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The case of *Artemia* spp. in nanoecotoxicology

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ABSTRACT

Artemia spp. is one of the most widespread saltwater organism suitable for ecotoxicity testing, but no internationally standardised methods exist. Several endpoints can be considered with *Artemia* spp. including short-term (24–48 h) and long-term (14 days) mortality, cysts and nauplii hatchability, biomass productivity, biomarkers' expression/inhibition and bioaccumulation on larvae as well as organisms' reproductive ability. Recently, *Artemia* spp. started to be used as a reference biological model in nanoecotoxicology with both inorganic and organic engineered nanomaterials (ENMs) also in combination with traditional environmental stressors looking for potential interactive effects. Criticisms were detected about the use of *Artemia* spp. in relation to the hatching phase, the toxicity test design, the occasional use only of reference toxicants and the way testing solution/suspensions were prepared thus potentially compromising the reliability of nanoecotoxicological results. A full list of compulsory information that must accompany *Artemia* nanoecotoxicity data is provided with positive feedbacks also for other toxicity bioassays.

1. Introduction

Artemia salina is a zooplanktonic crustacean and is one of the most widespread saltwater organism used in ecotoxicity testing. It is present in a variety of seawater systems and is able to filter a large amount of water per hour (Ates et al., 2013a,b,c). Bioassays can involve the use of cysts or be dependent on animals cultured in the laboratory. According to Nunes et al. (2006), cysts based assays are cheap, simple and reliable in routine screening, whereas cultured organisms can be more suited for basic scientific research because of their low genetic variability and thus homogeneity. Several endpoints can be considered with *Artemia* spp. including short-term (24–48 h) (Vanhaecke and Persoone, 1981.) and long-term (14 days) (Manfra et al., 2012) mortality, cysts and nauplii hatchability, growth of biomass production inhibition, including behavioural (such as swimming speed alteration) (Gambardella et al., 2014) and biomarker related ones (e.g. enzyme expression or inhibition, oxygen consumption rate) and reproductive ability (Nunes et al., 2006). *Artemia* spp. has been greatly used in ecotoxicological testing because of its adaptability to a wide range of salinities (5–300 g/L) and temperatures (6–40 °C) (USEPA, 2002), considering mortality and growth as the main endpoints (Sarabia et al., 1998). *Artemia* spp. has a short life cycle, high flexibility to adverse environmental conditions, high fecundity, bisexual/parthenogenetic reproduction strategy, small body size and adaptability to varied nutrient resources as it is a non-selective filter-feeder (Nunes et al., 2006). As such, these characteristics should make *Artemia* spp. as a really suitable organism for ecotoxicity testing, but several criticisms about its sensitivity have been presented according to a leaning-by-doing approach. The sensitivity towards a wide range of substances is really lower than that of other species, so that the possibility to underestimate potential effects may occur. *Artemia* spp. showed to be less sensitive than *Streptocephalus rubricatus* and *Streptocephalus texanus* (Crisinel et al., 1994), *Echinometra lucunter*, *Crassostrea rhizophorae* (Nascimento et al., 2000) and *C. gigas* (Libralato et al., 2007), *Selenastrum capricornutum* and *Dunaliella tertiolecta* (Gaggi et al., 1994), *Vibrio fischeri*, *Daphnia magna* and *Brachionus plicatilis* (Guerra, 2001), *Daphnia similis* (Silva et al., 2004), *Paracentrotus lividus* (Fichet et al., 1998), *Daphnia pulex* and *Thamnocephalus platyurus* (Nunes et al., 2006), and *Spirostomum ambiguum* and *Tetrahymena thermophila* (Nalecz-Jawecki et al., 2003). Other criticisms could be related to the use of cysts (Migliore et al., 1997) and cyst-hatched nauplii (Persoone and Wells, 1987) of *Artemia* for testing purposes that could introduce some variability in test results. Indeed, in the genus *Artemia* there is no cyclic bisexual versus

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parthenogenetic mechanism like for rotifers or cladocerans, that is generally on a seasonal basis, but cysts production may reflect the occurrence of genetic variation to increase adaptability; it is evident that genetic variability is not a desirable condition for ecotoxicity assays (Nunes et al., 2006). At the international level, the use of *Artemia* spp. in toxicity testing is subjected to a broad discussion with supporters and detractors (Nunes et al., 2006).

Recently, *Artemia* spp. has started to be used as a biological model in nanoecotoxicology. The aim of this paper is to review the existing approaches highlighting the main limits and criticisms in the investigation of engineered nanomaterials (ENMs) effects. Suggestions are made about the basic information to be provided to strengthen data quality and reliability in the perspective of future hazard and risk assessment.

1.1. *Artemia* spp. and nanoecotoxicology

Currently, sixteen papers about *Artemia* spp. and nanoparticles are available considering inorganic ($n = 10$), organic ($n = 1$) and mixtures ($n = 5$) of ENMs and other chemicals. They included C_{60} and 17α-ethinylestradiol (single substances and binary mixtures) (Park et al., 2010), nPb (Cornejo-Garrido et al., 2011), single walled carbon nanotubes (SWCNTs) associated with N-octyl-O-sulphate chitosan (Fatouros et al., 2011), C_{60} and $nTiO_2$ (Rajasree et al., 2011), bare and silica-encapsulated magnetic nanoparticles (Ashtari et al., 2012), nAg (Kumar et al., 2012), uncoated α-Al_2O_3 and γ-Al_2O_3 (Ates et al., 2013a), nZn and nZnO (Ates et al., 2013b), $nTiO_2$ (Ates et al., 2013c), carbon black Printex XE2, multi-walled carbon nanotubes (MWCNTs), molybdenum compound nanowires and molybdenum oxide nanowires (Baumerte et al., 2013), nAg coupled with squilla chitosan, cephadrin and piperacillin (Gohar et al., 2013), nAu (Karthik et al., 2013), nAg (Arulvasu et al., 2014), nano-SnO_2, nano-CeO_2 and nano-Fe_3O_4 (Gambardella et al., 2014), pristine graphene and pristine graphene monolayer flakes and graphene nano-powder grade (Pretti et al., 2014) and nAg and nAu (Vijayan et al., 2014).

All papers focused to the genus *Artemia*, including *A. salina* (Ates et al., 2012) or *Artemia franciscana* (Minetto et al., 2014). They were reviewed for methodological aspects in ascending chronological order adopting a step-by-step approach from the purchase/production of cysts to the final expression of toxicity data.

Park et al. (2010) studied the bioavailability of 17α-ethinylestradiol (EE2) in male adult *Danio rerio* exposed through the diet to *Artemia* spp. accumulating nC_{60}, EE2 and a mixture of nC_{60} and EE2 within their gastrointestinal tract. Thus the accumulation and quantification of nC_{60} in *Artemia* spp. were investigated as well. Cysts (6.8 g) of grade A brine shrimp eggs (Brine shrimp direct, Ogden, UT, USA) were hatched in 1 L saltwater (15 g/L of Instant Ocean®) for 24 h at an unspecified hatching temperature. After hatching, nauplii were transferred to 100 mL of brackish water. Stock suspension of C_{60} (600 mgC_{60}/900 mL of MilliQ water) and solution of EE2 (10 mg/L) using ethanol as carrier were prepared by continuous long-term stirring (6 months) and protected from light. Nauplii were contaminated spiking the living medium starting from stock suspensions. Spiked brine shrimps were rinsed several times with deionised water prior to feeding *D. rerio*. Positive controls were not mentioned. Exposure temperature and light and feeding regimes of nauplii during the spiking period were missing. *Artemia* spp. larvae showed to rapidly accumulate nC_{60} in the gastrointestinal tract already after 30 min of exposure. The accumulation of nC_{60} in brine shrimps after 2 h was seven-fold greater starting from a 10% v/v nC_{60} suspension rather than a 2% one. Fishes fed with EE2 spiked *Artemia* spp. showed the highest expression of vtg1A/B, whereas no significant changes ($p < 0.05$) were detected between the controls and nC_{60} or the mixture of nC_{60} and EE2 treatments.

Fatouros et al. (2011) assessed the potential toxicity of SWCNTs stabilised by alkyl-sulphate chitosan derivatives of different molecular weights with *A. salina*. Cysts from NT Labs (UK) were incubated in artificial seawater (Tropic Marin, Wartenberg, Germany) at 27 °C for 24 h with constant lighting until hatching. Between 20 and 25 nauplii in triplicate were exposed in 10 mL volume composed of 0.02 mL of phosphate buffer solution and 0.98 mL of *N*-octyl-*O*-sulphate chitosan (NOSC) associated to SWCNTs for 24 h at 27 °C; 48-well plates were used as incubation vessels. Besides the effects of NOSC (100, 200 and 300 mg/L) and microwave purified SWCNTs (100 mg/L) alone were considered as well. Changes in temperature and water composition were introduced compared to the declared reference protocol (Vanhaecke et al., 1981). *Artemia* larvae were counted by the use of a stereoscope. NOSC and SWCNTs alone showed no effects on the viability of nauplii. Authors observed that NOSC-SWCNTs were uptaken by brine shrimps and subsequently excreted in the form of "enveloped dark rod-like structures" without compromising their general mobility. Negative and positive controls were not clearly mentioned.

Cornejo-Garrido et al. (2011) investigated the acute toxicity induced by nano-sized lead in aqueous suspensions on *A. salina* mortality according to Petrobras (1996). Brine-shrimp cysts obtained from a pet shop were hatched in Petri dishes containing artificial seawater (3.7% w/v) from Instant Ocean® marine salts dissolved in deionised water (pH = 8); cysts were incubated at 25 ± 1 °C for 48 h. Nauplii were separated from their shells and the remaining cysts, and transferred to freshly-prepared seawater with a Pasteur pipette. Bioassays were carried on in 96-well culture plates. Ten larvae were hosted in 0.1 mL of artificial seawater. Suspensions were prepared in deionised water and added (0.1 mL) to the wells containing *A. salina*, but the final exposure salinity was not verified. Negative (seawater) and positive ($K_2Cr_2O_7$) controls were included. The percentage of larvae mortality was checked after 24 h contact time. Results evidenced that *A. salina* swallowed lead nanoparticles, but they did not cause mortality.

Rajasree et al. (2011) examined the acute lethal effect of nC_{60} and $nTiO_2$ on *A. salina* considering various larval stages: 1st Instar, 2nd Instar, zoea and metanauplii. Suspensions of nC_{60} and $nTiO_2$ were prepared according to Lovern and Klaper (2006) and Oberdorster (2004) via sonication and filtration. For each test about 100 mg of cysts were incubated in 100 mL of seawater (35%) at 27 ± 1 °C in a cylinder-conical tube with lateral illumination of 500–100 lux keeping them in suspension by gentle aeration. Seawater prior to use was filtered at 0.2 μm at checked for pH (7.5 ± 0.5) and a minimum oxygen content (90% saturation). Ten larvae at various developmental stages (1st Instar, 2nd Instar, zoea and metanauplii) were exposed in 10 mL at 27 ± 1 °C for 1, 6, 12, 24, 36, 48 and 96 h to test suspensions. Larvae were exposed to 40, 180, 260, 350, 440, 510, 700 and 880 μg filtered nC_{60}/L, to 0.5, 1, 2, 4, 5, 6, 7, 8, 9 and 10 mg sonicated nC_{60}/L, to 0.2, 1, 1.2, 2.5, 3.6, and 10.1 mg filtered $nTiO_2$/L and to 50, 100, 150, 200, 250, 300, 350, 400, 450 and 50 mg sonicated $nTiO_2$/L. Larvae were not fed during bioassays. No reference was made to a standardised protocol. Suspensions prepared via filtration showed higher toxicity levels than sonicated ones. The toxicity effects increased at subsequent developmental stages due to the fact that organisms feed themselves more voraciously. Apart from mortality, some sub-lethal responses were highlighted such as fast erratic movements with animals unable to move for prolonged periods potentially playing a role in increasing the predation risk. Negative and positive controls were not mentioned.

Ashtari et al. (2012) assessed the potential acute lethal toxicity of bare and silica-encapsulated magnetic nanoparticles using

A. salina according to Michael et al. (1956). Cysts were obtained from the Aquatic Animals Research Center of Urmia University (Urmia, Iran). Larvae were hatched in a bottle containing unspecified artificial seawater (38 g/L) for 28–30 h at room temperature. The experimental design included 10, 100 and 1000 mg/L of magnetic nanoparticles exposing ten nauplii per concentration. Testing vials were put horizontally on a shaker at 8 rpm for better aeration. Larvae mortality was checked after 24 h. Negative controls were not mentioned while gallic acid was used as reference toxicant ($EC_{50} = 20$ mg/L). Authors concluded that both the bare and silica-encapsulated magnetic nanoparticles had no significant toxicity on brine shrimps. The paper did not explain hatching and exposure temperatures, seawater characteristics and source, treatment of suspensions before the exposure, if any, and how the dynamic exposure regime (i.e. shaking vials for better aeration) influenced results (i.e. nanoparticle aggregation) rather than static conditions.

Kumar et al. (2012) assessed the effects of silver nanoparticles biosynthesised from extracts of *Sargassum ilicifolium* by *A. salina* mortality test. Nanoparticles were produced from 100 mL of filtrate (Whatman n. 1) (100 mg seaweed boiled at 80 °C in 100 mL of deionised water) and 0.1 mL of 1 M $AgNO_3$ solution. Nanoparticles were characterised by scanning electron microscope (SEM) and transmission electron microscope (TEM). Brine shrimp cysts were purchased by an unspecified purchaser and hatched (1 g in 1 L) in artificial saltwater (3.2 g of NaCl in 100 mL) for 48 h at 22–29 °C. After hatching, active nauplii were collected without distinguishing between Instar I and II larvae in order to perform the test. The exposure occurred in 2 mL vials in the dark with a 24 h contact time considering suspensions containing 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 nM of silver nanoparticles. No information is available about suspension preparation. The number of nauplii exposed per replicate and the number of replicates were missing. Negative controls with and without seaweed extracts were carried on. Positive controls with reference toxicants were not mentioned. Silver nanoparticles mortality as LC_{50} was about 1.08 mg/L.

Ates et al. (2013a) investigated the potential difference in the effect of uncoated α-Al_2O_3 and γ-Al_2O_3 nanoparticles of two sizes for both substances (50 nm and 3.5 μm and 5 nm and 0.4 μm, respectively). *A. salina* as reference biological model was considered for uptake, toxicity and depuration of nanoparticles after 24 h and 96 h exposure time. Suspensions from nanomaterials stored at room temperature were prepared in MilliQ water at a stock concentration of 20% w/v. They were vortexed (20 s at 2000 rpm) and ultra-sonicated (bath sonicator for 10 min). A magnetic stirrer was used to prevent their settlement while transferring aliquots to testing vessels already containing testing organisms. *Artemia* cysts (Artemia International LLC. Houston, TX, USA) were kept at 4 °C in a moisture-free container. Cysts were hatched in artificial seawater (3% m/v) (instant Ocean®, 24 h aeration and filtration at 30 μm Millipore cellulose filters) according to Persoone et al. (1989). Briefly, cysts were hydrated in distilled water at 4 °C for 12 h and washed to separate the floating ones from those sinking. The sinking cysts were collected on a Buckner funnel and washed with cold deionised water. About 3 g of pre-cleaned cysts were incubated for hatching in 1.5 L of seawater for 24 h at 30 ± 1 °C and 1500 lux providing aeration from the bottom of the hatching device. Experiments with *Artemia* nauplii included the exposure to 5, 10, 50 and 100 mg/L of both α-Al_2O_3 and γ-Al_2O_3 nanoparticles. Toxicity tests were carried on according OECD (2004) that is the *D. magna* immobilisation test. Negative controls were included, but positive ones were not mentioned. Exposures occurred in 500 mL of seawater with aeration provided from the bottom of the conical flask to prevent settling of nanoparticles (2.9–3% m/v salinity, light: dark regime 16: 8 h, water temperature 24 ± 2 °C and pH 7.6–8.8). Organisms were not fed during the tests. The number of larvae exposed counted under magnification lens after sequential dilutions before exposure was in the range $12.9–16.1 \times 10^3$. Batches of *Artemia* were analysed (a) to elucidate nanoparticles deposition at the end of the exposure period by phase contrast microscope, (b) to determine their total uptake and depuration levels after acid digestion by inductively coupled plasma mass spectrometry and (c) oxidative stress (lipid peroxidation) by the malondialdehyde assay. Larvae accumulated both aluminium nano-oxides to high levels, but a limited amount of the micro-sized ones. No acute 24 h toxicity was found, but mortality and lipid peroxidation increased after 96 h exposure. The conclusion is that both nano-oxides showed a marginal acute toxicity to *Artemia* that is mediated by oxidative stress after prolonged exposure periods.

Ates et al. (2013b) used the same procedures described in Ates et al. (2013a) to evaluate the impact of Zn and ZnO nanoparticles (10, 50 and 100 mg/L) on *A. salina* considering both the effect of particle size and solubility on toxicity. Results evidenced that Zn nanoparticles were more toxic than ZnO nanoparticles in similar exposure conditions. The effects were mainly related to the greater amount of Zn^+ released from Zn nanoparticles. Moreover, the size showed to contribute to the observed toxicities. Smaller Zn nanoparticles (40–60 nm) were more toxic than larger ones (80–100 nm), like 10–30 nm ZnO nanoparticles compared to 200 nm ones. Lipid peroxidation presented the highest levels after 96 h exposure.

Ates et al. (2013c) used the same procedures described in Ates et al. (2013a) to evaluate the impact of $nTiO_2$ (10, 50 and 100 mg/L) on *A. salina*. Nanoparticles showed to aggregate in saltwater to form microscale particles, but they had no effect on accumulation. Indeed, nauplii and adults accumulated the microscale aggregates in their gut within 24 h of exposure. No significant mortality was observed after 24 h exposure. Oxidative stress effects with slight mortality were observed after 96 h contact time due to the accumulation of nanoparticles in the gut cavity leading to starvation. Anyhow, authors stated that no food was provided during the exposure period so that the starvation was effective anyway.

Baumerte et al. (2013) assessed carbon black (CB) Printex XE2, MWCNTs, molybdenum compound nanowires and molybdenum oxide nanowires effects using acute *A. salina* toxicity. The Artoxkit M™ method was taken into consideration, but keeping water salinity next to the one of Baltic Sea (15‰). Larvae were hatched from dormant cysts at 25 °C for 36–48 h until Instar II stage. Exposure occurred in 24-well plates (3 mL) considering 6 replicates and 10 organisms per replicate at 20 ± 1 °C. Lethality was observed after 24 h and 48 h. The exposure scenarios were not clearly elucidated in the paper. After 48 h at 10 mg/L the toxicity was CB < MWCNT and molybdenum oxide < molybdenum compound. None of the particles caused significant effects to *A. salina* after 24 h.

Gohar et al. (2013) verified the toxicity of unspecified silver nanoparticles coupled with squilla chitosan, cephadrin and piperacillin against *A. salina*. No information was available about the source of cysts. The testing mixture was prepared in dimethylsulfoxide (DMSO) considering a 1:1:1:1 ratio and investigated in triplicate for potential acute lethal effects after 24 h of contact time at 2500, 2700, 2900, 3000, 3500, 3700, 4000 and 5000 mg/L exposing ten nauplii per replicate. Unspecified brackish water was used for both dilution water and negative controls; DMSO was verified to be non-toxic up to ten times the used concentrations. Other positive controls were not mentioned. The mixture EC_{50} was found between 3000 and 3500 mg/L. The paper did not provide hatching and exposure temperatures, seawater characteristics and the source and the procedure of preparation of suspensions before the exposure.

Karthik et al. (2013) studied the effect of gold nanoparticles. No information is available about testing organisms and exposure

conditions. Authors carried on the toxicity tests (lethality) according to Amat (1985) with unspecified minor modifications. *Artemia* showed no mortality up to 8 mg/L of gold nanoparticles. Negative and positive controls were not mentioned.

Arulvasu et al. (2014) assessed the toxicity effect of silver nanoparticles with *Artemia* spp. brine shrimps collected from salt pan of Kelambakkam (Chennai, India) using a 150–200 μm mesh net. Water cleaned cysts were dried on absorbing paper for one night before decapsulation in sodium hypochlorite without affecting the viability of the embryos. About 2 g of cleaned decapsulated cysts were incubated in 2 L of seawater in a conical plastic container at 30 ± 1 °C and 1500 lux providing aeration from the bottom of the hatching device. Under these conditions, cysts hatched after 24 h. *Artemia* nauplii with less than 24 h were exposed for 24 h and 48 h monitoring growth, viability and mortality under intermittent flow-through conditions. Negative controls were included, but positive controls were not mentioned. Bioassays were carried on in 12-well plates in 2 mL of seawater (33‰) exposing 10 nauplii per replicate. Larvae were exposed to 2, 4, 6, 8, 10 and 12 nM of silver nanoparticles in triplicate. The experimental setup aged 24 h in darkness before the addition of nauplii with no specifications about the storage temperature. Hatched larvae after exposure were checked for (a) mortality, (b) morphological variations compared to the control, (c) apoptosis with acrinidine orange staining and (d) DNA damage with Comet assay. Results evidenced that nauplii guts were filled with silver nanoparticles aggregates causing significant mortality after 24 h of exposure. After 48 h of exposure, mortality significantly increased. Moreover, it was evidenced that hatchability was inversely correlated to nano-silver concentration. The authors reported that "these effects were most likely due to the lack of food uptake", but they did not apparently supply any food to the testing organisms during the test. Indeed, it is not required to supply food to *Artemia* spp. for acute toxicity test (24–48 h) (APAT and IRSA-CNR, 2003; Artoxkit M™ method), but only for short-term acute (96 h) or long-term acute ones (14 days) (Manfra et al., 2012). Arulvasu et al. (2014) reported that at exposure concentrations greater than 2 nM the gut region started to be interested to nano-silver accumulation being completely filled at 12 nM inducing tissue degradation. Authors evidenced that apoptosis induced by silver nanoparticles significantly increased with exposure concentration as well as DNA damage that reached about 48% only at 12 nM of silver nanoparticles.

Gambardella et al. (2014) investigated the toxicity of nano-SnO_2, nano-CeO_2 and nano-Fe_3O_4 on the shrimp *A. salina* considering mortality (48 h) and behavioural (swimming speed alteration) and biochemical responses (enzymatic activities of cholinesterase, glutathiose-S-transferase and catalase). Commercially available dehydrated cysts of *A. salina* (500 mg) were incubated for 24 h at 28 °C under 16 h light, 8 h dark conditions and continuous aeration of the cysts suspension in seawater (37% salinity) to obtain Instar I larvae. Hatched larvae were separated by non-hatched ones and isolated in a beaker containing filtered natural seawater prior to testing. The toxicity tests were performed with 10–15 larvae in 24-well polystyrene plates exposed in triplicate to 1 mL of metal oxide nanoparticles (0.01, 0.1 and 1 mg/L). After 48 h of exposure, larvae were collected, rinsed and transferred into new plates with filtered seawater thus starting the determination of the various endpoints. Negative controls were included, but no positive control was mentioned. Suspensions were prepared from stock solutions of 1 g/L in 0.22 μm filtered natural seawater that were sonicated for 15 min at 100 W using a 50% on/off cycle in an ice bath. Results evidenced that metal oxide nanoparticles did not induce significant mortality effects in the investigated exposure range, but accumulated in the gut and determined detectable behavioural and biochemical changes.

Pretti et al. (2014) studied the ecotoxicity of pristine not-functionalised graphene (pristine graphene (PF), pristine graphene monolayer flakes (PGMF) and graphene nano-powder grade C1 (GNC1)) on *A. salina* (24 h). Toxicity tests were carried on according to Vanhaecke and Persoone (1981). Batches of five nauplii were exposed to treatments into Petri dishes (0.625–10 mg/L) incubated at 25 °C in the darkness for 24 h under gentle shaking (80 rpm). Mortality was assessed by light microscopy and expressed as percentage. Positive controls were carried on with potassium dichromate. Three independent experiments were performed. Besides mortality, *A. salina* was tested after 48 h exposure for oxidative stress, lipid peroxidation, and total glutathione peroxidase and catalase activity. Tests in triplicate with about 2000 nauplii per replicate were performed at 20 ± 1 °C under a photoperiod of 16 h light/8 h darkness (Nunes et al., 2006). All exposure experiments were carried on under stirring and the use of dispersing agents was avoided. Natural seawater (35 g/L salinity) was oxygen saturated and filtered at 0.21 μm. All ENMs showed significant biomarker-related effects compared to the controls at the highest exposure concentrations, but no acute toxicity was found.

Vijayan et al. (2014) assessed the effect of silver and gold nanoparticles against *A. salina* mortality (24 h). They were biosynthesised from the aqueous seaweed extract of *Turbinaria conoides* collected from the wild to be used in antifouling paints. Nanoparticles were produced separately from the authors using aqueous solutions of $AgNO_3$ and $HAuCl_4$ $3H_2O$ and the aqueous filtrate (Whatman n. 1 filter) of 5 g of seaweed powder boiled in 100 mL of sterile deionised water. Nanoparticles were characterised using field emission scanning electron microscopy (FESEM) equipped with energy dispersive X-ray spectroscopy (EDX) as well as with high-resolution transmission electron microscopy (HR-TEM). Brine shrimp cysts were hatched according to Harwig and Scott (1971). The toxicity test included several exposure concentrations – 40, 60, 80, 100, 120, 140 and 160 mg/L – for both nanoparticles. Exposures occurred in 24-well plates containing 1 mL of sterilised seawater with no indications about how nanoparticles were added as well as the final testing volume. Ten nauplii per well were exposed for 24 h at 25 °C, but the number of replicates was not reported as well as the light exposure condition and feeding regime. Negative controls were included, but no positive control was mentioned. Silver nanoparticles mortality was found to be proportional to increasing exposure concentrations (LC_{50} = 89 ± 5 μL/mL). No ecotoxicological data were reported about gold nanoparticles.

2. Discussion

In most of the cases, papers referred to a different protocol or, sometimes, they just did not mention it. This suggests the existence of several approaches that could not present common actions. The most cited testing methods chronologically ordered belong to Michael et al. (1956), Harwig and Scott (1971), Sorgloos et al. (1978), Vanhaecke et al. (1981), Vanhaecke and Persoone (1981), Persoone and Wells (1987), Solis et al. (1993), Petrobras (1996), APAT & IRSA-CNR (2003), Libralato et al. (2007), Manfra et al. (2012), Artoxkit® (2014), Gambardella et al. (2014). It is evident that the absence of a standardised method (ISO, ASTM or OECD) to refer to with *Artemia* spp. in nanoecotoxicology is a main gap that should be filled as soon as possible to make *Artemia* spp. an official standard biological model. All the mentioned approaches present from minor to major changes in at least one of the phase sequence test procedure including all materials in general (saltwater

composition), the hatching phase, and the handling and exposure of nauplii or adults (such as the number of exposed organisms). A full revision of these protocols, that is not possible in the present paper, will be essential to elucidate the main gaps and limits of each procedure in order to formulate a proposal for a standard protocol to be included in an international round-robin intercalibration experiment for validation and standardisation. Since this is not accomplished, authors should provide in very detail how they carry on their experiments. Actually, Soares et al. (1992) stated that several factors can alter the reliability of results in ecotoxicity testing such as the environmental conditions both related to non-genetic factors (i.e. temperature, pH and chemical composition of the hatching and exposure medium, oxygen, photoperiod and nutrients) as well as to their genetic characteristics (origin of the batch of cysts). Indeed, it is compulsory to reduce the variability as a fundamental requirement in ecotoxicity testing (Nunes et al., 2006).

The main criticisms about the use of *Artemia* spp. in nanoecotoxicology within the present literature revision process were detected during the hatching phase and the toxicity test design, and the way testing solution/suspensions were prepared. About the hatching phase, it was noticed that the following information are missing or incomplete:

- The origin of cysts and their storage/maintenance conditions;
- The characteristics of saltwater and its treatments (e.g. filtration); if it is natural seawater the sampling point is frequently not reported; if it is artificial seawater its brand or composition could be not clearly displayed;
- The water oxygen saturation, pH and conductibility as well as the hatching temperature and time, and the photoperiod duration;
- The indication of larval stage exposed to suspensions (e.g. Instar I after 24 h hatching of cysts); indeed, sensitivity may change on the basis of the exposed larval stage.

Moreover, the experimental design did not always show the tested concentrations, the number of replicates per suspension and the number of organisms exposed per replicate. Sometimes, the final suspension volume where nauplii are exposed resulted unknown like the total contact time and the test reading frequency. Little data were also provided for exposure temperature, photoperiod and larvae feeding regime. The gap analysis evidenced that most papers included the assessment of negative controls (i.e. hatching and dilution water) and/or dispersing agents/carriers effects. Anyhow, just very few ones accounted for positive controls to check the sensitivity of larvae at the exposure conditions against one or more reference toxicants. Positive controls are essential to obtain reliable toxicity data facilitating their use in comparative analyses and clarifying the sensitivity of various brine shrimp population genetic characteristics.

Another hot point is about the preparation of nanoparticles suspensions. No specifications are available about ageing (the time interval between the time of suspension preparation and the beginning of the test), storage and way of dispersion that are really very sensitive tasks to be accomplished to allow toxicity data to be used in hazard and risk assessment as also recently stated by OECD (2014). Currently, no speculation is possible about the use *Artemia* spp. in nanoecotoxicology. The available data cannot help to support or detract such a biological model. Anyway, as they are organised in the present review, they can contribute to effectively understand which information must be compulsorily provided in a scientific paper in order to make them usable such as in toxicity ranking activities or hazard assessment of ENMs.

3. Conclusions

Artemia spp. might be a suitable biological model in nanoecotoxicology, at least for screening purposes due to its cost-effectiveness, but a lot of gaps into the knowledge still exist. Several protocols and unspecified approaches have been used with various hatching and exposure procedures. It is strongly required the harmonisation of protocols followed by a standardisation activity. In the meanwhile, authors should provide a full description of their nanoecotoxicological activity with *Artemia* spp. in order to sufficiently elucidate their test preparation and exposure conditions. At the moment, only few nanoecotoxicity data originated from common exposure scenarios thus limiting their use for hazard and risk assessment.

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