absence of taxol. With taxol, ten of the fifty-five proteins identified in *A. franciscana* proteome appeared at both exposure times (0 and 12 h): all decreased except for one. Fructose 1,6-bisphosphate aldolase, enolase, HSPs and ATP-binding protein are represented in the undeveloped cysts as isoforms differing in both isoelectric point and molecular mass. In post-diapause *A. franciscana*, these proteins were present in reduced amount. Because only the 40S ribosomal protein S12 increased within the microtubule proteome during development (12 h) further in vitro analyses are required for physiological verification.

3.1.3. Teratogenicity test

Artemia spp. larval stages growth inhibition can be also considered as a teratogen test system based on disturbance of elongation development (relative to controls raised at the same time and conditions) from 24 to 48 h in animals cultured in medium containing a presumptive teratogen (Olson, 1979; Kerster and Schaeffer, 1983). Even though the protocol is not suited to particulates, substances volatilized from water solution at 25 °C or waters may have very low concentrations of teratogens such as municipal wastewaters or receiving waters. Growth length inhibition is calculated considering measurements between the well-pigmented eye and the poorly defined end of the tail by centering an animal in the stereomicroscope's field. With the exception of zinc, chemicals in the μg/L range were not teratogenic, whereas chemicals between 0.25 and 25 mg/L were teratogenic (Cd, Hg, Pb, Zn, bromoform, *n*-butylphthalate, 1,2-dichloroethane, nitrobenzene, tetrachloroethylene, toluene, 1,2,4-trichlorobenzene and 1,1,3-thrichloroethane). Nevertheless the limited data, Kerster and Schaeffer (1983) stated that the system is not very sensitive.

3.1.4. Acute larvae behavioural test

Data about *Artemia* spp. short-term acute behavioral test are summarized in Table 1. Swimming speed is the most frequently used behavioral endpoint of physiological status for aquatic organisms (Faimali et al., 2006). Locomotion, like swimming, is used as stress indicator in several ecotoxicological studies representing a sensitive measure of toxic stress for a wide range of environmental contaminants (Little and Finger, 1990). In the last ten years, the swimming behavior was studied as a response to invertebrates' organic or inorganic exposure to pollutants including *Artemia*. There is still a general lack of appropriate biocompatible automation, optoelectronic sensors and algorithms for behavioral data analysis that could represent a way to solve the major shortcomings not yet addressed in ecotoxicology (e.g. toxicity tests to be performed manually and with low-throughput) (Huang et al., 2015). Williams (1994a,b) applied a physical model regarding the appendages of *Artemia* to empirically determine force coefficients acting regimes relevant to developing larvae. This first analysis imported equations exploring changes in the mechanics of swimming in animals too small for direct measurement. Larvae are interesting because, compared to adults, they can undergo fluctuations in size and shape. *Artemia* develops from a small nauplius that ratchets along with one pair of oars to a multi-limbed adult gliding continuously through the water. As the animals approach the adult morphology growing and adding limbs along the trunk, their average swimming speed increases and the jerky swimming of hatchlings changes into the smooth gliding of adults. The gradual change in swimming does not correspond only to the trunk limbs increase, but also to energy budget supporting the movement (Williams, 1994b). Davenport and Healy (2006) assessed the relationship between physical parameters (medium salinity, body density, and buoyancy) and swimming in *A. franciscana* larvae, finding that the horizontal swimming speed was unaffected by salinity and viscosity (i.e. between 8.5–100‰), but the observed differences in vertical swimming rates are solely due to the relatively constant body density. Venkateswara Rao et al. (2007) studied the covered distance and swimming speed of *Artemia* nauplii survivors after 24 h exposure to 4 pesticides (acephate, chlorpyrifos, monocrotophos and profenofos). A computerized video tracking system (Ethovision, Noldus Information Technology, Wageningen, The Netherlands) was used for the automation of behavioral experiments. Twenty-five nauplii were placed individually into a 96 well culture plate and their behavior was recorded. Authors observed that the AChE enzyme activity might be inhibited by pesticides thus acetylcholine accumulated at neuromuscular level altering the locomotion behavior of organism interrupting the coordination between the nervous and muscular junctions. Larsen et al. (2008) analyzed the effect of temperature-dependent viscosity of ambient seawater (20‰) on the swimming velocity of brine shrimps using video-microscope recordings at various temperatures (between 10 °C and 20 °C) and viscosities (between 1×10^{-6} m^2/s and 1.6×10^{-6} m^2/s) (but keeping a constant temperature of 20 °C). They suggested that modifications in swimming velocity of *Artemia* were due to changes in kinematic viscosity. Recently, Garaventa et al. (2010) recorded the swimming speed alteration of *Artemia* together with mortality, using a video camera

with a macro-objective for recording the larval swimming paths. Larvae were dark-adapted for 2 min before the video start (i.e. the experimental time required to reach steady speed and uniform spatial distribution) and the images were analyzed using an advanced image processing software to reconstruct the individual paths/tracks. This system provided a suitable tool to detect linear swimming speed of *Artemia*, since authors obtained a velocity (3.05 mm/s) in accordance with other studies using the same organism. Alterations in swimming speed were detected for various compounds (Zn pyrithione, Macrotrol® MT-200, and eserine) <0.1–5% of their LC_{50} values. Gambardella et al. (2014) used the same system investigating the toxicity of metal oxide nanoparticles (MO-NPs) to *A. salina*, using swimming speed, and enzymatic activity alterations, and mortality as endpoints thus including continuous or periodic observation of single animals in a non-endpoint perspective. Although no mortality was found, MO-NPs decreased larvae swimming speed and biochemical responses, evidencing the sensitivity of non-lethal endpoints. Alyuruk et al. (2013) proposed another video-tracking method assessing whether monitored *Artemia* are dead or alive observing colors/textures of nauplii. Authors exposed *A. salina* nauplii at various concentrations between 7 and 100 mg/L of $K_2Cr_2O_7$ and *p*-coumaric acid. Considering results by microscopy observation, the proposed algorithm tracks and counts were robust relative to the number of motile nauplii. This kind of automated analysis was also able to provide additional quantitative data such as curvature of paths, number of stops and immobile time. Manfra et al. (2016) compared the swimming speed alteration to hatching and mortality exposing *Artemia* to $CuSO_4$, SDS and DEG. The system for measuring the swimming speed proposed by Garaventa et al. (2010) showed that swimming speed was more sensitive than mortality, with sensitivity similar and sometimes higher than cysts' hatchability.

Recently, Huang et al. (2015) proposed a proof-of-concept lab-on-chip platform capable of performing fully programmable time-lapse and video-microscopy of multiple samples for rapid *A. franciscana* ecotoxicity analysis. This method dynamically detected sub-lethal behavioral endpoints, such as changes in speed of movement or distance travelled by each animal, observing how they changed compared to the negative control exposure after 24 h contact time. Nevertheless these endpoints present promising perspectives compared to Artoxkit (2014), further research activity is strongly required to identify clear contaminant-response relationships.

3.1.5. Acute mortality test

Data about *Artemia* spp. short-term acute immobilization/mortality test are summarized in Table 2. The *Artemia* suitability for acute toxicity studies is well documented in the scientific literature over the past 80 years (Boone and Baas-Becking, 1931). The first general method for conducting toxicity tests with *Artemia* spp. can be attributed to Michael et al. (1956). This paper stimulated further research about the test development and definition of more detailed testing procedures. In 1975, this extensive research led to the creation of the *Artemia* Reference Center (ARC-test) at Ghent University (Belgium) and the release of the first short-term toxicity protocol (i.e. mortality) with brine shrimp larvae (Vanhaecke et al., 1981; Vanhaecke and Persoone, 1981; Vanhaecke and Persoone, 1984). The bioassay is carried out in static conditions for 24 h at $25 \pm 1\,^{\circ}C$ in darkness in covered glass Petri dishes (60 × 12 mm). A homogeneous population of Instar II–III nauplii hatched from cysts (100 mg) was exposed to SDS as reference toxicant. If the test meets the quality criteria it is considered as valid: negative controls ≤10%, and 13.3 mg/L < LC_{50} (SDS) < 19.9 mg/L, dissolved oxygen (DO) concentration after 24 h > 2 mg/L in the lowest concentration with 100% mortality of larvae (Vanhaecke and Persoone, 1981).

An international intercalibration exercise focused on Vanhaecke and Persoone (1981) protocol assessed its reliability, accuracy and precision considering $K_2Cr_2O_7$ and SDS as reference toxicants for intra- and inter-laboratory exercises. The study involved 67 laboratories (59 laboratories in Europe and 8 in US) (Vanhaecke and Persoone, 1984). For SDS, the interlaboratory coefficient of variation (CV) was 24.82% and 25.25% for European and US laboratories, in this order. Considering $K_2Cr_2O_7$ as reference toxicant, the CV was 34.89 and 18.49% for European and US, respectively (Vanhaecke et al., 1981; Vanhaecke and Persoone, 1984). Results evidenced that the toxicity test was carried out with little difficulties showing satisfactory and reliable results. Persoone and Castritsi-Catharios (1989a,b) used the approach of the *Artemia* Reference Center (ARC) (University of Gent, Belgium) test to set a standard protocol for routine toxicity testing of antifouling and other potentially hazardous paints. The effectiveness of this technique was assessed by further studies (Castritsi-Catharios et al., 2007, 2013, 2014). This protocol was engineered in a toxkit called Artoxkit (MicroBioTests Inc., Mariakerke (Gent), Belgium) (Artoxkit, 2014) including a batch of dormant eggs (cysts) of *A. franciscana* to generate "on demand" larvae eliminating the need for continuous culturing. In 1989, international intercalibration exercises involving 129 laboratories between Europe, USA and Canada investigated the reliability of Artoxkit on $CuSO_4$ as reference toxicant. Results were encouraging with only few participants reporting problems. The precision was not striking with a CV equal to 50% in Europe and USA, and 33% in Canada. Criticisms raised about the inexperience of some participants and the instability of $CuSO_4$ in seawater determining a decrease in toxicity over the course of few days and creating some misunderstanding about moribund and really dead nauplii (Persoone et al., 1993). Many authors used the Artoxkit since its commercialization (>180 results in Google Scholar searching for "Artoxkit" 18th Jan 2016) like Koutsaftis and Aoyama (2007).

Solis et al. (1993) suggested a modification of the acute toxicity test developing a new *A. salina* microplate cytotoxicity assay requiring a small amount of testing sample (0.6 mg) up to 1 mg/mL in artificial sea water. The use of 96-well microplates in 100 μL sea water enabled testing a large number of samples.

A detailed procedure to perform acute test with *Artemia* spp. was reported by Guzzella (1997) and APAT and IRSA-CNR (2003) using nauplii at II–III Instar stage. They reported the outcome of an interlaboratory study (acute 24 h mortality) between five Italian laboratories investigating SDS, $K_2Cr_2O_7$ and $CuSO_4$ as reference toxicants. Toxicity tests were carried out using as dilution water both ASPM artificial seawater (NaCl = 26.4 g, KCl = 0.84 g, $CaCl_2{\cdot}H_2O$ = 1.67 g, $MgCl{\cdot}H_2O$ = 4.6 g, $MgSO_4{\cdot}7H_2O$ = 5.58 g, $NaHCO_3$ = 0.17 g and H_3BO_3 = 0.03 g in 1 L of ultrapure water) and Instant Ocean. The CV was between 28% and 52% using ASPM and between 21% and 64% using Instant Ocean, suggesting that ASPM is more suitable than Instant Ocean.

Between 2006–2009, the 24 h mortality test with *A. franciscana* was intercalibrated together hatching assay (Manfra et al., 2015b). Three inter-comparison results constituted the dataset for calculating the 24 h LC_{50} mean and the repeatability/reproducibility coefficients on $CuSO_4$. The CV value and the *r* and *R* values indicated acceptable inter-laboratory test-reliability similar to ISO (1996). Guzzella (1997) and APAT and IRSA-CNR (2003) methods were used for cysts hatching. Larvae transfer from cyst hatching Petri dish to test chambers was operated minimizing as much as possible the dilution of test medium. Mortality was defined operationally as total absence of movement (i.e. swimming activity or movement of appendices) after mechanical stimulation (i.e. touching the larvae with the tip of a glass Pasteur pipette) for approximately 10 s of observation.

Table 3
Artemia spp. long-term toxicity tests.

References	Test	Toxicant	Concentrations (mg/L)	Exposure (h)	Cysts (mg)	Endpoint	T (°C)	Salinity (‰)	pH	Test light (lx)	Aeration	Dilution medium	Food (cell/mL)	Organisms per chamber	Replicates	Test vessel (mL)	Test mL	QC	Reference Toxicant	NOEC/LOEC/LC_{50}/EC_{50}
Browne and Wanigasekera (2000)	n.a.	Salinity	n.a.	21	n.a.	n.a.	15-24-30	60-120-180	n.a.	Fluorescent light	no	ASW (renewal every 3 d (M) d 7 d (R)	Yeast, and *Dunaliella* sp.	5/jar	10	jars	100 (M); 250 (R)	n.a.	n.a.	NOEC = 8 (parental SU) NOEC = 56 (parental G) NOEC = 56 (parental R) NOEC = 56 (F1 SU) NOEC = 56 (F1 G)
Cunningham (1976)	n.a.	Dimilin	0-0.001-0.002-0.005-0.010	21-28	n.a.	R	25 ± 2	25	8.2	n.a.	n.a.	ASW	Daily renewal	15	5	n.a.	n.a.	n.a.	n.a.	n.a.
Nováková et al. (2007)	n.a.	$CdCl_2$, $ZnSO_4$	Cd: 5-10-15-25-50-100-250; Zn: 50-100–250 mg	10	n.a.	M	20 ± 1	n.a.	n.a.	n.a.	n.a.	NSW	Glucose	10	n.a.	polystyrene Petri dishes	10	n.a.	n.a.	Synergistic or antagonistic effects
Gebhardt (1976)	n.a.	Cd, Cu, Hg	Cd: 0-1.0-3.3-33.0; Cu: 0-0.3-0.03-0.003; Hg: 0-0.001-0.003-0.05	25	n.a.	G (length), R (time (d) until nauplii production)	27 ± 2	150-320	n.a.	16 h fluorescent light	n.a.	NSW (renewal every 3 d)	*D. viridis* (40–50 cells/mm^3) every 3 d	5	n.a.	300	n.a.	n.a.	n.a.	n.a.
Manfra et al. (2012)	semi-S	SDS	1.56-3.12-6.25-12.5-25	14	n.a.	M	25 ± 2	35 ± 2	n.a.	900 ± 100 and 14 h light	n.a.	ASW*	*D. tertiolecta* (1 × 10^5 cells/mL)*	10	3	100	50	CM ≤ 20%	SDS	LC_{50} = 8.0 ± 5
Manfra et al. (2015a)	semi-S	DEG	12500-25000-50000-100000-200000	14	n.a.	M	25 ± 2	35 ± 2	n.a.	900 ± 100 with 14 h light	n.a.	ASW*	*D. tertiolecta* (1 × 10^5 cells/mL)*	10	3	100	50	CM ≤15%	n.a.	NOEC(14 d) = 25000
Manfra et al. (2015a,b)	semi-S	SDS	1.6-3.1-6.2-12.5-25.0	14	n.a.	M	25 ± 2	35 ± 2	n.a.	900 ± 100 with 14 h light	n.a.	ASW*	*D. tertiolecta* (1 × 10^5 cells/mL)*	10	3	100	50	CM ≤15%	$K_2Cr_2O_7$	EC_{50}(14 d) = 8.03 ± 1.11 (n = 5); EC_{50}(14 d) = 8.50 ± 3.34 (n = 24)
Savorelli et al. (2007)	semi-S	Dispersant	3.12-6.25-12.5-25	7-14	n.a.	M, G (carapace length)	25 ± 1	35 ± 1	n.a.	900 ± 100 with 14 h light	n.a.	ASW*	*D. tertiolecta* (1 × 10^5 cells/mL)*	10	3	100	50	CM ≤ 20%	SDS	NOEC(7 d) = 6.25 (M, G); NOEC(14 d) = 6.25 (M and G, *A. parthenogenetica*); NOEC(14 d) = 3.12 (M, *A. franciscana*) NOEC(7 d) dispersant = 5 (M, *A. franciscana*)

ASW = artificial seawater; CM = negative control mortality; DEG = diethylene glycol; EC50 = effect concentration for 50% population; G = growth; LC50 = lethal concentration for 50% population; LOEC = lowest observed effect concentrations; M = Mortality; n.a. = not available; NOEC = no observed effect concentrations; NSW = natural seawater; R = reproduction; S = static; SU = survival; SDS = sodium dodecyl sulphate; * = renewal after 2-5-7-9-12 d.

3.2. Long-term toxicity testing

Data about *Artemia* spp. long-term immobilization/mortality test are summarized in Table 3. Most efforts in developing toxicity tests with *Artemia* spp. have been focused on acute endpoints during the last 40 years (Sorgeloos et al., 1978; Vanhaecke et al., 1980, 1981; Persoone and Castritsi-Catharios, 1989a,b) and thus few (and still not internationally standardized) chronic protocols exist.

Gebhardt (1976) evaluated the chronic effects of Cd, Cu, and Hg on *A. salina* growth and reproduction using a static renewal test with medium and food replacement every 3 days. Natural seawater from the Great Salt Lake (Utah, USA) was used as testing medium, and brine shrimp were fed with *Dunaliella viridis*. Adults were investigated for immobilization, newly hatched nauplii for growth and reproduction inhibition and eggs for hatching experiments. Cu and Hg did not affect growth and reproduction at concentrations below acute mortality. Mating occurred both in negative controls, and Cu and Hg spiked media when brine shrimps were approximately 15 days old. The maximum length reached was of about 8 mm. Only Cd between 1.0 and 33 mg/L significantly inhibited growth and reproduction rate. Cd delayed mating of about two days compared to the controls. No mating occurred at 33 mg/L of Cd, and no nauplii were produced during the experiment at this concentration.

Cunningham (1976) investigated the effects of Dimilin (TH 6040) on various brine shrimp life stages considering a static renewal test. *Artemia* cultures consumed algae present in the jars and were supplemented with a few drops of yeast suspension daily. Brine shrimp reproduction was evaluated exposing pairs of organisms and monitoring the subsequent number of produced nauplii. After 21 days, adult survivorship in the negative control was approximately 90% and declined to 70% after 28 days. After 40 days, survival dropped below 50% and all shrimps were dead after 80 days.

Browne and Wanigasekera (2000) measured reproductive performance and survival at nine temperature–salinity (T–S) combinations (T = 15, 24 and 30 °C, and S = 60, 120 and 180‰) for four sexual (*A. franciscana*, *A. salina*, *Artemia sinica* and *Artemia persimilis*) and one parthenogenetic (*A. parthenogenetica*) species. During survival tests, *Artemia* was fed with yeast and *Dunaliella tertiolecta* once a day alternately. The number of dead individuals was counted after day 1, 2, 3, 5, 7, 14 and 21. At sexual maturity, surviving individuals were transferred to jars containing brine and algal culture and monitored throughout their life cycle recording eleven life-history traits for each species (i.e. number of reproductive females, female pre-reproductive and reproductive periods, life-span for reproductive females, offspring per brood, broods per female, offspring per female, offspring per day per female, inter-brood interval, percentage of offspring encysted, and male life-span). All species showed the highest reproduction rate at 24 °C and at 120‰ salinity for *A. parthenogenetica*, *A. sinica* and *A. franciscana*, and at 180‰ for *A. salina* and *A. persimilis*. Only at 24 °C and 120‰ salinity all species completed their whole life cycle. Results indicated that *A. franciscana* is both euryhaline and eurythermal reproducing at four T–S combinations. *A. salina* appeared cold-adapted and intolerant to low salinity (60‰) at any temperature. Reproductive performance and survival were optimal at 24 °C and 180‰ salinity.

Brix et al. (2003) determined the chronic (28 d) toxicity of As (Na_3AsO_4) to *A. franciscana* measuring in triplicate the effects on growth, reproduction, and survival under intermittent flow-through conditions considering a full life-cycle approach (Stephan et al., 1985). The study started considering 24 h old nauplii, and continued looking at the reproduction of the parental generation. Cysts were hatched in Great Salt Lake (Utah, USA) seawater (27 °C and 28 g/L of salts) that was used also as dilution water. After 11 d, brine shrimp sexually matured and began pairing for mating. At this time, a random subsample from each test concentration was collected and weighed (dry weight). Six adult pairs for each test concentration were then monitored for reproduction up to day 28 when surviving shrimps were measured for dry weight. Adult survival was the most sensitive endpoint, with growth and reproduction slightly less sensitive than survival. The no observed effect concentration (NOEC) for survival was 8 mg/L, and the lowest observed effect concentration (LOEC) was 15 mg/L of dissolved As. Growth and reproduction LOEC values were >56 mg/L that is the highest concentration tested. After the parental generation exposure to As, the F1 generation acclimated to As thus presenting a significant lower sensitivity than the parental generation.

Brix et al. (2004) investigated the use of *Artemia* for the derivation of a chronic site-specific water quality standard for Se in the Great Salt Lake (Utah, USA). The method previously described in Brix et al. (2003) was applied to *A. franciscana* exposed to different Se nominal concentrations (0, 3.8, 7.5, 15, 30, 60 mg/L). Growth of the parental generation on day 11 and reproduction on day 21 were the two most sensitive endpoints for *Artemia* with respect to the other species evaluated (i.e. brine fly and hypersaline algae). Brine shrimp was the Great Salt Lake's most sensitive species resident, and the direct effects of Se on resident biota were not the critical exposure pathways in deriving a site-specific water quality discharge limit for the Great Salt Lake.

Savorelli et al. (2007) evaluated the conditions for 7–14 d bioassay with the brine shrimp *Artemia*, using *A. franciscana* and *A. parthenogenetica*. Exposures to SDS and oil dispersant were carried investigating somatic growth (i.e. carapace length from the top front of the head up to the caudal furca) and mortality. Algal food and toxicant were renewed three times a week. Test chambers were incubated at 25 ± 1 °C, 900 ± 100 lux with a 14:10 h light/dark photoperiod. Savorelli et al. (2007) proposed the use of II–III instar stage nauplii, *Instant Ocean*® salt mixture, and *D. tertiolecta* as food source for the 14 d toxicity test (sensitivity increases between 7 d and 14 d) including a negative control success ≥80%. Mortality was selected as endpoint for the proposed method being more sensitive than growth for both *A. franciscana* and *A. parthenogenetica*.

Manfra et al. (2012) proposed a video presenting on a step-by-step basis the 14 d toxicity test protocol of Savorelli et al. (2007) including testing materials (cysts, dilution media, algal culture and toxicant), cyst activation, preparation of testing toxicant (SDS) concentrations, preparation of testing media and food renewal. The video showed how to transfer nauplii (n = 10) from Petri dishes to test chambers in order to reduce dilution of testing solutions. After 14 d, the number of surviving larvae was counted and LC_{50} calculated considering a threshold average mortality ≤20% and LC_{50}(SDS) = 8.0 ± 5 mg/L. Manfra et al. (2015a) considered a 14 d protocol with *A. franciscana* to investigate the toxicity of DEG as an anticorrosive agent frequently used in offshore oil drilling activities.

Manfra et al. (2016) published the results of the intercalibration exercise with SDS considering the 14 d toxicity test. As for the acute mortality test, eleven participating laboratories carried out three intercomparison assays calculating LC_{50} values and repeatability/reproducibility coefficients. Results complied with ISO (1996).

4. Discussion

After more than five decades of use in ecotoxicology, *Artemia* spp. demonstrated its ability mainly as pre-screening of toxic agents (Dvorak et al., 2012), thus *Artemia* spp. endpoints seem to respond to the market need of toxicity testing tools, even though no internationally standardised toxicity testing protocols currently exist according to OECD and ISO.

Among the short-term toxicity tests, biomarkers and teratogenicity are the less popular endpoints with just few papers and citations probably due to their limited sensitivity. Behavioral endpoints, especially swimming inhibition, have been investigated only recently and seem to have a great potential in the near future, mainly because results can be acquired via image and video analysis taking into account both continuous and periodic observations of single or groups of animals. Hatching and acute mortality are the most common endpoints with a different level of on-going standardization process. Hatching (48 h static test) was intercalibrated at Italian level (Manfra et al., 2015b), while acute mortality (24 h static test) was intercalibrated on the basis of the available standard (APAT and IRSA-CNR, 2003) at Italian (Manfra et al., 2015b) as well as European level (Persoone et al., 1993). Both provided data on $CuSO_4$ as reference toxicant. Among the long-term toxicity tests, the 14 d static-renewal mortality test was intercalibrated at Italian level (Manfra et al., 2015b) with SDS on the basis of Unichim protocol (2012).

To make *Artemia* spp. an official internationally recognized standard biological model in ecotoxicology and nanoecotoxicology, further efforts are necessary. The steps towards standardization should involve (i) national member (who then contacts ISO) upon a request by an industry sector or group for a standard; (ii) creation of a group of experts from all over the world negotiating all aspects of the standard, including its scope, key definitions and content; (iii) multi-stakeholder brainstorming and reviewing process including experts from the relevant industry, consumer associations, academia, non governmental organizations and government.

We highlight some tasks to be easily accomplished as a correct practice to ameliorate the feasibility, reliability and comparability of *Artemia* spp. toxicity data including all endpoints and potentially contributing to support official standardization. Authors did not report and reviewers did not require several key parameters. According to Tables S1, S2, 1–3, frequent omissions are present: pH, aeration conditions (DO), amount of hatched cysts, feeding rate, test container, quality criteria (QC) for negative controls and reference toxicant. In Table S1, the acceptability threshold for cysts hatchability is repeatedly missing like as the reference toxicant. Hatching toxicity tests with *Artemia* spp. never provided saltwater pH values (Table S2), and, just in few cases, data about light conditions during exposure, and positive and negative controls quality criteria for acceptability. In Table 1, the positive control is frequently absent like as the determination of EC50 values for the swimming velocity inhibition, thus limiting the comparability with other endpoints' sensitivity. Within the behavioral endpoints, a harmonization of protocols is strongly required also because authors proposed time-by-time specific approaches, apparatuses, tracking and recording devices. In Table 2, the database is quite complete probably because the 24 h short-term toxicity test is the most widespread. Missing information is generally related to the amount of hatched cysts and DO that represent interesting parameters to fully circumscribe the general test (pre-)conditions. Similarly, it was found for Table 3 where data about reference toxicants are very scarce too. Finally, it is not clear which is the cost effectiveness of each endpoint compared to its relative sensitivity and current readiness for the market.

In conclusion, we can provide the following order of easy-to-standardize toxicity tests: 24–48 h mortality > 14-28 d mortality > hatching test > behavioral endpoints > biomarkers.

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.2016.04.017.

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