AN INTERLABORATORY COMPARISON OF NANOSILVER CHARACTERISATION AND HAZARD IDENTIFICATION: HARMONISING TECHNIQUES FOR HIGH QUALITY DATA

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Abstract:

Within the FP7 EU project NanoValid a consortium of six partners jointly investigated the hazard of silver (Ag) nanoparticles (AgNPs) paying special attention to methodical aspects that are important for providing high-quality ecotoxicity data. Laboratories were supplied with the same original stock dispersion of AgNPs. All partners applied a harmonised procedure for storage, and preparation of toxicity test suspensions. Altogether ten different toxicity assays with a range of environmentally relevant test species from different trophic levels were conducted in parallel to AgNP characterisation in the respective test media. The paper presents a comprehensive dataset of toxicity values and AgNPs characteristics like

hydrodynamic sizes of AgNP agglomerates and the share (%) of Ag^+-species (the concentration of Ag^+-species in relation to the total measured concentration of Ag prior to separation). The studied AgNP preparation (20.4 ± 6.8 nm primary size, mean total Ag concentration 41.14 mg/L, 46-68% of soluble Ag^+-species in stock, 123.8 ± 12.2 nm mean z-average value in dH_2O) showed extreme toxicity to crustaceans *Daphnia magna*, algae *Pseudokirchneriella subcapitata* and zebrafish *Danio rerio* embryos (EC50 <0.01 mg total Ag/L), was very toxic in the *in vitro* assay with rainbow trout *Oncorhynchus mykiss* gut cells (EC50: 0.01-1 mg total Ag/L); toxic to bacteria *Vibrio fischeri*, protozoa *Tetrahymena thermophila* (EC50: 1-10 mg total Ag/L) and harmful to marine crustaceans *Artemia franciscana* (EC50: 10-100 mg total Ag/L). Along with AgNPs, also the toxicity of $AgNO_3$ was analysed. The toxicity data revealed the same hazard ranking for AgNPs and $AgNO_3$ (i.e. the EC50 values were in the same order of magnitude) proving the importance of soluble Ag^+-species analysis for predicting the hazard of AgNPs. The study clearly points to the need for harmonised procedures for the characterization of NMs. Harmonised procedures should consider: (i) measuring the AgNP properties like hydrodynamic size and metal ions species in each toxicity test medium at a range of concentrations, and (ii) including soluble metal salt control both in toxicity testing as well as in Ag^+-species measurements. The present study is among the first nanomaterial interlaboratory comparison studies with the aim to improve the hazard identification testing protocols.

Keywords: FP7 EU project NanoValid, nanomaterials' aging, dissolution, hydrodynamic diameter, toxicity, Ag salt

Abbreviations:

UL: University of Ljubljana, Slovenia

NICPB: National Institute of Chemical Physics and Biophysics, Estonia

CCMB: The Centre for Cellular & Molecular Biology, India

Eawag: Swiss Federal Institute of Aquatic Science and Technology, Switzerland

UFZ: The Helmholtz Centre for Environmental Research, Germany

FHG-IKTS: Fraunhofer Institute for Ceramic Technologies and Systems, Germany

1 INTRODUCTION

Hazard identification, i.e. the identification of the effects of concern, is an important step in assessing nanomaterial risk and is required under multiple regulatory frameworks worldwide (Hristozov et al., 2014). The first nanoecotoxicological studies emerged almost 10 years ago and ever since the field has considerably proliferated (Kahru and Dubourguier, 2010; Kahru and Ivask, 2013). For example, a search in the database Science Direct made in March 2009 yielded only 17 articles on keywords "silver nanoparticles and ecotoxicity" (Kahru and Dubourguier, 2010) while the same search done in July 2015 already revealed 268 records. According to Kahru and Ivask (2013), silver nanoparticles (AgNPs) are within the top five nanomaterials studied for their (eco)toxicological properties when considering nanomaterials listed in OECD Sponsorship Programme for the Testing of Manufactured Nanomaterials (OECD, 2010). Moreover, given that applications utilizing antimicrobial properties of AgNPs are likely to further increase, e.g., due to the applications related to providing safe drinking water in large regions of the world such as India (Sharma, 2011) and Africa (Simonis and Basson, 2011), the risk assessment of AgNPs must have a priority.

A number of comprehensive reviews have already been published with an attempt to draw general conclusions on the environmental hazard of AgNPs (Fabrega et al., 2011; Bondarenko et al., 2013; Chernousova and Epple, 2013; Ivask et al., 2013; 2014; Sharma et al., 2014; Baker et al., 2014). The obtained toxicity values vary considerably. For example, a 275-fold variation in the toxicity values was observed for different mammalian cells *in vitro* (25 values), 500-fold for different strains of bacteria (46 different median EC_{50}, LC_{50}or Minimal inhibitory values) and 40-fold difference for *Daphnia magna* (13 different 48h EC_{50} values) (data taken from Bondarenko et al., 2013, Ribeiro et al., 2014). One reason for this could be different types of AgNPs in terms of size, supplier and stabiliser/coating. Furthermore, dispersion procedures and exposure conditions varied between the studies.

The current view of the risk assessors is that although the information on ecotoxicity of various types of nanomaterials (NMs) is rapidly expanding, a limited number of high quality data is available for univocal hazard and risk assessment of NMs (Jackson et al., 2013; Oomen et al., 2014). There is an ongoing debate which criteria define high quality data and unification of these criteria is still needed (Krug, 2014). Several attempts have been made to define the criteria concerning physico-chemical characterisation (Mills et al., 2014; Kühnel et

al., 2014) and toxicity testing (Kühnel et al., 2014). The latter has been done by the DaNa project (Data and knowledge on nanomaterials - processing of socially relevant scientific facts; www.nanoobjects.info) which suggested the co-called Literature Criteria Checklist (Kühnel et al., 2014). The term quality toxicity data in the present paper means that the toxicity study has considered (i) the basic rules for toxicity study (relevant exposure concentrations, reference controls, impurities…), (ii) specific NMs properties (interferences with the assay, suitable dispersion agents,…), and (iii) sufficient and competent NM physico-chemical characterisation data (Hristozov et al., 2014; Jackson et al., 2013; Oomen et al., 2014; Kühnel and Nickel, 2014; Bondarenko et al., 2013; Krug, 2014).

To increase the quality of hazard identification data a number of international initiatives has been undertaken to harmonise, and standardise the toxicity testing protocols. Among these are: the OECD Working party on Manufactured Nanomaterials (Kühnel and Nickel, 2014), ISO technical Committee 229, and the NanoSafety Cluster Working group 10 (Oomen et al., 2014). The EU FP7 large-scale integrated project NanoValid (www.nanovalid.eu) aims to develop a set of reliable reference methods for hazard identification and exposure assessment of engineered NMs. Within this scope, a consortium of six NanoValid partners jointly investigated the hazard of AgNPs, paying special attention to methodical aspects that are important for providing high-quality ecotoxicity data as defined in the chapter above. To diminish the variability caused by different batches of AgNPs and different storage and preparation of suspensions for toxicity tests, the partners were supplied with the same original stock dispersion of AgNPs, and a harmonised procedure for handling of AgNPs was applied. For bioassays, organisms from different environments and trophic levels were chosen. In addition to the toxicity tests, the partners were also responsible for in-house characterisation of the hydrodynamic size of AgNPs agglomerates and the percentage (%) of Ag^+ and Ag^+-test medium ligand complexes in comparison to total Ag prior to separation (hereafter referred to as the share of Ag^+-species) in the respective test media.

The aim of this paper is to deliver the experience and recommendations from FP7 NanoValid consortium to improve the hazard identification of nanomaterials. We focused on the specific challenges associated with the characterisation of AgNP dispersion in toxicity test media in parallel to the toxicity testing using a wide array of environmentally relevant

test species. In particular, we addressed the importance of harmonised procedures for AgNP characterisation during the course of experiments.

2 MATERIALS AND METHODS

2.1 Case study set-up

Six NanoValid partner research institutions participated in the study: University of Ljubljana (UL, Slovenia), National Institute of Chemical Physics and Biophysics (NICPB, Estonia), The Centre for Cellular & Molecular Biology (CCMB, India), Swiss Federal Institute of Aquatic Science and Technology (Eawag, Switzerland), Helmholtz Centre for Environmental Research (UFZ, Germany), and Fraunhofer Institute for Ceramic Technologies and Systems (FHG-IKTS, Germany). The experimental set-up is schematically presented in **Fig. 1.** The partners were provided with the same stock dispersion of polyvinylpyrrolidone (PVP) stabilised AgNPs (see chapter 3.1 for characteristics).

All partners used the same procedure for the storage and preparation of test suspensions. First, the original stock was vortexed and then diluted to final tested concentrations without prior sonication. Always freshly prepared dispersions were used for the bioassays. Each partner performed the characterisation of AgNPs (hydrodynamic diameter and the share of Ag^+-species) in their respective test media as well as in dH_2O using different concentrations of AgNPs. Analyses of the share of Ag^+-species and toxicity tests were done at different time periods after the receipt of the stock dispersion. Details on the time of analyses were carefully recorded and are presented in **Fig.1.**

2.2 Toxicity tests

The following organisms from different taxonomic groups and a fish cell line were chosen: the rainbow trout (*Oncorhynchus mykiss*) intestinal cells (RTgutGC) *in vitro*, the naturally luminescent marine bacterium *Vibrio fischeri*, protozoa *Tetrahymena thermophila,* freshwater green alga *Pseudokirchneriella subcapitata*, freshwater crustacean *Daphnia magna,* marine crustacean *Artemia franciscana*, and freshwater zebrafish *Danio rerio*

(different early life stages). To investigate different toxicity of NPs for zebrafish due to potential time changes in NPs (aging), the tests with zebrafish were done by the same partner at two different time points: May 2013 and August 2014. All test media compositions are described in **Supplementary information (Table S1)**. In addition, in all toxicity tests the $AgNO_3$ was used as an ionic control for AgNP.

2.2.1 Rainbow trout intestinal cells *in vitro*

The assay was performed by Eawag, Switzerland. Rainbow trout intestinal cells (RTgutGC cells) were cultured as described previously (Kawano et al, 2001). For cytotoxicity investigation, 150000 cells were seeded per well of a 24 well plate (polystyrene) in 1 mL Leibovitz L-15 medium (L-15 supplemented with 5% FBS and 1% gentamycin) and grown for 48 h at 19°C. Prior to incubation with AgNPs, the cells were washed twice with 1 mL exposure medium (L-15/ex, Schirmer et al, 1997) and then incubated with 1 mL L-15/ex containing the indicated concentrations of AgNPs for 24 hours.

For determination of cytotoxicity, a combined assay involving three endpoints (metabolic activity, lysosomal and membrane integrity) was used (Schirmer et al. 1998). After incubation with AgNPs, the medium was removed and the cells washed twice with 1 mL phosphate buffered saline (PBS). The cells were then incubated with 400 µL PBS containing 5% (v/v) Alamar Blue and 4 µM 5-Carboxyfluorescein Diacetate, Acetoxymethyl Ester (CFDA-AM). After 30 min incubation, fluorescence (λ_{ex}=530 nm λ_{em}=595 nm for Alamar Blue, λ_{ex}=493 nm λ_{em}=541 nm for CFDA-AM) was quantified using a multiwell plate reader (Infinite M200, Tecan, Maennedorf, Switzerland). Cells were subsequently washed once with 1 mL PBS and then incubated in 400 µL PBS containing 1% (v/v) Neutral Red solution (final concentration of Neutral Red was 50 µg/mL). After 1 h incubation, cells were washed once with 1 mL PBS and then incubated for 10 min with the Neutral Red extraction solution (50% Ethanol absolute, 49% dH_2O, 1% acetic acid) on a shaker. Fluorescence of the Neutral Red was measured on a multiwell plate reader (λ_{ex}=530 nm λ_{em}=645 nm).

2.2.2 Naturally luminescent marine bacteria *Vibrio fischeri*

The kinetic *Vibrio fischeri* bioluminescence inhibition assay was performed in NICPB, Estonia, according to ISO 21338:2010 as described in Heinlaan et al. 2008. Briefly, the lyophilized *Vibrio fischeri* NRRL-B 11177 Reagent (Aboatox, Turku, Finland) was rehydrated by adding 13 mL of cold (4°C) Reagent Diluent (NaCl 20 g/L; $MgCl_2$*$6H_2O$ 2.035 g/L; KCl 0.3 g/L). Upon rehydration, bacteria were stabilized (i.e. bacterial luminescence value is stable) first at 4°C for 30 min and then at 20^{o}C for another 30 minutes. Since *V. fischeri* is a marine organism, the test medium is 2% NaCl (**Supplementary information Table S1)**. Sample pH was not adjusted since it did not fall beyond the range of 6.0 - 8.5 (i.e. not affecting the luminescence of bacteria). The testing was performed in polypropylene cuvettes at 20°C on automatic BioOrbit 1251 luminometer (ThermoLabsystems, Finland), connected to computer operated by Multiuse software (BioOrbit, Finland). The test bacterium was automatically dispensed on the sample using dispenser controller unit. Bacterial luminescence was measured every 0.6 seconds during 30 sec under continuous mixing. After 30 min of incubation, luminescence was measured again for 30 sec. Due to continuous mixing and simultaneous kinetic measuring of luminescence, every sample acts as its own reference and no color/turbidity correction is needed. Controls, both negative (2% NaCl) and positive (3,5-dichlorophenol), were always included in the runs. The inhibitory effects of chemicals on bacterial luminescence were calculated according to the following equations:

$$30\text{-}min\ \ INH\% = 100 - \frac{IT_{30}}{KF * IT_0} * 100\ \ ;\ \ KF = \frac{IC_{30}}{IC_0};$$

KF (correction factor) is characterising the natural loss of luminescence of the control (i.e. bacterial suspension in 2% NaCl). IC_0 and IT_0 are the maximum values of luminescence during first 5 seconds after dispensing of 100 µL of test bacteria to 100 µL of control or test sample, respectively. IC_{30} and IT_{30} are respective values after 30 minutes. 30-min EC50 is the concentration of a compound reducing the bioluminescence by 50% after 30 minute incubation with the compound.

2.2.3 Freshwater protozoa *Tetrahymena thermophila*

The *Tetrahymena thermophila viability* assay was performed in NICPB, Estonia, by measuring the ATP content in protozoa using the luciferin–luciferase method as described earlier

(Mortimer et al., 2010). Briefly, protozoan culture (*T. thermophila* strain BIII) was grown axenically in SSP medium supplemented with 250 µg/mL each of streptomycin sulphate and penicillin G. The cells were harvested during the exponential growth phase by centrifugation at 300 *g* for 5 min at 4 °C and washed twice with deionized water. For toxicity testing, the cell density was adjusted to 10^6 cells/mL (or OD600nm=2) by adding appropriate amount of deionised water to the cells.

For toxicity analysis, 100 µL of harvested and washed *T. thermophila* cells suspension (final density in the test 5×10^5 cells/mL) in deionised water was added to 100 µL of Ag-formulations (AgNP or $AgNO_3$) in deionised water in 96-well polystyrene plates. Each concentration was tested in at least triplicate. The test plates were incubated at 30 °C in the dark. For ATP extraction, upon 2 and 24 h of exposure, 50 µL of 1% TCA, 4 mM EDTA solution was added to 50 µL of cell suspension. The extracts were placed on ice for 5 minutes and then stored at -18 °C until further analysis. Prior to analysis the samples were thawed and 20 µL of each sample was transferred onto white 96 well polypropylene microplate. 80 µL of Tris-EDTA buffer (0.1 M Tris, 2 mM EDTA, adjusted to pH 7.75 with acetic acid) was added into each well to reduce TCA concentration to 0.1%. After measuring the background luminescence ($RLU_{background}$), 100 µL of 500-fold diluted ATP assay mix (FLAAM, Sigma-Aldrich) was added to each sample well, the plate was shaken and luminescence was measured (RLU_{sample}). Finally, 10 µL of thawed 10^{-5} M ATP standard (estimation of potential quenching of luminescence by NPs) was added to each sample well, the plate was shaken and luminescence was measured (RLU_{std}). Luminescence measurements were done by Orion II plate luminometer (Berthold Detection Systems, Germany). The amount of the ATP in each well was calculated according to the following equation:

$$C_{ATP,\,\mu mol} = \frac{RLU_{sample} - RLU_{background}}{RLU_{std}} * RLU_{\,standard\ in\ test,\ \mu mol}$$

The ATP concentrations in the samples were expressed as percentages of the non-treated controls.

2.2.4 Freshwater alga *Pseudokirchneriella subcapitata*

The algal growth inhibition assay was performed in NICPB, Estonia, according to OECD201 guidelines (OECD 2011) as initially described in Aruoja et al., 2009. The *P. subcapitata* stock culture originated from the commercial test system Algal Toxkit F (MicroBioTests Inc., Nazareth, Belgium). Briefly, exponentially growing algae were exposed to Ag NNV-003 and $AgNO_3$ and incubated at 24 ± 1°C for up to 72 h in 20-mL glass scintillation vials containing 5 mL of algal growth medium (OECD, 2011, **Supplementary information Table S1)**. All samples were run in duplicate with four controls distributed evenly on the transparent table that was constantly shaken and constantly illuminated from below with Philips TL-D 38W aquarelle fluorescent tubes. Algal biomass was measured at least every 24 hours using fluorescence of the algal pigment extract. For that, 50 µL of algal culture was transferred to 96-well black polypropylene plate (Greiner Bio-One), 200 µL of ethanol was added into each sample well and the plate was shaken for 3 h in the dark. Fluorescence was measured using microplate fluorometer (excitation 440 nm, emission 670 nm; Fluoroscan Ascent, Thermo Labsystems, Finland). The cell concentration of the control culture increased at least 16 times during 3 days. The variability between replicates was kept low by using the vials only once. The coefficient of variation of biomass density in replicate control cultures throughout the experiments did not exceed 5%. EC50 values (effective concentration leading to 50% reduction of biomass) were calculated from dose-response data as described below.

2.2.5 Freshwater crustacean *Daphnia magna*

Daphnia magna acute immobilisation assays were performed by two laboratories (UFZ and NICPB) according to the OECD202 (2004) standard. In UFZ, the crustaceans were cultivated under controlled conditions of 20 °C at a 16:8 h light:dark cycle. Daphnids were fed three times a week with algae and yeast flakes. Daphnia aged less than 24 h (neonates) were exposed to Ag and $AgNO_3$ in ADaM (Aachener Daphnien medium) for 48 h (**Supplementary information Table S1**). Dose-response relationships and EC_{50}-values were determined by the application of the log-normal model.

In NICPB, differently from the OECD 202 test procedure, neonates used for the toxicity tests did not originate from a laboratory culture but were hatched from ephippia (the product of MicroBio Tests, Inc., Mariakerke-Gent, Belgium). To obtain *D. magna*

neonates, *D. magna* ephippia were incubated in Petri dishes for 3-4 days at 20°C under continuous illumination of 6000 lux. Hatched neonates were collected for further testing. Prior to the initiation of exposure, the hatched neonates were "pre-fed" with living microalgae *Pseudokirchneriella subcapitata* during two hours. Then, daphnids were transferred into the samples *via* clean OECD202 test medium (**Supplementary information Table S1**) to reduce the carry-over of alga and potential modification of the nanomaterial toxicity to *D. magna*. Testing was conducted on 30-well polycarbonate test plate (MicroBio Tests, Inc., Mariakerke-Gent, Belgium) with 4 technical parallels of 5 daphnids per 10 mL sample. At least 3 independent assays were conducted. Upon 48 h of incubation at 21°C in the dark, the immobilisation (mortality) of daphnids was recorded by visual observation. The daphnid was considered immobilized if it did not resume swimming within 15 sec of gentle agitation. Immobilisation (mortality) percentage for each concentration was calculated as follows:

% dead/immobilized= dead/immobilized animals*100/total number of animals in the test
MS Excel macro Regtox was used for calculating the toxicity values.

2.2.6 Marine crustacean *Artemia franciscana*

The Artemia sp. assay was performed by CCMB, India. For every 1 liter culture, 1.0 gram of Artemia cysts were used (San Francisco Bay strain). These cysts are hydrated in distilled water under aeration for 45 minutes and then 'bleached' to remove the cysts wall using 20% sodium hypochlorite solution for 15 minutes. The 'bleached' Artemia eggs were then washed and allowed to hatch in 2.5% salt water (pH 8.0, NaCl, with continuous light and aeration for 24 hours (Sorgeloos, 1973) in the presence and absence of Ag NPs. The temperature was maintained at 28°C which is the optimum temperature for hatching of Artemia eggs.

The Artemia eggs were exposed to two different concentrations of nanomaterial prepared in 2.5 % NaCl (**Supplementary information Table S1)** (100 mg/L and 10 mg/L). The hatching rate and immobility (inability to swim after gentle agitation) were checked after 24 hours for each Artemia cultures exposed to nanoparticles. Three aliquots of 100 µL were taken from each flask after thorough mixing and counted under the stereo-microscope. Number of hatched Artemia (immotile vs. motile), umbrella stage and unhatched cyst were

counted. Hatching rate was expressed as percent of total number of fully hatched Artemia in comparison to total Artemia in the aliquot. Criteria for validity was no mortality in control.

2.2.7 Zebrafish *Danio rerio embryos*

Zebrafish (*Danio rerio*) embryos were cultivated and exposed to the respective nanoparticles according to the OECD 236 (2013) guideline. To obtain the embryos, the fish were cultured at 26 ± 1 °C at a 14:10 h light:dark cycle. Fish were fed daily with flake food and *Artemia spec. ad libitum*. For the egg collection, spawn traps covered with a wire mesh were placed into the fish tanks on the day prior to spawning. After selection of fertilised eggs, eggs were transferred to ISO-water (294.0 mg/L $CaCl_2*2H_2O$, 123.3 mg/L $MgSO_4*7H_2O$, 63.0 mg/L $NaHCO_3$, and 5.5 mg/L KCl dissolved in deionised water) and exposure experiments were started at a time point of 2, 28 and 72 hours post fertilisation (hpf) in ISO-water (**Supplementary information Table S1**) up to 48 h. The 28 hpf embryos were mechanically dechorionated with forceps prior to testing to estimate whether toxicity effects occur due to an enrichment of NPs at the chorion. The following parameters were used as lethal endpoints: coagulation, missing formation of somites, no detachment of tail and no heartbeat. In total 10 individuals per concentration were investigated and a minimum of 3 replicates were performed.

2.2.8 Data analysis

EC50 values for daphnids, algae, *V. fischeri, T. thermophila* and *Artemia franciscana* were calculated from the concentration-effect curves by the application of the log-normal model using REGTOX software for Microtox Excel™ (MSExcel macro REGTOX EV7.0.5.xls) unless stated otherwise. The REGTOX software is available online at: http://eric.vindimian.9online.fr/ (Vindimian, E., 2005). For calculation of EC_{50} values the Hill-model (non-linear fit) was used. The toxicity data obtained for the zebrafish embryos and *Artemia franciscana* were evaluated using GraphPad Prism software. The toxicity of AgNPs and Ag salt to test organisms was classified according to the EC50 values (based on compound concentration in case of AgNPs and Ag^+ concentration in case of $AgNO_3$) as: <0.1

mg/L=extremely toxic; 0.1–1 mg/L = very toxic; 1–10 mg/L = toxic; 10–100 mg/L = harmful; <100 mg/L = non-toxic to the aquatic organisms (CEC 1996; Sanderson, 2003).

2.3 Characterisation of the stock of AgNPs

The AgNPs (designated as NNV-003; batch number Parnasos_IG010305_Ag NAMA39_1202_Ag) were supplied by Colorobbia Italia Spa (http://www.colorobbiaitalia.it/). The NPs were supplied as aqueous suspension with a nominal particle concentration of 40 g Ag/L stabilised with the surfactant polyvinylpyrrolidone (PVP).

The total amount of Ag in supplied stock was measured with inductively-coupled plasma mass spectrometry (ICP-MS), and atomic absorption spectroscopy. Measurements were done in at least 3 replicates.

The share of Ag^+-species. Dissolution of NPs in general is assessed by separation of the particles from the suspension. The latter contains what is most commonly referred to as dissolved ionic Ag (Ag^+) (Ivask et al., 2014) or dissolved Ag (Odzak et al., 2014). However, this terminology is not entirely accurate, therefore we propose the term "the share of Ag^+/Ag^+-ligand complexes (Ag^+-species)" in comparison to the total Ag. This is because, not all measured Ag^+ dissolve from AgNPs, but some of it may remain from the synthesis *via* controlled reduction of Ag salts (Behra et al., 2013). Secondly, in the test medium dissolved Ag^+ become complexed with various ligands (Herrin et al., 2001, Behra et al. 2013, Levard et al., 2012) and the analytical instrumentation used in the current work does not differentiate between Ag^+ and Ag^+-ligand complexes.

Two different approaches were used to separate Ag^+ species from AgNPs in the stock dispersion: (i) filtration through Amicon Millipore filter units with molecular weight cut off 3 kDa by centrifugation at 4000 *g* for 20 min, or (ii) ultra-centrifugation for 30-60 min in a spin-out ultracentrifuge (Beckman L8-M) at 362 769 *g*. Ag^+-species in the supernatant after separation were measured using ICP-MS (after pore separation), and AAS after ultracentrifugation (accredited laboratory using the method EVS-EN ISO/IEC 17025:2005). Both approaches have previously been shown to be efficient in separation of NPs from ion species (Ivask et al., 2014; Navarro et al. 2008, Odzak et al. 2014, Yue et al., 2015).

2.4 Characterisation of AgNPs dispersions in the toxicity test media and in deionised water (dH_2O)

The following properties of AgNPs in toxicity test media and dH_2O were evaluated: the primary size using transmission electron microscopy (TEM, at 50 mg/L), the hydrodynamic diameter of AgNPs and the fraction of Ag^+-species in AgNPs dispersions (for the concentrations, see 2.4.1). Analyses were done by different partners at different periods after receipt of the stock dispersion (partner Labs and times of analyses are given in **Fig.1**). The methods used for the hydrodynamic diameter and Ag^+-species share analyses are described below.

2.4.1 Hydrodynamic diameter

The sizes of NPs agglomerates in aqueous test media were determined using dynamic light scattering technique (DLS). The hydrodynamic size of AgNPs was measured in 2% NaCl (*V. fischeri* medium), *D. magna* OECD202 medium (all Malvern Zetasizer Nano-ZS at 20 mg/L; 173° angle), *A. franciscana* medium (Horiba Nanopartica SZ100; 90° angle, 0.1-100 mg/L), *D. rerio* medium and *D. magna* ADaM (Zetasizer Nano; 173° angle, 0.5 mg/L) and L-15/ex exposure medium for RTgutGC cells (Malvern Zetasizer Nano-ZS, 173° angle, 1 and 10 mg/L). In dH_2O, which is a test medium for *T. thermophila*, the following nominal AgNPs concentrations were analysed: 4 and 40 mg AgNPs/L (Zetasizer Nano; 173° angle), 25 and 50 mg AgNPs/L (Horiba Nanopartica SZ100; 90° angle), 1 and 10 mg/L (Malvern Zetasizer Nano-ZS; 173° angle) and 40 mg/L (Particle Size Analyzer VASCO, Cordouan technologies; 135 ° angle). Each suspension was analysed at least thrice and according to ISO 22412:2008 the data are expressed as average particle diameter xDLS ± SD (further described as "z-average ± SD).

The chosen AgNP concentrations were selected on the basis of the toxicity data: for each test medium they were in the range of the toxicity values for the respective test organism under consideration of the detection limits of the methods. Dispersions were prepared in the same way as for the toxicity testing. The suspensions in the test media were analysed immediately after the preparation (time 0) and also after 24 h and 48 h at room temperature (excluding *A. franciscana* test medium and L-15/ex medium). The suspensions in dH_2O were measured immediately after the preparation (time 0 h). All the analyses were

done at laboratory ambient light conditions. The composition of test media is described in **Supplementary information (Table S1)**.

2.4.2 The share of Ag^+-species in comparison to total Ag

Partners did not use the same procedure for the determination of Ag^+-species in AgNPs toxicity test dispersions. The majority of the partners used ultracentrifugation to separate the Ag^+-species from AgNPs. The protocols were as follows: 60 min (the whole cycle) centrifugation at 362 769 *g* (NICPB) followed by AAS, 30 min at 16 000 *g* followed by ICP-MS (UFZ), 30 min at 70 000, 75 000 and 100 000 *g* followed by AAS (CCMB), and 30 min at 100 000 *g* and measured with FAAS (UL). In addition, one partner used dialysis membrane ultrafiltration with Amicon Millipore filter units with molecular weight cut off (MWCO) of 3 kDa) (4000 *g*, 20 min) combined with ICP-MS (Eawag).

The efficiency of ultracentrifugation to remove all NPs was checked in the case of centrifugation approaches below 100 000 *g*, but ultracentrifugation (362 769 *g*) and ultrafiltration centrifugation (3 kDa pores, 4000 *g*) have previously proven efficient in NPs vs ion separation (Ivask et al., 2014; Navarro et al. 2008, Odzak et al. 2014). Carefully decanted supernatants in each set were checked for the presence of NPs by DLS: Particle Size Analyzer VASCO, Cordouan technologies; 135 °; Horiba Nanopartica SZ100 and Malvern Zetasizer Nano-ZS, and transmission electron microscopy (JEOL 2010). This approach enables only to confirm the presence of particles. But due to low resolution of the method, negative signal does not necessarily indicate that the particles are not present.

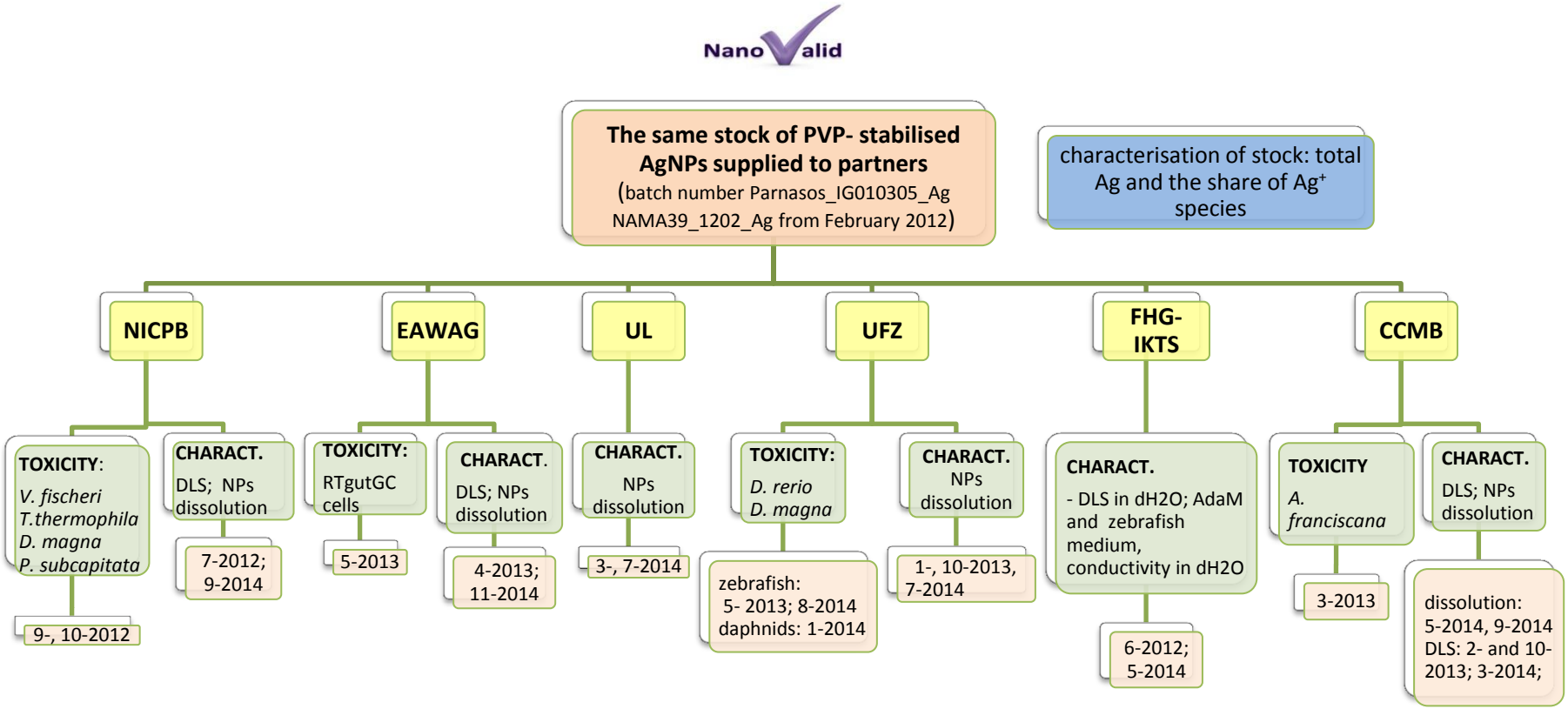

Fig.1. Experimental set-up of the NanoValid case study of AgNPs hazard identification. The yellow boxes present the institutions involved: National Institute of Chemical Physics and Biophysics (NICPB, Estonia), Eawag; Swiss Federal Institute of Aquatic Science and Technology (Switzerland), University of Ljubljana (UL, Slovenia); Helmholtz Centre for Environmental Research (UFZ, Germany); Fraunhofer Institute for Ceramic Technologies and Systems (FHG-IKTS, Germany) and Centre for Cellular & Molecular Biology (CCMB, India). The toxicity and characterisation studies performed by each partner are listed below (green boxes). Please note, that also the dates of performance are given in pink boxes (corresponding month and year are given as numbers, for example 1-2014 indicates January 2014). Photos on test species are illustrative (*V. fischeri* and *P. subcapitata* are from www.wikipedia.com).

3 RESULT

3.1 Characteristics of AgNPs in supplied stock

The stock suspension of nanosilver that was distributed to the NanoValid consortium contained PVP stabilised AgNPs dispersed in deionised water (dH_2O) (nominal concentration 40 g/L) (designated as NNV-003; batch number Parnasos_IG010305_Ag NAMA39_1202_Ag, February 2012; Colorobbia Italia Spa (Firenze, Italy). The concentration of PVP is considered confidential and could not be disclosed by the producer. These AgNPs have been previously characterised and the properties are described in Zou et al. (2015). In short, the X-ray diffraction pattern for AgNPs confirmed the presence of metallic silver and the XPS analysis on AgNPs without any sputtering indicated the presence of high amount of organic molecules on the surface, which could be attributed to PVP. The actual measured total Ag in AgNPs stock suspension (mean ± SD) obtained by three partners were: 38.3 ± 0.8 g/L (EAWAG, ICP-MS), 42.7 ± 5.12 g/L (NICPB, AAS) and 42.44 ± 1.52 g/L (UL, FAAS). All values are close to the nominal value provided by the supplier (40 g Ag/L). Two different approaches (ultrafiltration and 362 769 *g* ultracentrifugation) were used to analyse the share of Ag^+-species in the provided stock: 46% of was measured by ultracentrifugation and 68% by ultrafiltration approach (**Fig.3**).

3.2 Characteristics of different concentrations of AgNPs in dH_2O

3.2.1 The size of AgNPs

According to TEM the AgNPs were highly faceted and polydisperse in shape. Mean primary particle size (50 mg/L) was: 20.4 ± 6.8 nm determined by transmission electron microscopy (**Supplementary information Fig.S1**). This is coherent with the data for the same batch of AgNPs: mean TEM size 21 ± 8 nm reported by Zou et al. (2015).

The mean hydrodynamic diameters of AgNPs in dH_2O are presented in **Fig. 2.** The data were generally comparable between the laboratories with very low polydispersity indexes (**Supplementary information Table S2)**. However, one partner obtained values, which were slightly higher and more variable than the others (CCMB). This partner used a

significantly different angle of measurement (90°) than the rest of the partners (173°). The mean hydrodynamic diameter (z-average value) from all partners was 123.8 ± 12.2, which is in general agreement with the data reported by Zou et al (2015) who showed that the mean hydrodynamic diameter of the particles when suspended in deionised water was 117 ± 24 nm, and the zeta potential was −20 ± 9 mV. No concentration related changes in AgNPs diameter can be elucidated from presented data. Also no changes were observed at different times of analysis.

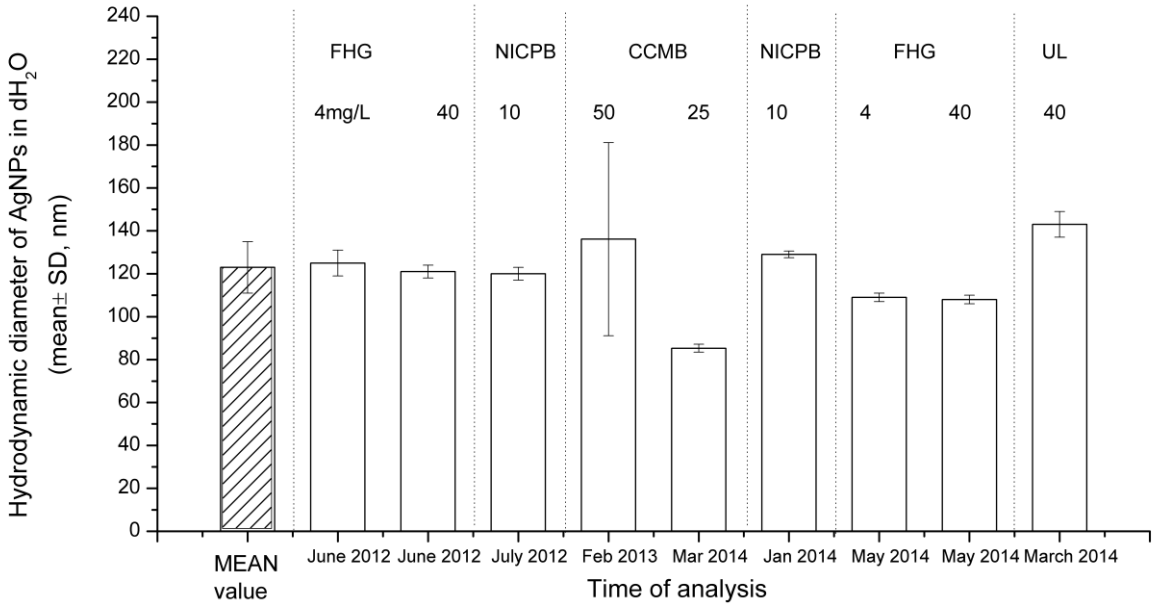

Fig. 2. Hydrodynamic diameter (z-average value ± SD) of AgNP agglomerates in deionised water (dH_2O) at the time of preparation of different AgNP concentrations from stock dispersion (time 0 h). AgNPs were diluted to the concentrations given above the bars and their hydrodynamic size was determined immediately (n≥3). Data are presented according to the time of analysis. Mean value from all partners is also shown. Providers of the reported values: NICPB, CCMB, UL and FHG-IKTS.

3.2.2 Share of Ag^+-species

All the results on the share of Ag^+-species are presented in Figure 3: they are ranged according to the (a) concentration of total Ag, and b) to the time of analysis and by partner labs to evaluate the influence of AgNPs aging on the Ag^+-species. In Figure 3 all results on the quantification of Ag^+-species in AgNP suspensions in dH_2O are grouped according to the techniques used in different laboratories (four of them used centrifugation up to 100 000 *g*, one partner applied ultracentrifugation above 300 000 *g* and one ultrafiltration for the separation of the particulate silver from the Ag^+-species). Each laboratory used several dilutions of AgNPs as well as the assays were done at different time points during 1.5 years using the same stock of AgNPs. Throughout the data are presented as the share of Ag^+-species (%) in the AgNPs suspensions prepared in dH_2O in relation to the total measured concentration of Ag prior to separation (**Fig. 3a and b**). In the upper panel of **Fig. 3 (a)** the data are arranged by increasing concentration of total Ag in the analysed suspension, i.e. from 0.025 mg Ag/L to 115 mg/L. Also, the data for the stock suspension of the AgNPs (40 mg Ag/L) are added (**Fig 3a**). The same data are presented in the lower panel according to the analysis time, from July 2012 till December 2014, i.e. the time-span of 'aging' of the stock suspension was about 1.5 years (**Fig.3b**). It is known that the solubilisation rate of metallic particles depends on the ratio of the particles and solvent (in our case dH_2O) (Kasemets et al., 2009). Indeed, there was a general tendency for increase of the share of Ag^+ species with lower particle: dH_2O ratio, i.e. the share was decreasing with the increase of the nominal concentration of the suspension of AgNPs, however the correlation was not highly significant (R^2=0.24) (**Fig.4**).

However, we observed that the same method used for particle separation usually yielded the same share of Ag^+-species even when the concentration of total silver in initial suspensions largely varied. For example, when the data of different partners were analysed separately (**Supplementary information Fig. S2**) we observed that UL (used 100 000 *g* centrifugation as a separation step) obtained share of Ag^+-species from 39-53 % (average 48 %) while the nominal total concentration of Ag in suspensions varied from 2.7 to 115 mg/L. UFZ (16 000 *g* centrifugation) obtained almost the same share of Ag^+ for 0.025 mg Ag/L, 2.7 mg/L as well as for 43 mg Ag/L (60-64%). NICPB obtained 46-49% share of Ag^+-species for 9.8 mg Ag/L and 40 000 mg Ag/L suspensions when using the highest centrifugation force (362 769 *g*). Analogously, EAWAG team who used ultrafiltration and centrifugation as separation techniques, obtained 52-68% share of Ag^+-species for concentrations spanning from 1 to

40 000 mg Ag/L. This initiated further studies to find the source of variability. We found out, that centrifugation up to 100 000 *g* (30 min) was insufficient to separate AgNPs from Ag^+-species, because NPs were found in the supernatant. In this case the use of DLS to check the efficiency of separation was appropriate, since we have detected NPs in the supernatant. In the case of ultracentrifugation at 362 769 *g* (30-60 min) and 3 kDa pores a SP-ICP-MS approach was used and no NPs were found after separation (Ivask et al., 2014, Navarro et al. 2008, Odzak et al. 2014). However, the efficiency of NPs separation could not entirely explain the variability of data obtained by different partners. For example, comparing the data for 10 mg AgNPs/L the values obtained with the insufficient separation method (100 000 *g*) were lower (39 %) than those of ultracentrifugation (52 %), even though particles remaining in the supernatant should lead to a higher silver share. Due to the reasons described above, we conclude that the absolute values in **Fig. 3** may be overestimated and therefore a conclusions on the time- related modification of AgNPs in stock dispersion ("aging") cannot be deduced based on these data.

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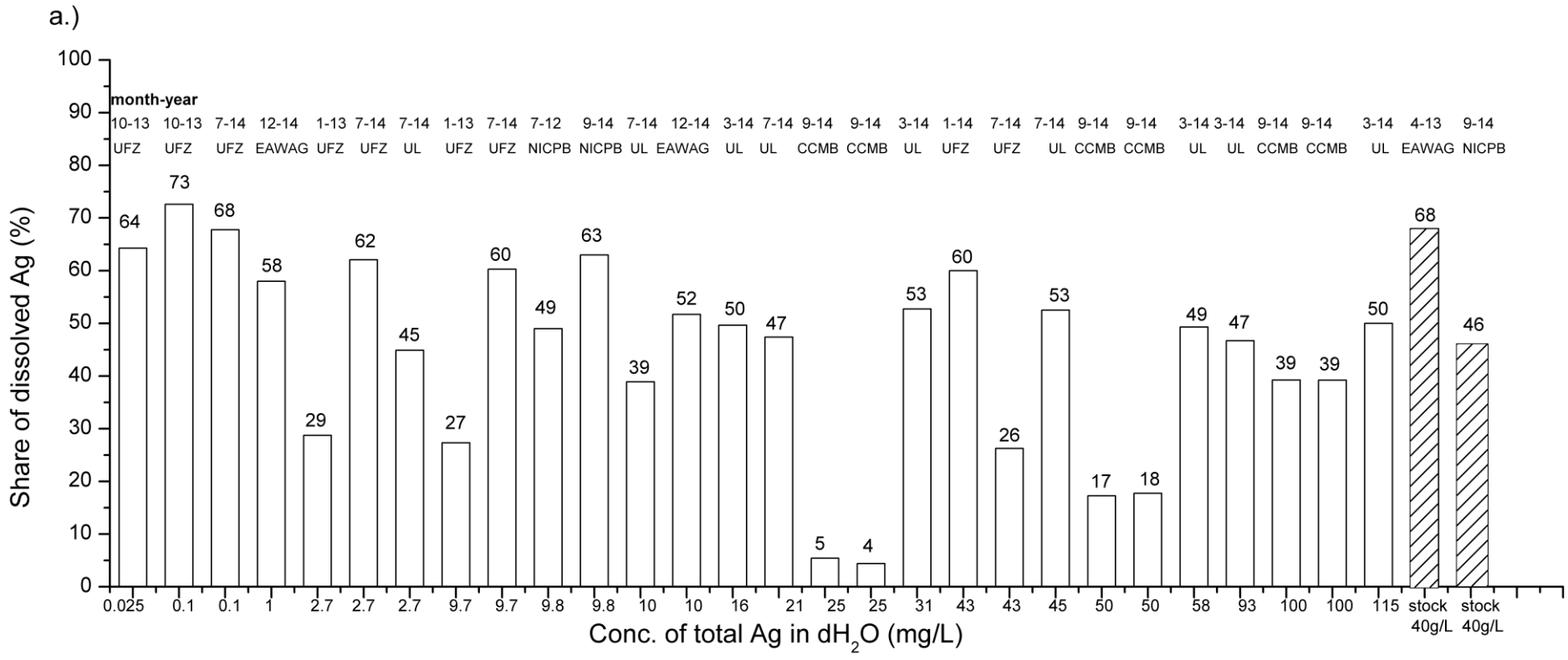

UFZ: 16 000 g; NICPB: 362 769 g; EAWAG: 3kDa pores, 4000 g
UL: 100 000 g; CCMB: 100000 g

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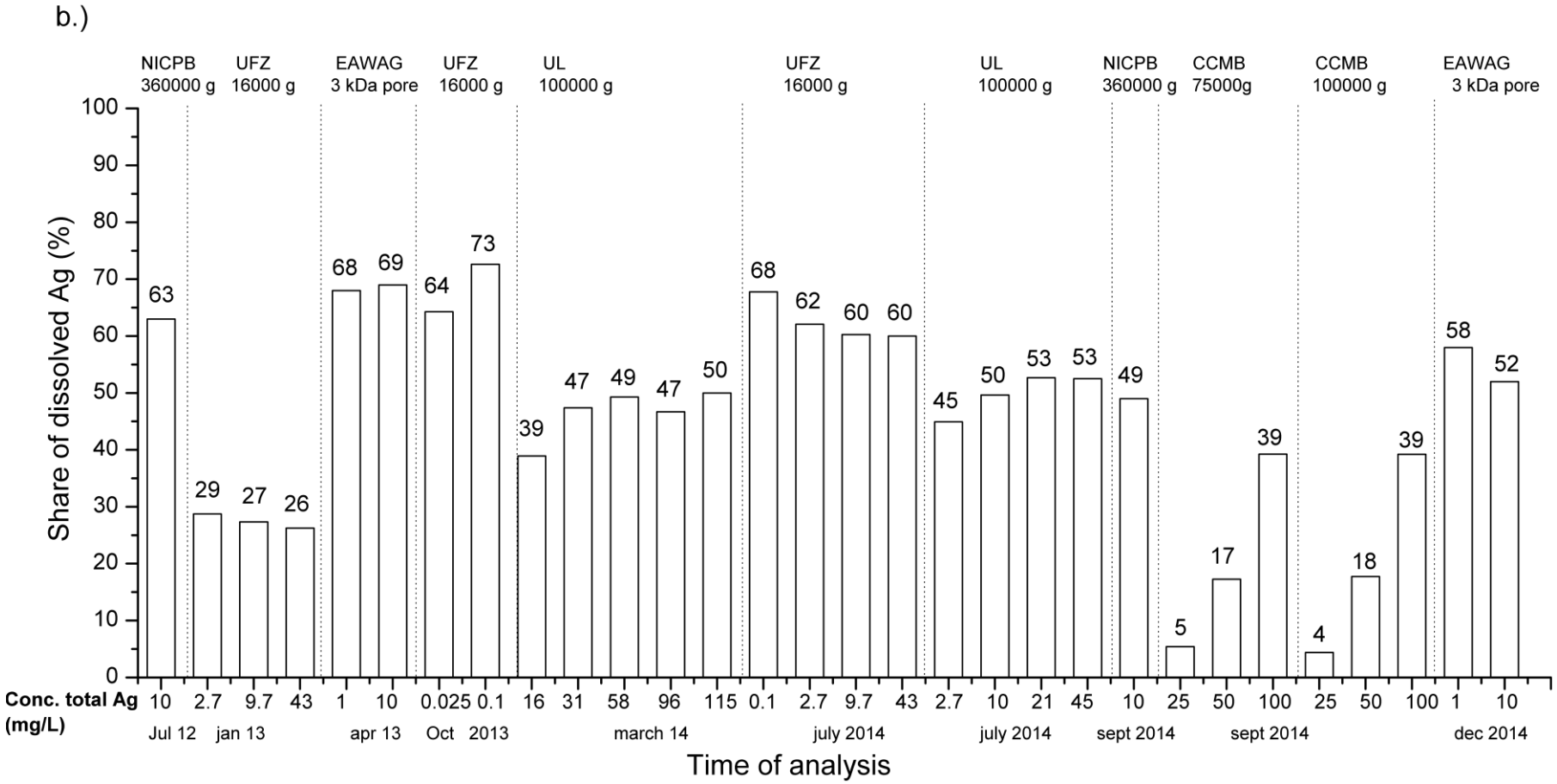


Fig 3. The share of Ag^{+}-species in comparison to measured total Ag in dH_2O performed by different partners: a) ranged according to the concentration of total Ag, and b) ranged according to the time of analysis and by partner labs. Stock AgNPs suspension is also shown (Fig.3a). The measured concentrations of total Ag (mg/L) are shown on x-axis. Mean values are shown on the top of the column.

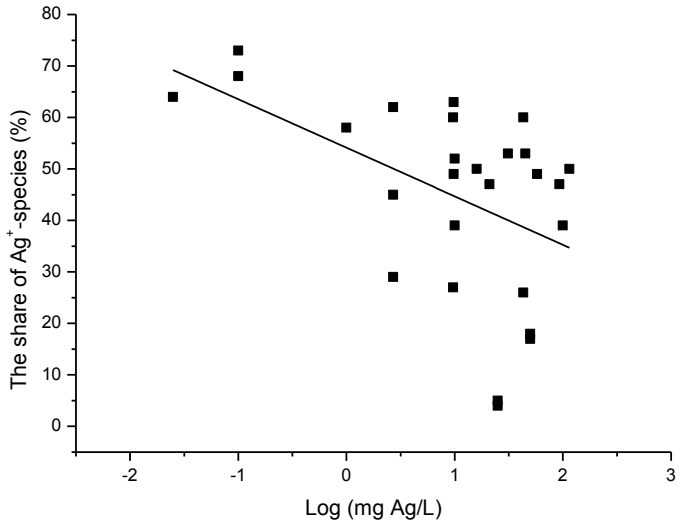


Fig. 4. Share of Ag^+-species versus total concentration of Ag in the suspension (log values). Data are plotted from Fig. 3a whereas the data for stock suspensions (40 000 mg Ag/L) are excluded.

3.3 Characteristics of different AgNPs concentrations in toxicity test media

3.3.1 The hydrodynamic size of AgNP agglomerates

The hydrodynamic diameters of the AgNPs agglomerates in different toxicity test media were quite comparable, ranging from 103 nm to 136 nm **(Fig.5)**. Similarly as in the case of dH_2O, the value provided by a partner using a significantly different angle of measurement (90°) than the rest of the partners (173°) yields the data with higher SD. The sizes of AgNP agglomerates in the toxicity test media were similar to those in dH_2O (123.8 ± 12.2 nm).

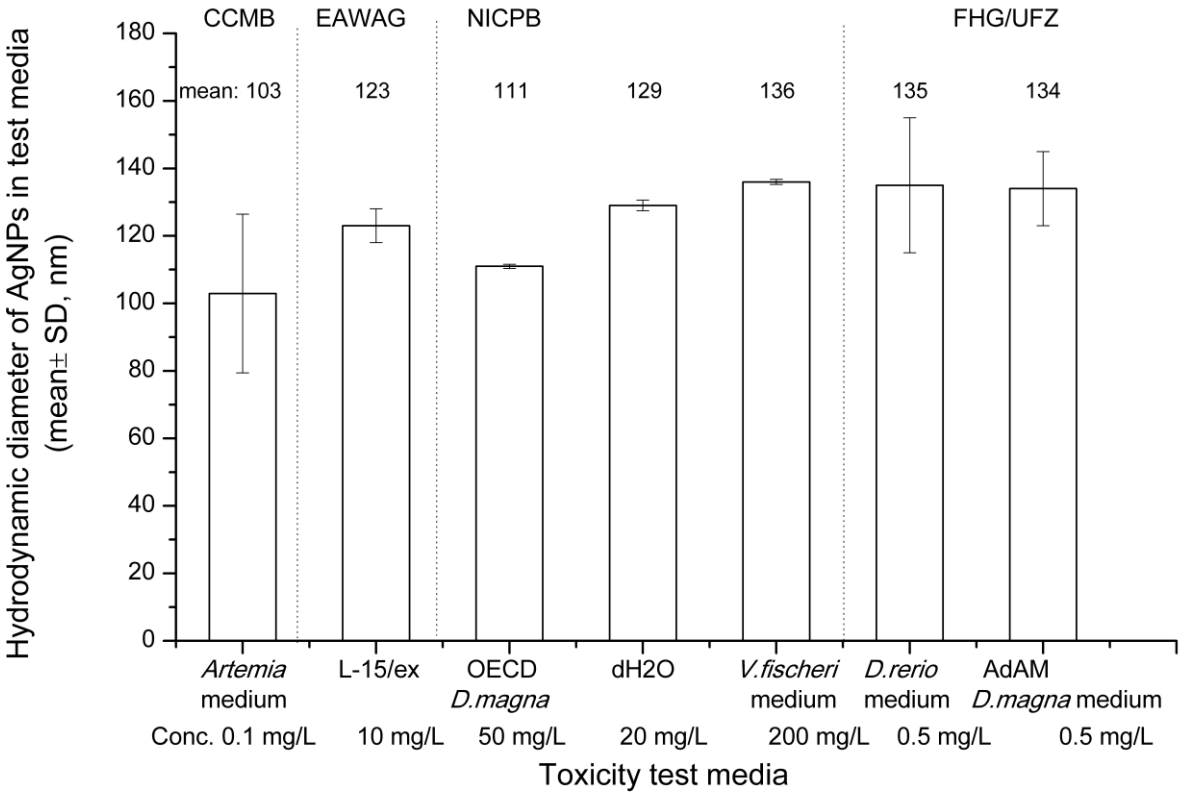


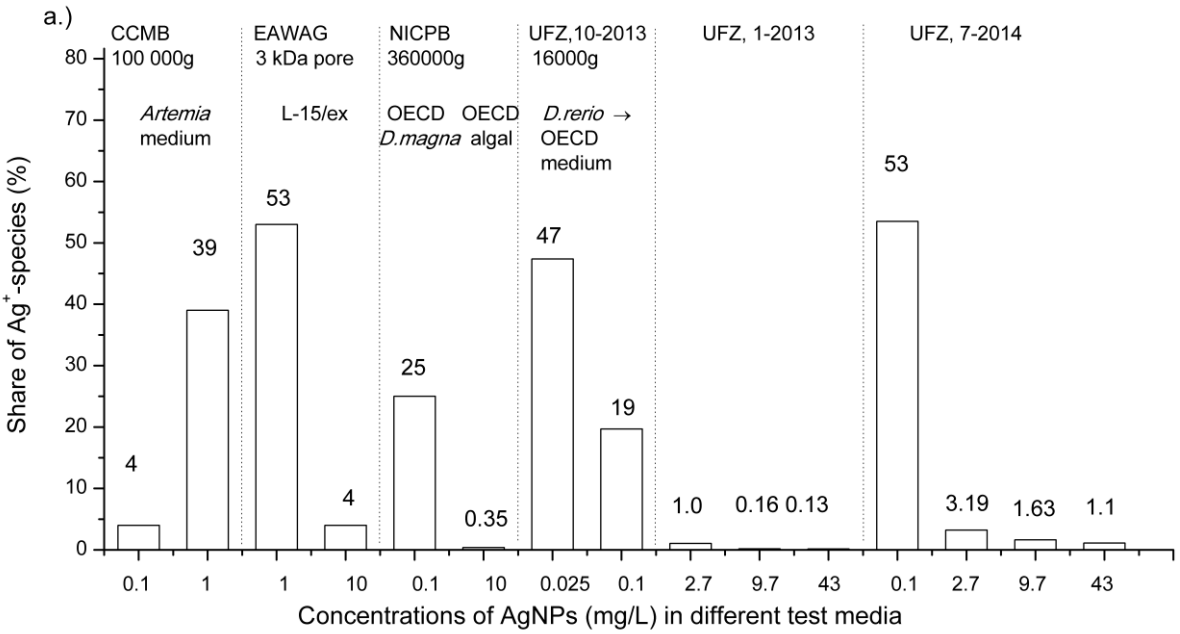
Fig. 5. Hydrodynamic size (z-average value ± SD, n≥3) of AgNPs agglomerates in different toxicity test media (time 0 h). AgNPs stock suspension was diluted to the concentrations given on x-axis and the particle size was determined immediately. Test media are described on x-axis: Artemia- *Artemia sp.* Assay medium, L-15/ex - exposure medium for RTgutGC cells, *V. fischeri*- medium for *Vibrio fischeri*, *D. rerio*- Zebrafish OECD medium, ADaM and OECD media for *Daphnia magna*. Values are shown on the top of the bars.

3.3.2 Share of Ag^+-species

The share of Ag^+-species (%) in comparison to measured total Ag in toxicity test media is shown in **Fig. 6.** When comparing the data from the partners who tested a range of AgNPs concentrations in zebrafish embryo OECD medium or L-15/ex medium always applying the same separation technique, we can conclude, that the presence of Ag^+ species (dissolution) depends on the concentration: a significantly larger share was measured at lower exposure concentration of AgNPs (**Fig. 5a**). That is coherent with data obtained for the share of Ag^+-species in deionised water (**Supplementary information Fig. S2**) and the data previously shown for solubilisation of CuO and ZnO NPs (Kasemets et al., 2009).

The effect of test medium composition on the amount of Ag^+-species (%) can be evaluated from **Fig 5b**, which shows the data obtained at approximately the same total Ag concentration (10 mg/L of algal media, 14 mg/L for L-15/ex, 9.7 mg/L in zebrafish medium, 10 mg/L in dH_2O). For discussion purposes, we also added the values for Luria Bertani bacterial growth medium (LB medium: tryptone and yeast extract), and yeast peptone dextrose (YPD medium: yeast extract, bactopeptone and glycose). The data show that significantly less Ag^+-species (%) were measured in complex algal, L-15/ex and zebrafish embryo OECD medium in comparison to LB, YPD and dH_2O.

The amount of Ag^+-species in toxicity test media was analysed immediately after the preparation (time 0 h), and also after 24 h and 48 h at room temperature. In none of the cases (daphnia, algae and zebrafish media) the share of Ag^+-species was different from those at the beginning of the test (data not shown). This indicates that the total amount of Ag^+-species does not change during the experimental exposure period used.



b.)

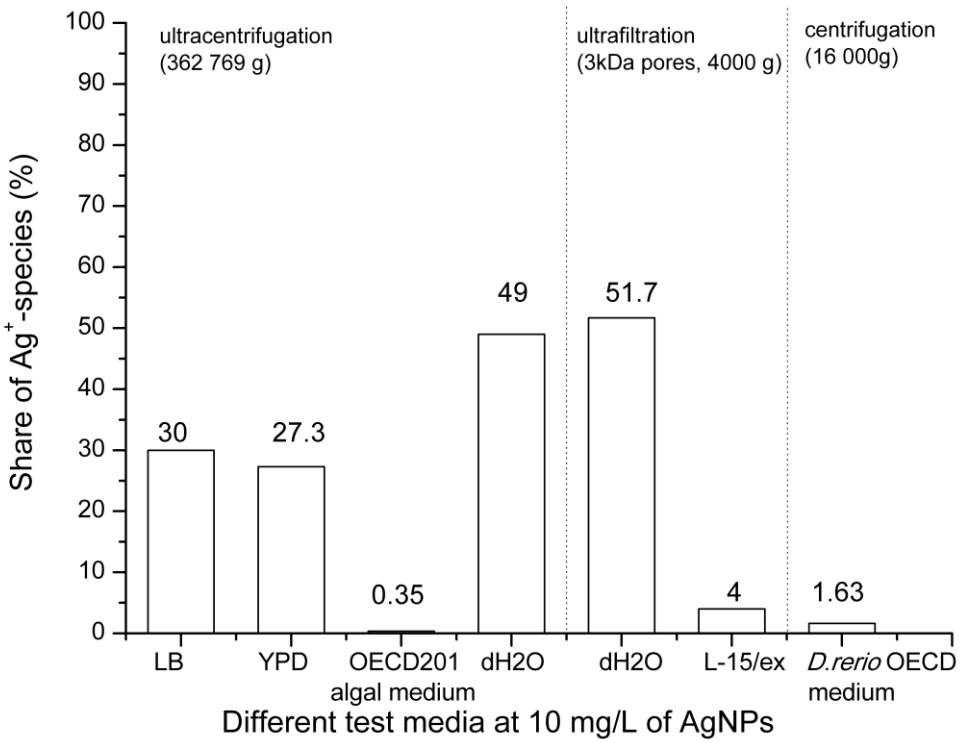


Fig. 6. The share of Ag^{+}-species (%) in comparison to measured total Ag in toxicity test media analysed by different partners. Data for all toxicity test media are shown in Fig 6**a**, while in Fig. 6b we present the data only for 10 mg/L of AgNPs. Data for LB (Luria Bertani bacterial growth medium), and YPD (yeast extract peptone dextrose) are added for comparison. Abbreviations: OECD201 test medium (for algal assays), L-15/ex: exposure medium for RTgutGC cells, and zebrafish *Danio rerio* embryo OECD medium. Numerical values are shown on the top of the bars.

3.4 Toxicity of AgNPs

Toxicity data for AgNPs are summarised in **Table 1**. When we apply the toxicity classification used by Bondarenko et al. (2013) AgNPs need to be considered extremely toxic to crustaceans *D. magna,* microalgae *P. subcapitata* and zebrafish embryos; very toxic to RTgutGC cells *in vitro*, toxic to bacteria *V. fischeri, protozoa T. thermophila,* and harmful to marine crustaceans *A. franciscana*.

179 The toxicity to *D. rerio* embryo (age at exposure 2hpf) was tested at two different
180 times over the course of 15 months. The toxicity measured in August 2014 (48h EC50= 0.061
181 mg/L) was the same as in May 2013 (*D. rerio*) (48 h EC50= 0.066 mg/L).

Table 1: Toxicity of AgNPs and $AgNO_3$ towards the different test organisms. EC50 values are arranged from the least sensitive test organism to the most sensitive based on the exposure protocols used. Toxicity of AgNPs and $AgNO_3$ were ranked to different hazard categories according to EC50 values. For the ranking, we applied the criteria used in Kahru and Dubourguier (2010) and color coding applied by Bondarenko et al. (2013): <0.1 mg/L=extremely toxic (dark red); 0.1–1 mg/L = very toxic (red); 1–10 mg/L = toxic (orange); 10–100 mg/L = harmful (yellow). *D. rerio* test was done at two different times (May 2013, and August 2014) and different developmental stages (hpf-hours post fertilisation). Cartoons depicting the organisms are symbolic (*V. fischeri* and *P. subcapitata* are from www.wikipedia.com).

Test organism		**Biological organization**	**Toxicity Endpoint (biomarker)**	**International standardization**	**Effect value (mg AgNPs/L)**	**Ranking (AgNPs)**	**Effect value mg Ag^+/L ($AgNO_3$)**	**Ranking (Ag^+) salt**
Artemia franciscana		Marine crustacean, arthropoda	Immobility	no	24h EC50=1.3 48h EC50=10.8		48h EC50=15.0	
			Hatching success (% of hatched artemia)	no	24h EC50=1.3			
Tetrahymena thermophila		Freshwater protozoa	Viability (ATP content)	no	2h EC50= 3.2 24h EC50=3.9		2h EC50= 2.9 24h EC50=2.9	
Vibrio fischeri		Bacteria, prokaryotic microorganisms	Viability (luminescence)	ISO 21338:2010	30min EC50=2.6		30 min EC50=1.4	
RTgutGC cells		Rainbow trout's (fish) gut cells *in vitro*	Viability (metabolic activity)	no	24h EC50=0.551			
			Viability (membrane integrity)	no	24h EC50=0.732			
			Viability	no	24h EC50=0.576			

			(Lysosomal integrity)					
Danio rerio, 2 hpf embryo (May 2013)		Freshwater fish	Lethality (lethal malformations)	OECD 236: 2013	24h LC50= 0.084 48h LC50= 0.066			
Danio rerio, 2 hpf embryo (August 2014)		Freshwater fish	Lethality	OECD 236: 2013	24h LC50=0.061 48h LC50= 0.061		24h LC50= 0.045 48h LC50= 0.032	
Danio rerio, 28 hpf, dechorionated (May 2013)		Freshwater fish	Lethality	no	24h LC50=0.0324 48h LC50= 0.0334			
Danio rerio, 72 hpf, hatched embryos (May 2013)		Freshwater fish	Lethality	no	24h LC50=0.0493		24h LC50=0.018 48h LC50= 0.014	
Pseudokirchneriella subcapitata		Freshwater green microalgae	Growth (cell density)	OECD 201:2011	72h EC50=0.0086		72h EC50= 0.0071	
Daphnia magna, ADaM medium		Freshwater crustacean, arthropoda	lethality (Immobility)	OECD 202:2004	48h EC50=0.0034		48h EC50= 0.0014	
Daphnia magna, OECD medium		Freshwater crustacean, arthropoda	lethality (Immobility)	OECD 202:2004	48h EC50= 0.0025		48h EC50= 0.001	

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The relation between the toxicity of AgNPs and Ag^+-species from salt ($AgNO_3$) was evaluated based on the comparison of hazard ranking (**Table 1**) and linear regression analysis (**Fig.6)**. The hazard ranking according to 24 h and 48 h EC50 values was for AgNPs and Ag^+-species was exactly the same (**Table 1; Fig. 7**). The linear fitting revealed significant correlation between the effect values for AgNPs and Ag^+-species with coefficient of determination of R^2= 0.998 and a slope of 1.5 (**Fig.7**). From these data it is evident that the toxicity of AgNPs may be explained by Ag^+-species action in addition to AgNPs/AgNP-complexes.

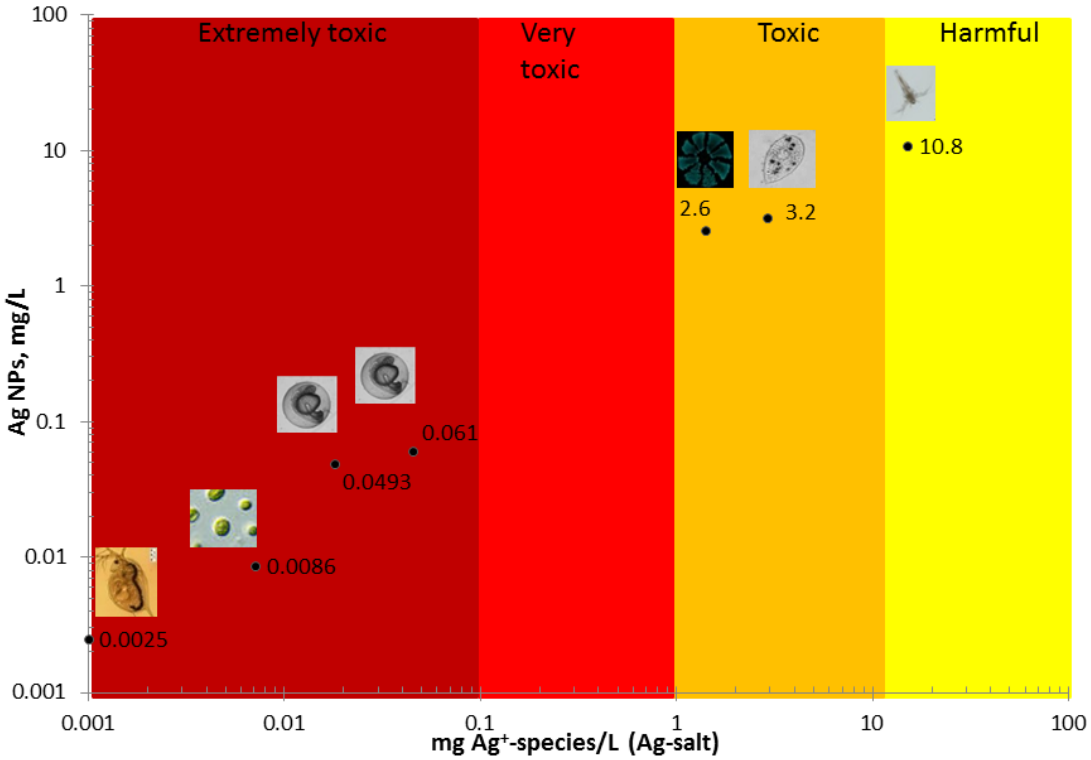

Fig.7. The correlation between the toxicity of AgNPs and Ag^+-species to different test organisms. Those values from Table 1 were included, which were available for both AgNPs and $AgNO_3$ (eight of them) (for *D. magna* two values in two different test media are reported). The numerical values for AgNPs toxicity are indicated for each test near the pictogram. The hazard ranking criteria are as described in Kahru and Dubourguier (2010) and color coding as in Bondarenko et al. (2013): <0.1 mg/L=extremely toxic (dark red); 0.1–1 mg/L = very toxic (red); 1–10 mg/L = toxic (orange); 10–100 mg/L = harmful (yellow). Cartoons depicting the organisms are symbolic (*V. fischeri* and *P. subcapitata* are from www.wikipedia.com).

4 DISCUSSION

In this paper, the experimental data on the hazard identification of AgNPs within the NanoValid consortium have been systematically collected and analysed in terms of the methodological approaches. The six involved partner laboratories investigated the toxicity of the same stock of AgNP suspension using a set of test organisms from different environments and trophic levels. Altogether, ten different tests with different exposure regimes were performed. In parallel, all the partners assessed the characteristics of AgNPs in test media, such as the hydrodynamic size of NPs and the share of Ag^+-species (free and ligand-complexed). Based on these data and the hazard ranking criteria used by some of the authors earlier (Bondarenko et al., 2013) we demonstrate the sensitivities of different organisms to nanosilver and compare that to silver ions. The prime focus of this paper, however, is to give recommendations for hazard identification of NPs, and to demonstrate the importance of using harmonised procedures for NPs characterisation.

The need for harmonised protocols for the share of Ag^+ species in test media. The toxicity of silver nanoparticles has frequently been associated to Ag ions (Bondarenko et al., 2013; Ivask et al., 2014). Therefore, the share of Ag^+-species in the exposure media is crucial for the understanding of the mechanism of toxicity of different nanosilver preparations. In several metal-based nanoparticles, the dissolution of metals plays a great role in the interpretation of observed toxicity and solubility is a very important property of the nanomaterials to be considered in nanoregulation (Tantra et al., 2015). In this work, we adopted the term "the share of Ag^+ species" instead of dissolved Ag to indicate that in addition to "free" Ag^+ also their soluble ligand complexes are quantified with the applied techniques.

In this study very variable data on the share of Ag^+-species in dH_2O were obtained by different partners (Figure 3), ranging from 4 to 73 %. The variability can be attributed to different methods used to separate dissolved species from particles (Ivask et al., 2014; Odzak et al., 2014). Two main separation approaches have been applied: (ultra)centrifugation and ultrafiltration. We confirmed that among the protocols tested, only two approaches are efficient in AgNPs separation from Ag^+-species: the ultracentrifugation (362 769 *g*; 30-60 min) (Ivask et al, 2014) and 3 kDa pores ultrafiltration (4000 *g*, 20 min) (Navarro et al. 2008). However, the efficiency of separation alone could not explain the

observed variability of Ag^+-species data, since different results were observed by ultrafiltration and ultracentrifugation approaches: ultracentrifugation of stock Ag suspension (40 g/L) resulted in significantly lower share of Ag^+ species (46 %) than ultrafiltration (68%). This indicates that also other factors influence the outcome of the soluble Ag^+-species measurement. Tantra et al. (2015) recently concluded that the nanoparticle dissolution process is governed by dynamic relationships between a number of variables. Among these surface tensions, hydrodynamic conditions in terms of mechanical agitation, and mass of the particle exposed (aggregation/agglomeration) may significantly differ depending on the separation method applied. In fact, Odzak et al. (2014) suggested that the force applied during the process of ultrafiltration may increase the separation of the Ag-NPs and their PVP coating, thus exposing more Ag-NPs and increasing the release of Ag ions from the particles.

The experience obtained in this study clearly implies the need for a harmonised protocol for metal species measurements in NPs dispersions. Our suggestion is to check the efficiency of separation techniques prior to quantification, both for the (i) impact of the procedure on the particle dissolution itself, and (ii) the kind of metal species separated from each other (free ions, complexes and particles) (the latter may be done by application of electrochemical approaches, Romih et al., 2015). Another option is to use a quantification method which is able to clearly distinguish between particles from the background, such as for example single particle ICP-MS. It is far beyond this paper to discuss the suitability of certain separation and detection techniques (this has been reviewed elsewhere, Tantra et al., 2015), but we support the recommendation that a complementary approach applying a suite of techniques is needed.

Test medium composition alters Ag^+-species concentration. Our data showed that the medium composition significantly altered the presence of Ag^+-species in the AgNPs suspension. Namely, less Ag^+-species was measured in complex algal medium, L-15/ex and zebrafish embryo medium in comparison to LB, YPD and dH_2O. Since Ag^+ was already present in the stock dispersion (68% and 46%, depending on the analytical method used for separation of NPs and soluble Ag-species) we would expect that even without additional dissolution of AgNPs more Ag^+ would be present in algal medium, L-15/ex and zebrafish embryo. This suggests that Ag^+ is ligand- complexed in L-15/ex, algal and zebrafish embryo medium and these complexes are settled out during ultracentrifugation and ultrafiltration (L-15/ex). For L-15/ex medium, such a complexation of Ag^+ was recently shown by Yue et al.

(2015). When 1 µM of silver is introduced in L-15/ex medium, only 0.03% of the total silver is present as free Ag^+, while most of the silver is complexed by chloride forming $AgCl_2^-$ and $AgCl_3^{2-}$ complexes. L-15/ex, algal and zebrafish embryo medium have both high sulfate (SO^-_4) and chloride (Cl^-) content, while this is not the case for in LB, YPD and dH_2O. It is well known, that depending on the Cl/Ag ratio, Ag^+ react with chloride to form solid or aqueous Ag−Cl species (Levard, 2012, Behra et al., 2013, Yue et al., 2015). Sulfate (SO^-_4) is also a possible ligand for Ag^+ (Xiu et al., 2011, Choi et al., 2009), however it has a lower stability constant to form Ag-ligand complexes or precipitates and hence are less likely to occur (Choi et al., 2008, Levard et al., 2012). Algal medium also contains EDTA, which chelates Ag^+ (Fiorucci et al., 2000). Our results are in accordance with those by Ivask et al. (2014), who found, that 48 h and 72 h incubation of $AgNO_3$ in OECD 202 artificial freshwater (daphnids medium) and algal medium significantly decreased the amount of measured Ag by 78% and 18%, respectively, while a 100% recovery was found in the case of Ag-salt in dH_2O (at 390 000 *g*, 30-60 min). Based on these data we conclude that extrapolation of metal species data across toxicity test media is not possible and suggest that metal species measurements should be done in each respective toxicity media. Soluble metal controls should be included in parallel to account for the metal complexing by the media components.

The need for harmonised protocols for the NPs hydrodynamic size. All hydrodynamic size measurements of AgNPs were done using a DLS technique and were comparable. The angle of measurement and the concentration of particles in the range of 4-50 mg/L did not significantly change the z-average values obtained for the diameter of particles. This is the case only for dispersions with narrow size distributions. Hence, in general, the effect of the angle on the measurement needs to be further elaborated. It is considered, that the scattering angle used for measuring the particle size affects the size determination; e.g. the measure of particle size will be smaller at higher angle (Takahash et al., 2008).

The consideration for the aging of nanomaterials. One of the important properties of nanomaterials is their modification, e.g. aging; during the storage and the toxicity testing (Kittler et al. 2010, Tejamaya et al., 2012). In the present study, we were unable to estimate the time-dependent modification of AgNPs (in terms of Ag^+-species concentration) during the storage period (between January 2013 and September 2014) due to different separation methods applied. We have shown that AgNPs do not provoke different toxic effect on *D.*

rerio embryo within a 1.5 years difference, but still further experiments using appropriate techniques are needed to confirm that the AgNPs have not aged. The tests on the different Ag^+- species ratios should be done. Aging of AgNPs has previously been evidenced by other authors. Tejamaya et al. (2011) found significant changes in surface chemistry, shape, aggregation and dissolution of monodisperse (10 nm) citrate, PVP and polyethylene glycol (PEG) capped AgNPs in OECD *Daphnia sp*. medium after 21 days. We therefore suggest that NP characteristics are carefully monitored throughout the experimental period and the characterisation should be done at the same time as the performance of toxicity test to take into consideration the potential aging of stock NPs. Within the NanoValid project several activities aim at harmonising test procedures. One example is the development of decision trees to better structure test procedures, where the aging will be considered (Kühnel and Nickel, 2014).

Comprehensive set of toxicity data obtained confirms that toxicity of AgNPs is governed by Ag^+-species. This paper provides a comprehensive set of toxicity data on the AgNPs using a set of environmentally relevant aquatic test species from different food-web levels. AgNPs were found to be extremely toxic to crustacean *D. magna,* microalgae *P. subcapitata* and zebrafish embryo *D. rerio*; very toxic to RTgutGC cells, toxic to *V. fischeri, T. thermophila,* and harmful to *A. franciscana* (**Table 1, Fig.7.**). This is in line with previous reports, which found *D. magna* and algae to be very sensitive regarding silver NP toxicity (Kahru and Dubourguier, 2010, Bondarenko et al. 2013, Ivask et al., 2014). The linear regression analysis revealed that the toxicity of AgNPs was significantly correlated to those observed for Ag^+-species from Ag-salt as was also previously reported (Bondarenko et al., 2013; Ivask et al., 2014; Xiu et al., 2012, Groh et al., 2015). This finding supports the necessity of metal species measurements in NPs dispersions. Preferably, the share of different silver species (free ions, different complexes, particles) should be determined in the respective test medium. The data also indicate that in the case of metallic NPs, the toxicity testing should include the corresponding soluble metal salt. This information could serve as a reference for the estimation of the bioavailable metal ions and/or ligand metal-complexes that induce certain effect.

CONCLUSIONS

The NanoValid consortium concludes that high quality hazard identification of nanomaterials should be based on harmonised protocols for toxicity testing as well as characterisation of nanomaterials. We find it important that the measurement of metal ion-species is done for each toxicity test medium separately and measured at a range of toxicity test concentrations. Also data on metal species (free ions, different complexes, particles) in the respective test medium should be provided. Soluble metal salt control should be an integral part of nanomaterial testing, both during toxicity testing as well as for Ag^{+}-species measurements.

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References:

Aruoja, V., Dubourguier, H.C., Kasemets, K., Kahru, A., 2009. Toxicity of nanoparticles of CuO, ZnO and TiO_2 to microalgae *Pseudokirchneriella subcapitata*. Sci. Total Environ. 407: 1461-1468.

Baker, T.J., Tyler, C.R., Galloway, T.S., 2014. Impacts of metal and metal oxide nanoparticles on marine organisms. Environ. Pollut. 186:257-71.

Behra, R., Sigg, L., Clift, M.J., Herzog, F., Minghetti, M., Johnston, B., et al, 2013. Bioavailability of silver nanoparticles and ions: from a chemical and biochemical perspective. J. R. Soc. Interface. 10:20130396.

Bondarenko, O., Juganson, K., Ivask, A., Kasemets, K., Mortimer, M., Kahru, A., 2013. Toxicity of Ag, CuO and ZnO nanoparticles to selected environmentally relevant test organisms and mammalian cells in vitro: a critical review. Arch.Toxicol. 87: 1181-1200.

CEC (1996): CEC (Commission of the European Communities) technical guidance document in support of commission directive 93/67/EEC on risk assessment for new notified substances. Part II, Environmental Risk Assessment. Office for official publications of the European Communities, Luxembourg

Chernousova, S., Epple, M., 2013. Silver as antibacterial agent: ion, nanoparticle, and metal. Angew. Chem. In.t Ed. Engl. 52:1636-53.

Choi, O., Clevenger, T.E., Deng, B., Surampalli, R.Y., Ross, L. Jr, Hu, Z.,2009. Role of sulfide and ligand strength in controlling nanosilver toxicity. Water Res. 43:1879-86.

Fabrega, J., Luoma, S.N., Tyler, C.R., Galloway, T.S., Lead, J.R., 2011. Silver nanoparticles: behaviour and effects in the aquatic environment. Environ. Int. 37:517-31.

Fiorucci, A.R., Saran, L.M., Cavalheiro, E.T.G, Neves, E.A., 2000. Thermal stability and bonding in the silver complexes of ethylenediaminetetraacetic acid. Thermochim. Acta 356: 71-78.

Groh, K.J., Dalkvist, T., Piccapietra, F., Behra, R., Suter, M.J., Schirmer, K., 2015. Critical influence of chloride ions on silver ion-mediated acute toxicity of silver nanoparticles to zebrafish embryos. Nanotoxicology 9:81-91.

Heinlaan, M., Ivask, A., Blinova, I., Dubourguier, H-C., Kahru, A., 2008. Toxicity of nanosized and bulk ZnO, CuO and TiO2 to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. Chemosphere 71:1308 – 1316.

Herrin, R.T., Andren, A.W., Shafer, M.M., Armstrong, D.E., 2001. Determination of silver speciation in natural waters. 2. Binding strength of silver ligands in surface freshwaters. Environ. Sci. Technol. 35:1959-66.

Hristozov, D.R., Zabeo, A., Foran, C., Isigonis, P., Critto, A., Marcomini, A., et al., 2014. A weight of evidence approach for hazard screening of engineered nanomaterials. Nanotoxicology 8:72-87.

ISO 21338:2010(E). Water quality - Kinetic determination of the inhibitory effects of sediment, other solids and coloured samples on the light emission of *Vibrio fischeri* (kinetic luminescent bacteria test).

Ivask, A., Juganson, K., Bondarenko, O., Mortimer, M, Aruoja, V., Kasemets, K., et al. 2013. Mechanisms of toxic action of Ag, ZnO and CuO nanoparticles to selected ecotoxicological test organisms and mammalian cells in vitro: A comparative review. Nanotoxicology 8: 57-71

Ivask, A., Kurvet, I., Kasemets, K., Blinova, I., Aruoja, V., Suppi, S., et al., 2014. Size-Dependent Toxicity of Silver Nanoparticles to Bacteria, Yeast, Algae, Crustaceans and Mammalian Cells In Vitro. PLoS ONE 9: 102-108.

Jackson, P., Jacobsen, N.R., Baun, A., Birkedal, R., Kühnel, D., Jensen, K.A., et al., 2013. Bioaccumulation and ecotoxicity of carbon nanotubes. Chem. Cent. J. 7: 154.

Kahru, A., Dubourguier, H-C., 2010. From ecotoxicology to nanoecotoxicology. Toxicology 269:105-119

Kahru, A. Ivask, A., 2013. Mapping the Dawn of Nanoecotoxicological Research. Accounts of Chemical Research. Acc. Chem. Res. 46:823-833

Kasemets, K., Ivask, A., Dubourguier, H.C., Kahru, A., 2009. Toxicity of nanoparticles of ZnO, CuO and TiO_2 to yeast *Saccharomyces cerevisiae*. Toxicology in vitro 23: 1116-1122

Kawano, A., Haiduk, C., Schirmer, K., Hanner, R., Lee, L.E.J., Dixon, B., Bols, N.C., 2011. Development of a rainbow trout intestinal epithelial cell line and its response to lipopolysaccharide. Aquacult. Nutr. 17, 241–252.

Kittler, S., Greulich, C., Diendorf, J. , 2010. Toxicity of silver nanoparticles increases during storage because of slow dissolution under release of silver ions. Chem. Mater. 22: 4548:4554.

Krug, H.F., 2014. Nanosafety research-are we on the right track? Angew Chem Int Ed Engl. 53:12304-19.

Kühnel, D. and Nickel, C., 2014. The OECD expert meeting on ecotoxicology and environmental fate - towards the development of improved OECD guidelines for the testing of nanomaterials. Sci. Total Environ. 472:347-53

Kühnel, D., Marquardt, C., Nau, K., Krug, H.F., Mathes, B., Steinbach, C., 2014. Environmental impacts of nanomaterials: providing comprehensive information on exposure, transport and ecotoxicity - the project DaNa2.0. Environmental Sciences Europe 26:21.

Levard, C., Hotze, E.M., Colman, B.P., Dale, A.L., Truong, L., Yang, X.Y., et al. 2013. Sulfidation of silver nanoparticles: natural antidote to their toxicity. Environ Sci Technol. 47:13440-8

Mills, K.C., Murry, D.A., Guzan, K. A., Ostraat, M. 2014. Nanomaterial Registry: database that captures the minimal information about nanomaterial physico-chemical characterisitcs. J. Nanopart. Res. 16:2219.

Misra, S.K., Dybowska, A., Berhanu, D., Luoma, S.N., Valsami-Jones, E., 2012. The complexity of nanoparticle dissolution and its importance in nanotoxicological studies. Sci Total Environ. 438:225-32.

Mortimer, M., Kasemets, K., Kahru, A., 2010. Toxicity of ZnO and CuO nanoparticles to ciliated protozoa *Tetrahymena thermophila*. Toxicology. 269:182-189

Navarro, D.A., Watson, D.F., Aga, D.S., Banerjee, S., 2009. Natural organic matter-mediated phase transfer of quantum dots in the aquatic environment. Environ. Sci. Technol. 43:677-82.

Odzak, N., Kistler, D., Behra, R., Sigg, L., 2014. Dissolution of metal and metal oxide nanoparticles in aqueous media. Environ. Pollut. 191:132-8.

Oomen, A.G., Bos, P.M., Fernandes, T.F., Hund-Rinke, K., Boraschi, D., Byrne, H.J., Aschberger, K., Gottardo, S., von der Kammer, F., Kühnel, D., Hristozov, D., Marcomini, A. et al., Migliore, L., Scott-Fordsmand, J., Wick, P., Landsiedel, R. 2014. Concern-driven integrated approaches to nanomaterial testing and assessment--report of the NanoSafety Cluster Working Group 10. Nanotoxicology. 8:334-48.

OECD 2004: Guidelines for the testing of chemicals. Section 2: Effects on Biotic Systems. Test No. 202: Daphnia sp. acute immobilisation test. Organisation for Economic Co-operation and Development, Paris, France.

OECD 2011. Guideline 201 for the Testing of Chemicals, Alga, Growth Inhibition Test. Organisation for Economic Cooperation and Development, Paris, France.

OECD 2013: Guidelines for the testing of chemicals. Section 2: Effects on Biotic Systems. Test No. 236: Fish Embryo Acute Toxicity (FET) Test. Organisation for Economic Co-operation and Development, Paris, France.

OECD, 2010. List of Manufactured Nanomaterials and List of Endpoints for Phase One of the Sponsorship Programme for the Testing of Manufactured Nanomaterials: Revision.ENV/JM/MONO(2010)46.

OECD 2014. Ecotoxicology and Environmental Fate of Manufactured Nanomaterials: Test Guidelines. Series on the Safety of Manufactured Nanomaterials No. 40, available online, accessed 28-6-2015.

http://www.oecd.org/science/nanosafety/publicationsintheseriesonthesafetyofmanufacturednanomaterials.htm

Ribeiro, F., Gallego-Urrea, J.A., Jurkschat, K., Crossley, A., Hassellöv, M., Taylor, C., et al. 2014. Silver nanoparticles and silver nitrate induce high toxicity to *Pseudokirchneriella subcapitata, Daphnia magna* and *Danio rerio*. Sci. Total Environ. 466-467:232-41.

Romih, T., Drašler, B., Jemec, A., Drobne, D., Novak, S., Golobič, M., et al. 2015. Bioavailability of cobalt and iron from citric-acid-adsorbed CoFe2O4 nanoparticles in the terrestrial isopod *Porcellio scaber*. Sci. Total Environ. 508:76-84.

Sanderson, H., Johnson, D.J., Wilson, C.J., Brain, R.A., Solomon, K.R., 2003. Probabilistic hazard assessment of environmentally occurring pharmaceuticals toxicity to fish, daphnids and algae by ECOSAR screening. Toxicol Lett 144:383–395

Sarm, S.D, 2011. Life cycle of a nanosilver based water candle filter: examining issues of toxicity, risks, challenges and policy implications. J. Biomed Nanotechnol. 7:83-84.

Schirmer, K., Chan, A.G., Greenberg, B.M., Dixon, D.G., Bols, N.C. 1997. Methodology for demonstrating and measuring the photocytotoxicity of fluoranthene to fish cells in culture. Toxicol. in vitro 11:107-119.

Schirmer, K., Dixon, D.G., Greenberg, B.M., Bols, N.C., 1998. Ability of 16 priority PAHs to be directly cytotoxic to a cell line from the rainbow trout gill. Toxicology 127:129-41.

Sharma, V.K., Siskova, K.M., Zboril, R., Gardea-Torresdey, J.L. 2014. Organic-coated silver nanoparticles in biological and environmental conditions: fate, stability and toxicity. Adv Colloid Interface Sci. 204:15-34.

Simonis, J.J., Basson, A., 2011. Evaluation of a low-cost ceramic micro-porous filter for elimination of common disease microorganisms. Phys. Chem. Earth 36, 1129-1134.

Sorgeloos, P. 1973. First report on the triggering effect of light on the hatching mechanism of Artemia salina dry cyst. Mar. Biol. 22:75-76.

Takahash, K., Kato, H., Saito, T., Matsuyama, S., Kinugasa, S., 2008. Precise Measurement of the Size of Nanoparticles by Dynamic Light Scattering with Uncertainty Analysis. Part. . Part. Syst. Char. 25, 1: 31–38.

Tantra, R., Bouwmeester, H., Bolea, E., Rey-Castro, C., David, C.A., Dogné, J.M. et al., 2015. Suitability of analytical methods to measure solubility for the purpose of nanoregulation. Nanotoxicology 22:1-12

Tejamaya, M., Römer, I., Merrifield, R.C., Lead, J.R., 2012. Stability of citrate, PVP, and PEG coated silver nanoparticles in ecotoxicology media. Environ. Sci. Technol. 46:7011-7.

Xiu, Z.M., Zhang, Q.B., Puppala, H.L., Colvin, V.L., Alvarez, P.J., 2012. Negligible particle-specific antibacterial activity of silver nanoparticles. Nano Lett. 12 :4271-5

Xiu, Z.M., Ma, J., Alvarez, P.J., 2011. Differential effect of common ligands and molecular oxygen on antimicrobial activity of silver nanoparticles versus silver ions. Environ. Sci. Technol. 45:9003-8.

Yue, Y., Behra, R., Sigg, L., Freire, P.F., Pillai, S., Schirmer, K., 2015. Toxicity of silver nanoparticles to a fish gill cell line: Role of medium composition. Nanotoxicity 9, 54-63.

Zou, J., Hannula, M., Misra, S., Feng, H., Labrador, R.H, Aula, A.S., et al, 2015. Micro CT visualization of silver nanoparticles in the middle and inner ear of rat and transportation pathway after transtympanic injection. J. Nanobiotechnology 27:5

Vindimian, E., 2005. MSExcel macro REGTOX EV7.0.5.xls. Online available at: http://eric.vindimian.9online.fr/