Environmental Pollution 213 (2016) 173–183

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

A case study to optimise and validate the brine shrimp *Artemia franciscana* immobilisation assay with silver nanoparticles: The role of harmonisation☆

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ARTICLE INFO

Article history:
Received 21 December 2015
Received in revised form
5 February 2016
Accepted 5 February 2016
Available online xxx

Keywords:
Inter-laboratory exercise
Illumination regime
Ag speciation
FP7 NanoValid
Marine environment
Risk assessment

ABSTRACT

Brine shrimp *Artemia* sp. has been recognised as an important ecotoxicity and nanotoxicity test model organism for salt-rich aquatic environments, but currently there is still no harmonised testing protocol which would ensure the comparable results for hazard identification. In this paper we aimed to design the harmonised protocol for nanomaterial toxicity testing using *Artemia franciscana* and present a case study to validate the protocol with silver nanoparticles (AgNPs). We (i) revised the existing nanotoxicity test protocols with *Artemia* sp. (ii) optimised certain methodological steps based on the experiments with AgNPs and potassium dichromate ($K_2Cr_2O_7$) as a soluble reference chemical and (iii) tested the optimised protocol in an international inter-laboratory exercise conducted within the EU FP7 NanoValid project. The intra- and inter-laboratory reproducibility of the proposed protocol with a soluble reference chemical $K_2Cr_2O_7$ was good, which confirms the suitability of this assay for conventional chemicals. However, the variability of AgNPs toxicity results was very high showing again that nanomaterials are inherently challenging for toxicity studies, especially those which toxic effect is linked to shed metal ions. Among the identified sources for this variability were: the hatching conditions, the type of test plate incubation and the illumination regime. The latter induced variations assumingly due to the changes in bioavailable silver species concentrations. Up to our knowledge this is the first inter-laboratory comparison of the *Artemia* sp. toxicity study involving nanomaterials. Although the inter-laboratory exercise revealed poor repeatability of AgNPs toxicity results, this study provides valuable information regarding the importance of harmonisation of all steps in the test procedure. Also, the presented AgNPs toxicity case study may serve as a platform for further validation steps with other types of NMs.

1. Introduction

The number of published nanosafety studies has exponentially increased over the past decade (Kahru and Ivask, 2013). Despite a large number of available nanomaterial (NMs) hazard data the risk assessors often do not consider them enough reliable for risk assessment (Jackson et al., 2013; Oomen et al., 2014). One of the main gaps is the lack of harmonised testing protocols (Krug, 2014; Kühnel and Nickel, 2014; Petersen et al., 2015; Potthoff et al., 2015; Jemec et al., 2016). This suggestion is also in line with implementation of nanometrological principles in toxicity testing as

Abbreviations: UL-BF, University of Ljubljana, Biotechnical Faculty, Slovenia; UL-FKKT, University of Ljubljana, Faculty of Chemistry and Chemical Technology, Slovenia; NICPB, National Institute of Chemical Physics and Biophysics, Estonia; CCMB, The Centre for Cellular & Molecular Biology, India; UFZ, The Helmholtz Centre for Environmental Research, Germany.

☆ This paper has been recommended for acceptance by B. Nowack.

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http://dx.doi.org/10.1016/j.envpol.2016.02.015
0269-7491/

advised by the European Commission funded project CO-NANOMET (www.co-nanomet.eu). This project produced a foresight review document that surveys the nanometrology requirements and future strategies to support the development of European nanotechnology (Leach et al., 2011) recognizing significant deficit of awareness and level of priority in biological nanometrology, e.g. in toxicity testing.

Concerning the aquatic ecotoxicity of nanomaterials the majority of the available data concern freshwater species, mostly *Daphnia magna*, and there is remarkably less information on marine species (Juganson et al., 2015). Among the latter, brine shrimp *Artemia* sp. (Crustacea, Branchiopoda: *Anostraca*) (Supplementary information, Fig. S1) has been recognized as a suitable biological model in ecotoxicology (Nunes et al., 2006) and nanoecotoxicology (Libralato, 2014) due to good knowledge of its biology and ecology, and ability to provide various types of toxicity information. In addition, the tests with *Artemia* sp. can be performed without prior breeding of the adult animals in the laboratory (nauplii are hatched from the commercially available dormant cysts on demand) that also allows determination of the exact age of nauplii used for the experiments (Manfra et al., 2015), are easy to perform and cost-effective.

Despite the past efforts to standardise the *Artemia* toxicity assay protocol (Vanhaecke and Persoone, 1984; Persoone and Wells, 1987; Solis et al., 1993), the main challenge for the implementation of this assay for nanomaterials is the absence of a harmonised testing protocol (Libralato, 2014; Manfra et al., 2015). Several different protocols with various preparatory steps (hatching of the cysts) and toxicity test designs have been used until now for *Artemia* sp (reviewed in Table S1, Supplementary information). Libralato (2014) recently suggested *Artemia* sp. as a very suitable test organism for nanomaterials but pointed out a number of critical points in test design that may introduce variability to the test results: (i) origin of the cysts, (ii) age of larval stage upon exposure to the toxicant(s), (iii) composition of the test medium, (iv) exposure conditions (temperature, photoperiod), (v) experimental set-up in terms of the number of nauplii per unit volume of medium, the mode of exposure and test vessels, and (vi) the use of positive control.

To advance the use of *Artemia* sp. immobilisation assay for the purpose of nanomaterial hazard assessment for marine ecosystems we made an effort to validate the protocol with silver nanoparticles as these NPs are probably most challenging NMs for toxicity testing in high salinity test environment, mostly due to complicated speciation of bioavailable fraction of silver. For this purpose we (i) revised the existing nanotoxicity test protocols with *Artemia* sp., (ii) optimised certain methodological steps based on the experiments with silver nanoparticles (AgNPs), (iii) designed a protocol considering all sources of variability pointed out by previous authors (Libralato, 2014) and finally (iv) tested the procedure in an international inter-laboratory exercise with AgNPs and potassium dichromate (a standard positive control for aquatic toxicology assays). We chose *Artemia franciscana* as a test species for this protocol as suggested previously (Ruebhart et al., 2008; Manfra et al., 2015). To our knowledge this is the first attempt to validate the *Artemia* sp. immobilisation assay with nanomaterials.

2. Materials and methods

2.1. Chemicals

Silver nanoparticles (AgNPs) (designated as NNV 003; batch number Parnasos_IG010305_Ag NAMA39_1202_Ag) were supplied by Colorobbia S.p.A (Firenze, Italy; http://www.colorobbia.com). within the framework of the EU FP7 NanoValid project. Salts (NaCl, KCl, $MgCl_2$, $MgSO_4$, $CaCl_2$, $NaHCO_3$) for the test medium for *A. franciscana* (synthetic salt water; SSW) and reference chemical ($K_2Cr_2O_7$) were of analytical grade and purchased from Sigma–Aldrich Co. (St. Louis, USA).

2.2. Physico-chemical characterisation of silver nanoparticles (AgNPs)

AgNPs were supplied as an aqueous suspension in distilled water with a nominal particle concentration of 40 g/L (the mean measured total Ag concentration was 41.14 g/L, flame atomic absorption spectrometry, FAAS, Perkin Elmer AAnalyst 100, Waltham, Massachusetts, USA (Jemec et al., 2016). The AgNPs were stabilized with polyvinylpyrrolidone as a surfactant. The exact concentration of surfactant is considered an intellectual property of the supplier and cannot be revealed. The same batch of AgNPs has been previously characterized and described in Zou et al. (2015), Böhme et al. (2015) and Jemec et al. (2016). The morphology and size of AgNPs were measured by field emission scanning electron microscope (at 15 kV; FEI Nova NanoLab 600). A small drop of AgNPs solution was casted on carbon adhesive tab attached to a pin mount stub and subsequently air-dried in a desiccator a day before microscopy. The distribution of AgNP sizes was a non-symmetric function, therefore we provide the median value of the distribution: 19 nm (standard deviation: 21 nm) (Supplementary information, Fig. S2). The share of Ag^+-species in the stock suspension as defined by ultracentrifugation (30–60 min at 300 000 rpm, spin-out ultracentrifuge Beckman L8-M) was 46% (Jemec et al., 2016).

Dynamic light scattering (DLS) measurements of the hydrodynamic size of the AgNPs in synthetic salt water test medium, SSW (24.7 g/L NaCl, 0.54 g/L KCl, 2.15 g/L $MgCl_2$, 3.072 g/L $MgSO_4$, 1.14 g/L $CaCl_2$, 0.2 g/L $NaHCO_3$ in deionised water, Table 1) were performed using the Particle Size Analyzer VASCO (Cordouan technologies, Pessac, France). The suspensions were prepared by vigorous vortexing from the stock AgNP suspension and no additional sonication was applied. Average values of hydrodynamic diameter (z-value) of AgNPs in SSW in the dark at the beginning of the experiment (number of independent measurements = 1 and 2 in case of 10 mg/L, and 1 and 100 mg/L of AgNPs, respectively). Data are expressed as average particle diameter xDLS ± SD (further described as z-average ± SD). The z average values were: 169 ± 14 nm (1 mg/L), 144 nm (10 mg/L) and 145 ± 8 nm (100 mg/L).

The physico-chemical properties of AgNPs in SSW prepared in darkness (16 lux) in at the beginning of experiment and after 48 h incubation under illumination (6000 lux) and darkness (16 lux) were analysed. The following parameters were measured: the total Ag concentration in AgNPs suspension, pH, conductivity (mS/cm), and oxidation-reduction potential (ORP) (mV) (Thermo Scientific Orion Star A215 Benchtop pH/Conductivity Meter). The AgNPs suspension was digested in 20% aqua regia overnight (suspension/acid ratio 1:10 v/v) and measured by flame atomic absorption spectrometry (FAAS; Perkin Elmer AAnalyst 100, Waltham, Massachusetts, USA).

2.3. The design of a protocol with A. franciscana for inter-laboratory comparison

Many different experimental protocols in terms of hatching, exposure and toxicity endpoints have been employed in existing nanotoxicity protocols with *Artemia* sp (Table S1, Supplementary information). Based on the literature data and additional experiments with potassium dichromate ($K_2Cr_2O_7$) as a reference chemical commonly used in toxicity testing and AgNPs we designed a protocol that was further tested in inter-laboratory study.