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Effects of salinity on the toxicity of ionic silver and Ag-PVP nanoparticles to *Tisbe battagliai* and *Ceramium tenuicorne*

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Abstract

The toxic effects of polyvinylpyrrolidone (PVP) coated silver nanoparticles (Ag-NP_{PVP}) and ionic Ag, to *Tisbe battagliai* (Tb) and *Ceramium tenuicorne* (Ct) were investigated and the usefulness of standardised marine guidelines for ENP risk assessment were assessed. The toxicity of Ag-NP_{PVP} [$CtEC_{50}$ = 26.6 µg/L, $TbEC_{50}$ = 7.9 µg/L] and Ag^{+} [$CtEC_{50}$ = 2312.2µg/L, Tb EC_{50} = 90.9 µg/L] to both test species differed, with the silver ENPs being more toxic. In contrast to Ag^{+} the toxicity of Ag-NP_{PVP} increased significantly with increasing salinity, however, after thorough characterisation it was not possible to correlate the behaviour of the particles with an increase in toxicity and salinity. The results suggest that the observed toxicity is being elicited by the free ionic silver complexing in solution and also from an unknown potential particle related effect.

Keywords: silver nanoparticles, marine ecotoxicity, salinity, *Tisbe battagliai*, *Ceramium tenuicorne*

1 Introduction

Silver nanoparticles are one of the most widely used engineered nanomaterials (NMs) employed in commercial applications and their wide use in consumer products has led to them becoming a potentially important factor in environmental risk assessment (ERA) and monitoring (Klaine et al., 2008; Meyer et al., 2010). Naturally occurring nanoparticles have always existed in the environment without documented adverse effects (Murr et al., 2004; Handy et al., 2008). Despite this, the risks associated with engineered nanoparticles (ENPs) in the environment are becoming a significant concern (Moore, 2006; Ju-Nam and Lead, 2008; Tiede et al., 2009).

The ERA of NMs is a relatively new field and many questions about appropriate assessment methods exist (Tiede et al., 2009; Bhatt and Tripathi, 2011). At present there is inadequate information on the levels of nanomaterials in the environment (Paterson et al., 2011). Most work to date has focused on laboratory based evaluation of risk, where few interactions between the ENPs and natural environmental constituents occur. It is only in recent years that scientists have started to incorporate realistic environmental exposures into their laboratory work (Diegoli et al., 2008; Van Hoecke et al. 2011). Despite recent changes to a more "environmentally relevant" approach in ENP ecotoxicity and fate assessment, it can still be stated that, at present there exists no robust, reliable method for the quantification or assessment of ENPs in the environment.

Most ENP ecotoxicological assessments have, up to now, focused on the potential effects of ENPs in freshwater environments covering a wide array of species including fish (Farkas et al., 2011; Farkas et al., 2010; Bilberg et al., 2010), daphnids (Naddy et al., 2007), nematodes (Meyer et al., 2010), chironomids (Nair et al., 2011) and microalgae (Petit et al., 2010). Due to the increased use of silver ENPs in consumer products there is a likelihood that they will also end up in marine, estuarine and coastal environments. The possible adverse

56 effects elicited by these compounds must therefore be assessed employing suitable marine
57 test systems. A small number of studies employing marine species in the laboratory have
58 been performed (Galloway et al, 2010; Ringwood et al. 2010; Canesi et al., 2010), but there is
59 still a paucity of available data. Fabrega et al (2011) demonstrated that biofilm succession
60 was impeded on silver ENP treated biofilms, which in turn affected the abundance of major
61 bacterial groups that may cause further long term effect on the marine bacterial community.
62 In contrast Miao et al (2009) observed that silver ENPs formed non-toxic aggregates (>
63 0.22 μm) in seawater. Assessing the effects of ENP exposure to marine organisms poses new
64 and complex issues, hence, the importance of a realistic environmental assessment of both the
65 behaviour and effects needs to be emphasised.

66 The high ionic strength of seawater is likely to affect the behaviour of ENPs and may
67 prevent the introduced ENPs from becoming completely dispersed within the marine and
68 estuarine environment due to agglomeration (Gebauer and Treuel, 2011). There have been
69 some recent studies investigating the effects of salinity on ENPs revealing that only minute
70 changes in salinity may drastically affect the behaviour, effects and fate of ENPs (Matson,
71 2009). In the case of an estuarine environment, it is of even more importance that the
72 behaviour of ENPs and the effects of salinity be understood. Estuaries are constantly
73 changing and highly dynamic environments, in which organisms have to adapt to and tolerate
74 rapid changes in physico-chemical conditions over short periods of time. Estuaries and
75 coastal environments are also the most likely of the marine ecosystems to be impacted by
76 potential ENP contamination (Kaegi et al., 2008).

77 Silver ENPs are of particular interest in the marine environment, not only because of
78 their abundant use and potential entry into this ecosystem, but also because the speciation
79 behaviour of silver in saline waters increases bioavailability (Luoma et al., 1995). Upon
80 entering the marine environment, silver speciation becomes a significant factor (Cowan et al.,

1985). Therefore, in the marine and estuarine environment, silver is unlikely to remain in the form in which it was introduced. Another potential effect on the behaviour of ENPs in saline media, is the fact that their tendency to aggregate/agglomerate may increase with increased salinity.

The primary production of micro and macro algal species in the marine environment is of paramount importance. Effects on primary production within an ecosystem could lead to devastating effects on ecosystem functioning. Sub-lethal effects on these organisms may also lead to transfer and biomagnification of metal contaminants up the food chain (Quigg, 2008). At present, research on the interactions and toxicity of ENPs to macrophytes is lacking for both the terrestrial and aquatic ecosystems. Due to this lack of information on higher plants, *Ceramium tenuicorne*, a marine rhodophyte, was selected for use in this study. *C. tenuicorne* was selected as it represents a primary producer in both the marine and estuarine environment (i.e. euryhaline species) and could be used to assay the ENPs under a wide range of salinities. *Ceramium* species are found world-wide and are particularly abundant in temperate climates. The marine clone employed in this study originated from the Oslofjord (20 – 25 ‰), an inlet in the south-east of Norway, and has been maintained as a laboratory culture for over 30 years and can be adapted to a wide range of environmentally relevant salinities (ISO, 2010).

In order to augment the multi-trophic nature of this study, a marine harpacticoid copepod *Tisbe battagliai*, was incorporated into the testing. *T. battagliai* represents a primary consumer within the marine food web and is of environmental significance because, along with nematodes, copepods are the most abundant multicellular organisms on earth (Humes, 1994). It is also a standard test species in regulatory testing and employed in ERA. To the best of our knowledge, no data regarding the effects of ENPs to *T. battagliai* and *C. tenuicorne* exist in the literature. Therefore, the information gained in this work is considered

105 to contribute to the knowledge gap in understanding the effects of ENPs to these marine
106 species.

107 Although there are many studies in the literature on the ecotoxicological assessment
108 of ENPs, there is no standardisation in the methodologies used between different laboratories.
109 Significant factors, such as sample preparation and ENP characterisation, vary significantly
110 between laboratories. This variation leads to considerable differences in results for similar
111 particles and incomparable inter-laboratory data, for both ecotoxicological tests and
112 characterisation (Roebben et al., 2011, Petersen and Henry, 2012). The applicability of
113 standard guidelines for the assessment of ENPs in the environment is of great interest at
114 present. Several major international standards organisations (e.g. OECD and ISO) are
115 currently investigating the applicability of the standard methods for use in ERA and
116 regulation of ENPs. Due to the potential for more rigorous ERA of ENPs in the future, where
117 possible the studies performed in this work were performed in line with standard regulatory
118 guidelines (ISO 14669, ISO 10710) and according to GLP (Good Laboratory Practice, NIVA
119 has a GLP certified ecotoxicological laboratory), any deviations are described in full.

120 The main objectives of this study were to: (1) investigate the toxicity of silver nitrate
121 ($AgNO_3$) and polyvinylpyrrolidone coated silver nanoparticles (Ag-NP_{PVP}) to two marine
122 species and to fully support all ecotoxicological studies with a thorough characterisation of
123 the ENPs in all environmental media; (2) to investigate species specific differences in
124 sensitivity; (3) evaluate the potential modifying effects of varying salinity on the toxicity of
125 $AgNO_3$ and Ag-NP_{PVP} to *C. tenuicorne* and (4) to place the results in an environmentally
126 relevant context and to discuss the applicability of these standardised test guidelines for use
127 in the regulatory assessment of engineered NPs within the marine and estuarine environment.

128

2 Materials and Methods

2.1. Nanoparticles and test chemicals

The silver nanoparticles employed in this study were coated with polyvinylpyrrolidone (PVP). These PVP (Ag-NP_{PVP}) capped ENPs were kindly prepared and provided in a solution of MilliQ water by the University of Manchester, UK. Post analysis of the stock solution by ICP-MS, indicated that the concentration was 348 mg/L, based on a measurement of total silver.

Four different types of media were used in these studies: non-enriched natural seawater (NSW) for the *T. battagliai* assays and an enriched algal media for *C. tenuicorne* assays (Eklund, 2005, ISO, 2010), at three different salinities (to be referred to as CT10 ‰, CT20 ‰, and CT30 ‰). Natural seawater, collected from 60 m depth at Solbergstrand, Norway (ca. 34 ‰), was employed in the preparation of all media. All media were filtered to 0.2 µm prior to use. Test solutions of ENPs for use in the ecotoxicity assessments were prepared by diluting the stock solution in the appropriate saline media. Dilutions were made directly from the stock solution, where possible, in order to reduce the effects of potential aggregation/agglomeration during serial dilution. All test solutions were stirred for 3 hours at 100 rpm prior to characterisation and use in ecotoxicology tests.

The reference chemical, analytical grade potassium dichromate ($K_2Cr_2O_7$; CAS Registry No 778-50-9) was obtained from Sigma Aldrich. Polyvinylpyrrolidone (PVP; CAS Registry No 9003-39-8) also obtained from Sigma Aldrich, was employed as a control for the nanoparticle capping agent.

2.2. Nanoparticle characterisation

There are many techniques for measuring the size of nanoparticles, all of which are based on fundamentally different scientific principles. It has previously been reported that no single technique is sufficient to accurately estimate the size of nanoparticles in all media types (Domingos et al., 2009 Scalf and West, 2006). Each individual technique has intrinsic biases and artefacts produced during sample preparation, or from instrumental interference (Domingos et al. 2009). Therefore, in this study, a battery of different characterisation techniques was employed to investigate the size and behaviour of the Ag-NP_{PVP} in saline media.

2.2.1. Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) was used to measure the hydrodynamic diameter (Z-average), the intensity based size distribution and the zeta potential of Ag-NP_{PVP} in MilliQ water and all 4 media types. The particle size distributions (PSD) of the Ag-NP_{PVP} were analysed using a Malvern Instruments Zetasizer Nano Series (Malvern Instruments, UK). Concentrations of 100 µg/L were prepared in all media and in MilliQ water. Samples were analysed at 20 ºC. Typically, 1 mL of solution was placed into a disposable sizing cuvette and samples for all media were prepared and analysed in triplicate. Six measurements of each triplicate sample were taken for all media.

Using the same Malvern Instrument, zeta potential measurements were also performed on the same solutions described above. Folded capillary cells with ca. 3 mL of each solution were employed. Measurements were conducted at 20 ºC, using a concentration of 100 µg/L silver ENP and 6 measurements on triplicate samples were made.

2.2.2. Atomic Force Microscopy (AFM)

Atomic force microscopy (AFM) was used to resolve particles and groups of particles by offering visualisation in three dimensions. Samples of Ag-NP_{PVP} were prepared in all media types and MilliQ water at a concentration of 10000 µg/L. Samples were drop cast onto silicon wafers which were subsequently rinsed with de-ionised water and imaged in tapping mode with a high performance AFM microscope (Asylum MFP-3D-BIO™).

2.2.3. Transmission Electron Microscopy (TEM)

Solutions of the highest working concentrations of silver ENPs were prepared in all test media. The size and shape of the particles were determined by TEM. The ENP suspensions were prepared on the day of analysis in appropriate test media and applied to carbon coated grids (5 µl pipetted directly onto the grids). The grids were allowed to dry and images were captured with a Philips CM 100 electron microscope. Using image analysis software (Digimizer Version 3.4.1.0) to analyse micrograph images, 100 ENPs were measured and the mean particle size and PSD of Ag-NP_{PVP} in all media described.

2.3. Ecotoxicity testing

2.3.1. *Tisbe battagliai* culturing and testing

T. battagliai were cultured in the laboratory at NIVA in accordance with standard procedures (ISO, 1999) using NSW of ca. 34 ‰. Cultures were fed a single algal diet consisting of *Rhodomonas baltica* once per week (additional feeding may be required depending on the ability of the animals to clear the food) during culture water renewal.

197 *T. battagliai* toxicity tests were conducted with slight modifications according to the
198 ISO method (ISO, 1999). Toxicity tests were conducted with copepodids 6 ± 2 days old. The
199 bioassays were performed in 12-well polystyrene tissue culture plates (NUNC®) that had
200 been pre-treated overnight with appropriate exposure solutions. After initial range finding
201 studies, both Ag-NP_{PVP} and $AgNO_3$ were tested over the following range 1, 3.2, 5.6, 10, 32,
202 56, 100 µg/L (7 concentrations plus a negative control [NSW]). Controls for PVP were run
203 alongside to confirm that any observed toxicological effects with Ag-NP_{PVP} were not due to
204 the presence of the capping agent. All concentrations were run in quadruplicate (2.5 mL of
205 exposure solution per replicate with five test organisms per replicate, a total of 20 animals per
206 test concentration). Test plates were incubated in a temperature controlled room at 20 ± 2 °C
207 and a 16:8 hour light:dark photoperiod. Experiments were performed on three independent
208 occasions.

209 A reference substance, potassium dichromate ($K_2Cr_2O_7$), was run alongside the tests
210 in order to verify the sensitivity of the copepods and to ensure the test conditions were
211 reliable (ISO, 1999). At test initiation and termination, dissolved oxygen concentrations, pH
212 and salinity were measured in the control, top and bottom concentrations for all tests. Control
213 species mortality of less than or equal to 10 % was required as a further validation parameter.

214

215 **2.3.2 *Ceramium tenuicorne* adaptation**

216 A sample *of C. tenuicorne* (ITM, Stockholm) was supplied for use in this study (20
217 ‰.), with a pre-testing adaptation period, to ensure good growth of the clone at all proposed
218 test salinities.

219 Adaptation of the plants to 10 and 30 ‰ was conducted over several weeks prior to
220 testing. This was done by successively transferring the algae every three days to an

221 increase/decrease of approximately 3 ‰. The algae were then cultivated for at least two
222 weeks in the final test salinity prior to test initiation.

223 Prior to testing, the growth rate of the algae at the varying test salinities was
224 determined to ensure sufficient growth during the test period. This was carried out by
225 conducting a 7 day growth experiment according to the ISO guideline (ISO, 2010) with
226 exponentially growing female tips (0.6 – 1.2 mm start length) in all media without test
227 chemical. All media growth experiments were conducted in triplicate and measurements, at
228 test initiation and termination, were taken using an Olympus 1x71 fluorescent microscope
229 (without fluorescent mode) (Tokyo, Japan) and Olympus Soft Imaging system: Cell^D,
230 Version 3.2 (Build 1700).

231

232 **2.3.3 *Ceramium tenuicorne* silver toxicity and salinity effects on growth rate**

233 The toxicity of Ag-NP_{PVP} and $AgNO_3$ as well as the effects of salinity on these
234 toxicities, to the female plants, were studied using pre-adapted *C. tenuicorne* (section 2.3.2).

235 After initial range finding studies, definitive toxicity tests at all salinities were
236 conducted within the range 1, 5, 10, 50, 100, 500, 1000, 5000, 10000 µg/L (9 concentrations
237 and appropriate negative controls), for both Ag-NP_{PVP} and $AgNO_3$. As with the *T. battagliai*
238 assay, a PVP control was incorporated into the test design. All toxicity testing was conducted
239 according to modified standard methods (Eklund, 2005; ISO, 2010). Due to the small amount
240 of test material available for Ag-NP_{PVP}, exposure volumes were reduced to 2.5 mL per
241 replicate (4 replicates/concentration). 12-well pre-treated tissue culture plates (NUNC®) were
242 used for the exposures. All concentrations were run in quadruplicate with two algal plants per
243 replicate. Microplates were incubated for seven days at a temperature of 22 ± 2 ºC, a light
244 intensity of 70 ± 10 % µmol m^{-2} s^{-1} and a continuous light regime. In order to prevent
245 evaporation (due to the small volume size) plates were sealed with parafilm. Experiments

were conducted for all salinities, for both silver compounds, on three independent occasions to ensure reproducibility and increase statistical power. A reference chemical (potassium dichromate) was run alongside to ensure performance and reliability of test methods. At test initiation and termination, dissolved oxygen concentrations, pH and salinity were measured in the control, top and bottom concentrations.

2.4 Statistical analysis

The EC_{10}/EC_{50} (concentration that elicits an estimated 10 %/50 % toxic effect (i.e. growth inhibition, mortality) values for Ag-NP_{PVP}, $AgNO_3$ and $K_2Cr_2O_7$ were calculated using REGTOX-EV6.xls (Èric Vindimian http://eric.vindimian.9online.fr/), a curve fitting macro for Microsoft® Excel. Toxicity data for the algal and copepod tests were fitted to a sigmoidal curve and the Weibull (algal assays) and Hill (copepods assays) models were used to calculate Lethal Concentration (LC) and Effective Concentration (EC) values respectively. Statistical analyses were carried out using analyses of variance (ANOVA) followed by Dunnett's multiple comparison test. From these data, Lowest Observable Effects Concentrations (LOEC) and No Observable Effect Concentrations (NOEC) were calculated. These data analyses were performed using MINITAB® release 15 (MINITAB Inc. PA, USA). Statistical significance was accepted at $p \leq 0.05$.

3 Results

3.1. Nanoparticle characterisation

3.1.1. Dynamic Light scattering (DLS)

The average particle sizes of Ag-NP_{PVP} in all media and MilliQ water, as measured by DLS, are shown in Table 1. Ag-NP_{PVP} in MilliQ water showed hydrodynamic peaks at around 6 and 60 nm (Z-average value of 56.9 nm). In the case of the saline media, the values varied and there appeared to be no average particle size increase/decrease trend with increasing salinity. The CT10 ‰ media showed peaks at around 40 and 80 nm (Z-average value of 149 nm). The CT20 ‰ media showed two peaks in the size distribution of approximately 5 and 60 nm (Z-average value of 57.28 nm). The CT30 ‰ media showed two peaks in the size distribution at ca. 40 and 120 nm (Z-averge value of 105.01 nm). The highest salinity media (NSW) showed two main size distribution peaks, at around 28 and 154 nm (Z-average value of 76.44 nm). All size determination results are summarized in Table 1. Although the polydispersity index was below 0.5 for all samples measured, indicating that Z-average is a suitable mean size to use to compare these samples, the presence of multiple peaks, indicates that a degree of aggregation/agglomeration occurs in the media and the Z-average as determined by DLS may not be the most appropriate technique for representing the size distribution of these particles in solution

For zeta potential measurements, it was only possible to obtain usable values in the MilliQ, CT10 ‰ and CT20 ‰ media samples, as the high conductivity of the samples with a salinity above 20 ‰ caused interference in the readings. The zeta potential of the particles measured in the MilliQ water and *C. tenuicorne* media at 10 ‰ and 20 ‰ were -39.17 mV, -8.68 mV and 10.32 mV respectively. The low zeta potential values are consistent with a tendency for the nanoparticles to agglomerate or aggregate, as indicated by the size distribution measurements.

3.1.2 Atomic Force Microscopy (AFM)

AFM images were captured and analysed for drop cast samples of Ag-NP_{PVP} from all media and MilliQ water. Figure 1 shows an example of an AFM image and corresponding topography of a cross section for Ag-NP_{PVP} from CT10‰. As seen from Figure 1, the silver nanoparticles form isolated aggregates when precipitated from the media. The cross section of the image shows one of these aggregates with a height greater than 150 nm and a base diameter of > 0.5 µm. However, a significant number of features of dimensions ~10nm are also observed, indicating the presence of isolated nanoparticles. The average size and particle size distribution are described in Table 1.

3.1.3 Transmission Electron Microscopy (TEM) analysis

While both DLS and AFM indicate a significant degree of aggregation/agglomeration in the dispersions, TEM images indicate the presence of particles in the nanometer range (< 100 nm, ASTM International, 2006) in the stock solution (Farkas et al., 2010) and in the analysed exposure solutions 10000 µg/L. All TEM measurements are described in Table 1. The presence of nanoparticles is clearly seen in the MilliQ water sample, in which approximately 48 % of particles were < 5nm, while, 30 % were between 5 – 10 nm in size. Of the exposure media, the smallest average size, based on TEM analysis, was for CT10 ‰ media with an average size of 16.0 (± 5.8) nm. The average size for the CT20 ‰ media, CT30 ‰ media and the NSW (ca. 34 ‰) were 20.2 (± 4.1), 19.3 (± 6.3) and 18.9 (± 5.2) nm respectively. As with the DLS analysis, there was no specific trend in particle size based on increasing/decreasing salinity. The Ag-NP_{PVP} in all saline media (except in CT30 ‰, where only dispersed particles were seen) were observed to form loose agglomerates at a concentration of 10000 µg/L as well as being present as monodisperse particles in solution (Figure 2). In the calculated PSD (Figure 3), results indicated that there were no particles less

316 than 5 nm in diameter in any media type. The size distributions of the analysed particles were
317 similar for all media, 7.9 – 33.7, 11.4 – 36.3, 9.7 – 39.6 and 9.9 – 34.9 nm respectively, for
318 CT10 ‰, CT20 ‰, CT30 ‰ and NSW. For all media types between 87 - 98 % of the
319 measured particles were in the range 10 – 30 nm in diameter (Figure 3).

320

321 **3.2 Ecotoxicity testing**

322 ***3.2.1 Tisbe battagliai***

323 The mean 48 hour EC_{50} value for the reference chemical potassium dichromate with
324 *T. battagliai* was 7.2 (95 % CI = 5.8 – 9.1) mg/L, which corresponds to previously published
325 values for this species and chemical (Macken et al., 2008). No deleterious effects of PVP
326 were observed, therefore, toxicity due to the PVP capping agent in these experiments can be
327 ruled out.

328 Toxicity data for Ag-NP_{PVP} and $AgNO_3$ to *T. battagliai* are shown in Figure 4a and b
329 and Table 2. The LC_{10} and LC_{50} values indicate that the toxicity of Ag-NP_{PVP} was statistically
330 greater than the $AgNO_3$ toxicity. Despite the calculation of these values it can be seen from
331 Figure 4 that the silver salt had a greater toxic effect at higher concentrations, while the silver
332 ENP appeared to be more toxic at lower concentrations (see Figure 4c for a graphical
333 comparison of the mortality data). For all experiments, the test vessels were treated with
334 solutions of suitable concentrations overnight prior to test initiation and rinsed with MilliQ
335 water. This was done so as to remove any potential binding sites for the silver test solutions
336 on the plastic test vessel surfaces. The control mortality and physico-chemical parameters
337 measured at the start and end of all experiments were within the recommended limits (ISO,
338 1999).

339

340 **3.2.2 *Ceramium tenuicorne***

341 The mean 7 day EC_{50} value for the $K_2Cr_2O_7$ with *C. tenuicorne* was 3.9 mg/L (95 %
342 CI = 2.6 - 5.7), which was consistent with previous studies with this species (Macken,
343 unpublished) and no toxicity was observed with the PVP capping agent. The mean increase in
344 length over a 7 day test period, of six algal pieces, in the three proposed test salinities was
345 measured. Growth rate was observed to increase with increasing salinity, 1.12, 1.46 and 1.62
346 respectively for 10, 20 and 30 ‰ media. All salinities allowed for good growth of the algae,
347 sufficient to meet all validity criteria for standard guideline testing (ISO, 2010), however, for
348 continuous cultures, it was observed that the 20 ‰ cultures were the best performing for
349 long-term culture maintenance.

350 The control growth rates met with the validity criteria in all experiments (increase in
351 length over seven days by a factor of greater than 3, compared to the starting length [ISO,
352 2010]), therefore implying that the use of parafilm and reduced sample volume had no
353 adverse effects on the test system. Table 2 summarises the ecotoxicity data for all test
354 salinities with both Ag-NP_{PVP} and $AgNO_3$. As was observed with the *T. battalgiai* assays, the
355 toxicity of the silver ENPs was greater than that of $AgNO_3$ (Table 2 and Figure 5) to the test
356 organism when looking at EC values. Despite this, when NOEC/LOEC values were
357 calculated, the difference in toxicity changed and the ENPs can only be considered more
358 toxic at the highest salinity (CT30 ‰). The toxicity of the Ag-NP_{PVP} increased with
359 increasing salinity (EC_{50} values of 1872.5 (95 % CI = 954.5 – 3757.6), 393.5 (95 % CI = 954.5 –
360 3757.6) and 26.7 (95 % CI = 12.6 – 69.8) µg/L, at CT10 ‰, CT20 ‰ and CT30 ‰,
361 respectively). The observed toxicity of $AgNO_3$ to *C. tenuicorne* at all salinities was similar,

362 with a slight, but not statistically significant, increase in toxicity between 10 ‰ and 20 ‰
363 (based on EC values).

364

365 **4 Discussion**

366

367 The toxicity of both the ionic silver and Ag-NP_{PVP} assayed in this study were high
368 compared to other similar studies in the literature. No comparable toxicity data for *T.*
369 *battagliai* and silver or silver ENPs are available, however, there is some information on
370 other marine copepods. Pedroso et al. (2007) found that at 30 ‰ the 48 h EC_{50} for *Acartia*
371 *tonsa* was 173 µg/L (dissolved silver) indicating the harpacticoid, *T. battagliai* was more
372 sensitive to silver toxicity (48 h EC_{50} at 34 ‰ = 90.9 µg/L). In relation to species specific
373 differences, there are limits to identifying the most sensitive of the two species assayed in this
374 study. Different salinities, exposure periods and endpoints (e.g. lethality and growth) are
375 being assessed. However, it is clear that the sensitivity of the two species differs depending
376 on these factors and it is important to incorporate a battery of endpoints and exposure periods
377 for ERA in order to allow for the protection of the marine and estuarine environment.

378 The higher toxicity of silver ENPs compared to ionic silver, with *T. battagliai* may be
379 indicative of a potential particle specific mode of action on the toxicity and has been
380 observed by other authors (Griffitt et al., 2008; Navarro et al., 2008). The increase in toxicity
381 could be as a result of increased surface area and therefore increased capacity to release ionic
382 silver from the surface of the particle.

383 *C. tenuicorne* was also observed to be slightly more sensitive to Ag-NP_{PVP} than ionic
384 silver, however, differences in sensitivities were not as evident as those observed with

385 *T. battagliai* at all salinities. After exposure in algal media of 10 ‰, the EC_{10}/EC_{50} values
386 were similar for both Ag-NP_{PVP} and $AgNO_3$. The difference in sensitivity between the two
387 test chemicals became much more pronounced with increasing exposure media salinity.
388 These differences were particularly pronounced at 30 ‰. There did not appear to be a marked
389 increase in toxicity of $AgNO_3$ with increasing salinity. In contrast, there was a consistent
390 increase in toxicity up the salinity gradient with Ag-NP_{PVP}. The toxicity of metals in aquatic
391 systems is influenced by a variety of factors such as organic matter content (Nadella et al.,
392 2009) and salinity (Verslycke et al., 2003). These results may indicate that an increase of
393 chloride ions or other inorganic ligands within the media are changing the behaviour and
394 bioavailability of the silver ENPs to the test species. Previously published work, investigating
395 the effects of salinity on the toxicity of contaminants in the marine environment to
396 invertebrates, have observed an increase in toxicity with decreasing salinity (Verslycke et al.,
397 2003; Kwok and Leung, 2005; Pedroso et al. 2007). Ytreberg et al. (2011) investigated the
398 effects of salinity and organic matter on the toxicity of Cu to *C. tenuicorne* and concluded
399 that the effects of salinity on Cu toxicity were not clear, as both a positive and negative effect
400 was observed, a decrease in toxicity with increasing salinity in the presence of organic matter
401 but no decrease in the absence of organic matter.

402 As in this work, an increase in toxicity with increasing ionic strength has also been
403 observed in the literature (Erikson et al., 1998). This is the opposite of what would be
404 expected if only the free silver ions were responsible for the toxic response. Due to the high
405 ionic strength of seawater and the prevalence of inorganic ligands such as chlorine, silver
406 complexation will not be dominated by NOM, as in freshwater, but instead speciation is
407 dominated by the formation of strong chloro-complexes (Cowan et al., 1985; Cowan et al.,
408 1993; Reinfelder and Chang, 1999). Without definite information on the speciation and
409 complexation of silver in the media and seawater in our study, it is not possible to make clear

410 conclusions on the causes of the observed toxicity or the effects of changing ionic strength.
411 Unfortunately due to the low levels of silver, limitations in analytical capabilities and small
412 volumes of test material, speciation could not be investigated in greater detail in this
413 particular study.

414 As well as understanding the speciation of silver within seawater, an understanding of
415 the chemical nature of the exposure medium is also important in determining the behaviour
416 and bioavailability of silver and silver nanoparticles. In this study all of the exposure
417 solutions were prepared from NSW, however, the algal media was also enriched with a
418 variety of trace metals, salts and vitamins, including nitrogen, phosphorus, iron, and trace
419 amounts of cobalt, copper, zinc and manganese (ISO, 2010) One important factor for
420 consideration is the presence of chelating agents within exposure media (e.g. EDTA). In this
421 work the ISO *C. tenuicorne* media contained low levels of EDTA (final concentration of
422 approximately 300 μg/L). Despite this, it is unlikely that the presence of EDTA can
423 completely explain the difference in sensitivity between the two species, as EDTA has been
424 shown to have very a low affinity for silver (Zuiderveen and Birge, 1995).

425 In order to further try and explain the results of our study we have considered the
426 potential physiological susceptibility of the algae itself under differing environmental
427 regimes. Russell (1985) conducted an investigation of macroalgae in the Baltic Sea and found
428 *C. tenuicorne* was the least likely to cope with any large increase in salinity. Despite, the
429 algae used in our study going through an adaptation period of several weeks, this may help to
430 explain the increased sensitivity of this species with increasing salinity. In order to respond to
431 external salinity changes this particular seaweed, has to alter its water content and the
432 concentration of inorganic ions (Lobban and Harrison, 1994; Ferguson and Hogstrand, 1998).
433 Due to the fact that the Ag-NP_{PVP} toxicity increased with increasing salinity and the $AgNO_3$

toxicity did not increase, there could also be some sort of ENP effect that is enhanced in the presence of increasing Cl^-.

It is believed that no single technique for ENP characterisation is without its artefacts or can be employed in all cases for nanoparticle characterisation (Domingos et al., 2009). Therefore, in order to try and counteract this problem a battery of characterisation techniques were employed in this study. The dispersion system employed in this study was intended to reflect the realistic conditions and behaviour of the ENPs on entry into the marine environment and so no manipulations to force the ENPs into solution were conducted (e.g. use of solvents) (Kato et al., 2009).

In recent years, TEM has allowed scientists to obtain images of individual nanoparticles. However, during this analysis only a small fraction of the total sample is characterised. Therefore, the results may not be representative of the total sample being assayed and it is easy to acquire incorrect information on exposure solutions (Nowack and Bucheli, 2007). There are several potential artefacts associated with TEM and ENP characterisation, e.g. meniscus-based artefacts and co-precipitation with inorganic salts (Domingos et al., 2009). In saline media, the same effect could cause interference with the visual analysis of the sample, as any impurities, or salt in the sample, could make it difficult to distinguish the ENPs from these contributions on the air-dried grid.

Compared to the TEM results, the results of DLS measurements of the average particle size of Ag-NP_{PVP} in all media types, were far greater. Similar results have been observed by other authors (Domingos et al., 2009; Farkas et al., 2010; Hassellöv et al., 2008). It is important to note, that in DLS the diameter being measured is the hydrodynamic diameter, which is not only dependent on the core of the ENP but also on any surface

457 structures (i.e. PVP capping agent) and the concentration of ions in the medium. Therefore,
458 the particle size can be larger than that measured by electron microscopy techniques.

459 In this study, some of the raw data from the DLS measurements did not meet the
460 quality criteria (data generally showing high polydispersity). This polydispersity may have
461 been due to the fact that, with the addition of the silver ENPs to seawater, silver chloride
462 precipitated out of solution and caused the ENPs to sediment out. Immediately following the
463 addition of ENPs there was no visible sedimentation of particles in the test exposure
464 solutions, however, after microscopical examination (light microscope) at test termination
465 there was apparent fall out of particles on the bottom of the test wells in all experiments. For
466 zeta-potential measurement salinities above 20 ‰ were unable to provide usable,
467 reproducible values. The high conductivity of the saline samples was most likely the cause of
468 the interference in the readings. In the *C. tenuicorne* media there was a shift in zeta-potential
469 from a negative (CT10 ‰ = -8.68) to a positive (CT20 ‰ = 10.32) value with increasing
470 salinity. This shift of zeta-potential towards a positive value at higher salinities suggests that
471 a possible charge repulsion mechanism is becoming less dominant (Oo and Ong, 2010). It has
472 also been shown that inorganic ions (e.g. Cl^-) in solution can interact with charged surfaces
473 by non-specific or specific ion adsorption, which will affect the isoelectric point. In some
474 situations, as shown in this study, specific ion adsorption can lead to charge reversal of the
475 surface (Zeta Sizer Nanoseries, 2004).

476 In order to conduct a more thorough risk assessment of nanoparticle exposure it is
477 important to have some specific information. Within the environment their quantity,
478 persistence and dispersion is of vital importance. Without this information it is impossible to
479 conduct a comprehensive risk assessment and herein lies the problem. As previously
480 mentioned, measured total/bulk silver levels in the marine and estuarine environment are very
481 low, 0.04 – 31 ng/L (Kramer et al., 2002) and it is, at present, impossible to distinguish bulk

482 silver released into the marine environment from silver ENPs present. Therefore, we have no
483 true understanding of what the realistic levels of these materials in the aquatic environment
484 are. It is also likely that the exposure levels used in the laboratory are far higher than the
485 natural levels in the environment (Ward et al., 2006). Potentially, these high exposure
486 concentrations, used to cause a toxic effect in some laboratory experiments, may result in
487 different uptake mechanisms and rates than those that would occur at environmentally
488 realistic levels of silver or ENP silver (Fortin and Campbell, 2000). Therefore, making the
489 results of laboratory based ENP studies difficult to incorporate into nanoparticle risk
490 assessment at present, however, studies on the sensitivity and effects of species exposed to
491 ENPs can help provide information that may, in time, lead to a better understanding and more
492 appropriate use of toxicity information for ERA of these materials. In addition, the guidelines
493 need to be clarified to incorporate definitive guidance on the characterisation of ENPs in
494 environmental media. Guidance on the preparation of ENP solutions for ecotoxicological
495 assessment needs to be harmonised in order to yield comparable interlaboratory results for
496 future ERA. The development of additional nano-specific guidance documents are at present
497 being discussed but it remains a complicated and developing area of regulatory
498 ecotoxicology.
499

500 **5 Conclusion**

501 Silver ENPs have the potential to cause toxic effects to marine macrophytes and
502 invertebrates. Given the sensitivity of the two species used in this study, and their widespread
503 distribution in the marine environment, their susceptibility is of utmost importance. In
504 developing methodologies for the assessment of ENP toxicity, it is vital that the most suitable
505 species and endpoints be identified. At present definitive guidelines on the assessment of the
506 risks associated with ENP release and effects in the environment do not exist. Considering

that increased salinity was observed to have such a marked effect on the toxicity of the ENPs employed in this study, it may not be appropriate to use freshwater data to extrapolate risk within the marine environment. In addition, as the ISO methods stand, they may not be completely suitable for the assessment of ENPs. Despite the test design, organism's sensitivity and environmental relevance, the methods lack specific information on sample preparation, dosing and quantification of exposure concentrations. Although the characterisation techniques employed were unable to correlate ENP behaviour and salinity it is apparent that some process is causing an increase in toxicity with increasing salinity. As similar increases in toxicity of $AgNO_3$ with increasing salinity were not observed, it can be concluded that it is not merely the chemical nature of the ENPs responsible for the effect. Therefore, the observed increase in toxicity is most likely due to a combination of the surface properties of the ENPs and their reactivity within highly saline media, and the behaviour of the silver ions and complexation. There is a definite need for a more thorough understanding of the modifying effects of changing environmental parameters. This knowledge, along with suitable guidelines, is required in order to contribute more accurately to an ERA of ENPs introduced into the marine environment.

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770

771 **Figures**

772

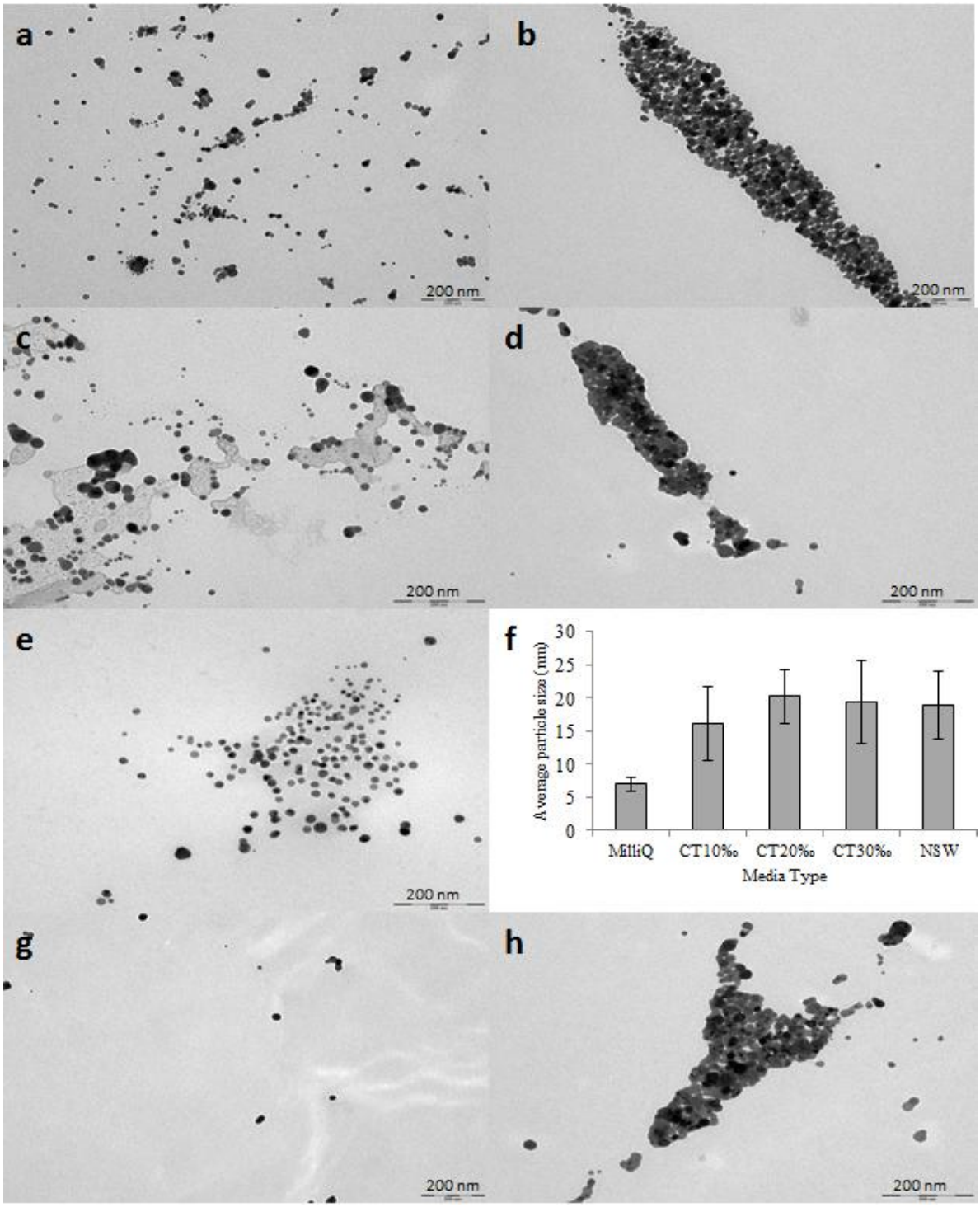
773

774

775 **Figure 1** An example of AFM image of Ag-NP_{PVP} in CT10 ‰ with underlying topographic

776 profile.

777

778 

779 **Figure 2** TEM images (scalebar: 200 nm) of Ag-NP_{PVP} in NSW (a - b), CT10 ‰ (c - d),

780 CT20 ‰ (e), CT30 ‰ (g - h) and the corresponding average size in all media (f).

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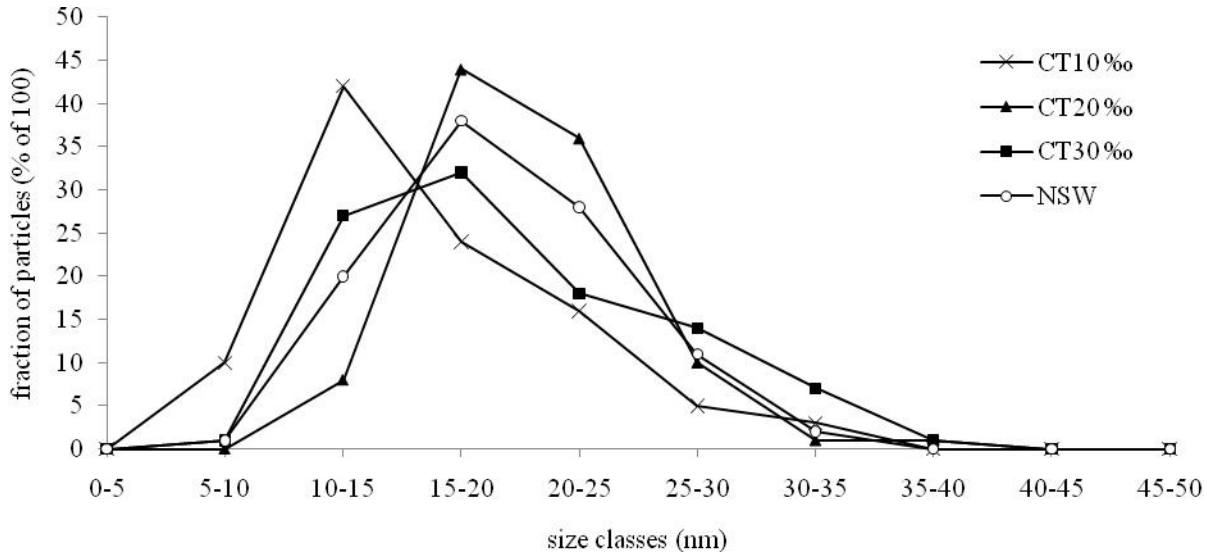
782



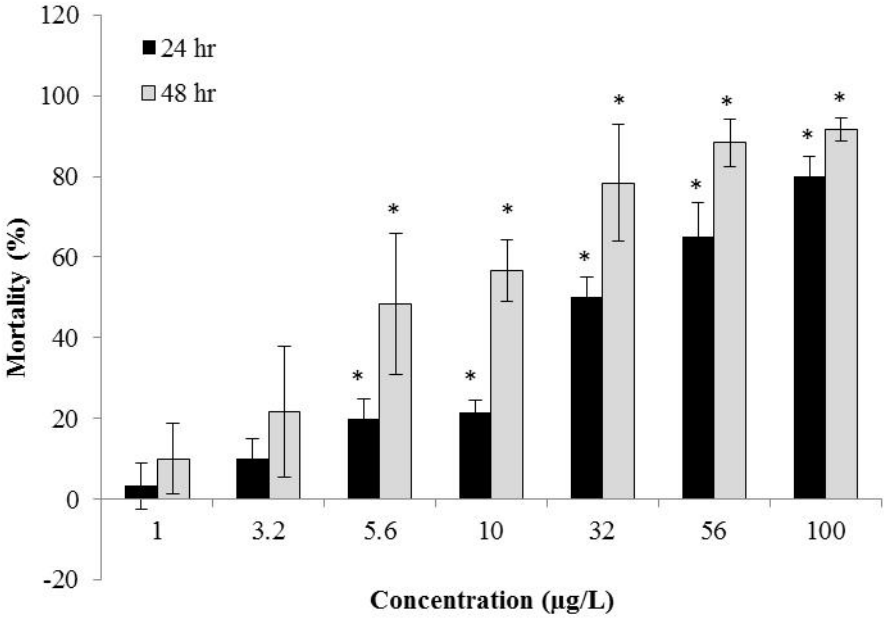
783

784 **Figure 3** Particle size distribution (PSD) from TEM image analysis of Ag-NP_{PVP} in different

785 media (n = 100 for measurements in all media).

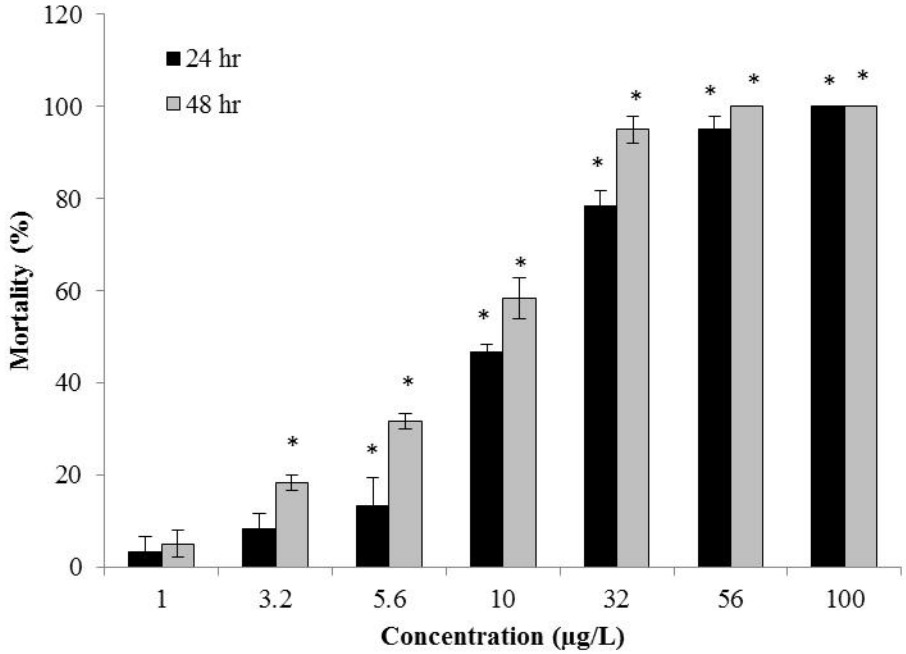
786

787 (a)



788

789 (b)



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792

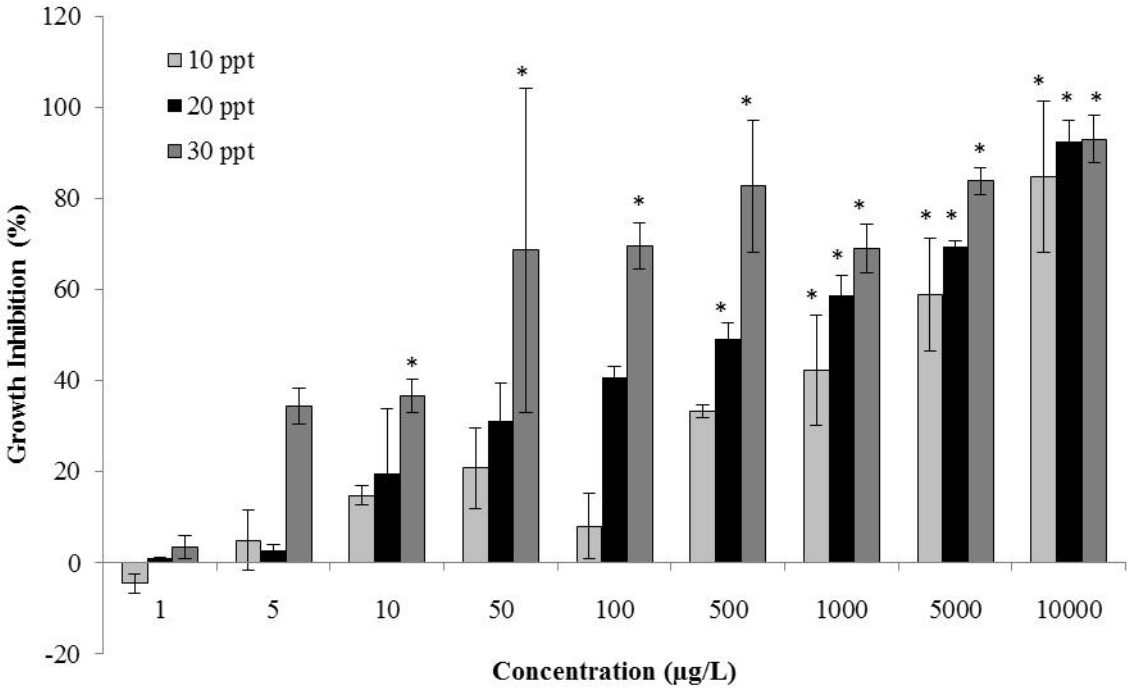
793 **Figure 4** Toxicity of (a) Ag-NP_{PVP} (b) $AgNO_3$ to *Tisbe battagliai* exposed for ■ 24 hours and

794 ▫ 48 hours.* indicates statistical significance from the control ($p \leq 0.05$). Data are mean ± SD

795 (n = 3). CV for the controls ranged from 0.00 – 2.94 %.

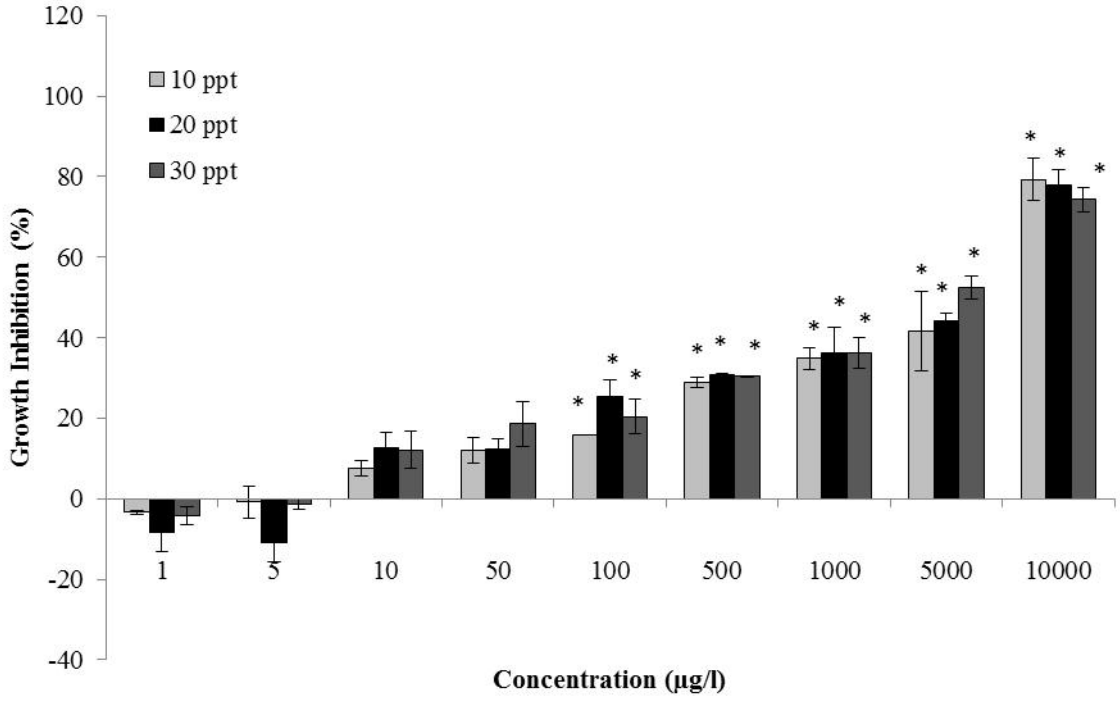
796

797 (a)



798

799 (b)



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801

802 **Figure 5** Growth inhibition of *Ceramium tenuicorne* after 7 days exposure to Ag-NP_{PVP} (a)
803 and $AgNO_3$ (b) at 10 ‰ (□), 20 ‰ (■) and 30 ‰ (▩). * indicates statistical significance from
804 the control ($p \leq 0.05$). Data are mean ±SD (n = 3). CV for the control ranged from 0.15 –
805 1.33 % (10 ‰), 5.29 – 13.07 % (20 ‰) and 15.7 – 16.65 % (30 ‰)
806

807 **Table 1** Particle size analysis and characterisation results for Ag-NP_{PVP} in all test media.

Media type	Method	Concentration (mg/L)	Mean PS (nm)	SD (nm)	Min (nm)	Max (nm)
MilliQ water	DLS	0.1	56.9	7.8	-	-
	TEM	10	7.0*	-	1.0*	60.0*
	AFM	10	18.5	6.2	5.9	38.2
10 ‰ 2A Media	DLS	0.1	149.0	29.8	-	-
	TEM	10	16.0	5.8	7.9	33.7
	AFM	10	24.7	38.7	6.5	280.1
20 ‰ 2A Media	DLS	0.1	57.3	3.2	-	-
	TEM	10	20.2	4.1	11.4	36.3
	AFM	10	13.8	7.1	5.5	56.4
30 ‰ 2A Media	DLS	0.1	105.0	18.5	-	-
	TEM	10	19.3	6.3	9.6	39.6
	AFM	10	24.0	23.7	5.7	120.3
NSW	DLS	0.1	76.4	39.2	-	-
	TEM	10	18.9	5.2	9.9	34.9
	AFM	10	12.2	7.8	55.4	71.7

808 SD = Standard deviation, NSW = Natural Seawater, DLS = Dynamic Light Scattering, TEM = Transmission Electron Microscopy, AFM =
809 Atomic Force Microscopy
810 * Approximately 48 % of particles were < 5nm, while, 30 % were between 5 – 10 nm in size (Farkas et al., 2010)
811

812 **Table 2** Ecotoxicity data for *Tisbe battagliai* and *Ceramium tenuicorne* assayed with silver nanoparticles ($Ag\text{-}NP_{PVP}$) and silver nitrate ($AgNO_3$).

Test Substance	Test Species	Salinity (‰)	Duration	EC_{10}/LC_{10}[a] (µg/L)[a]	EC_{50}/LC_{50}[b] (µg/L)[b]	NOEC[c]/LOEC[d] (µg/L)
$Ag\text{-}NP_{PVP}$	*T. battagliai*	34	24 h	3.8 (2.4 – 5.6)	30.8 (26.2 – 35.7)	3.2/5.6
		34	48 h	0.9 (0.6 – 1.7)	7.9 (6.2 – 10.3)	3.2/5.6
$Ag\text{-}NP_{PVP}$	*C. tenuicorne*	10	7 d	32.5 (4.0 – 129.6)	2120.4 (1199.9 – 3316.8)	100/500
$Ag\text{-}NP_{PVP}$	*C. tenuicorne*	20	7 d	1.5 (0.1 – 11.0)	373.9 (179.8 – 782.7)	50/100
$Ag\text{-}NP_{PVP}$	*C. tenuicorne*	30	7 d	0.1 (0.007 – 1.4)	26.7 (12.6 – 69.8)	5/10
$AgNO_3$	*T. battagliai*	34	24 h	12.7 (0.8 – 258.7)	167.3 (52.8 – 481.8)	32/56
		34	48 h	8.7 (2.0 – 102.5)	90.9 (38.0 – 238.9)	10/32
$AgNO_3$	*C. tenuicorne*	10	7 d	42.2 (6.4 – 143.8)	3606.5 (2318.2– 5378.6)	10/50
$AgNO_3$	*C. tenuicorne*	20	7 d	10.9 (0.6 – 81.4)	2246.6 (1020.8 – 4056.4)	50/100
$AgNO_3$	*C. tenuicorne*	30	7 d	11.4 (0.4 – 105.6)	2312.2 (1038.4 – 4819.6)	500/1000

813 [a] EC_{10}/LC_{10} values and corresponding 95 % confidence intervals
814 [b] EC_{50}/LC_{50} values and corresponding 95 % confidence intervals
815 [c] NOEC, no observed effect concentration, the highest observed concentration at which no significant effect ($p \leq 0.05$) was detected
816 [d] LOEC, lowest observed effect concentration, the lowest concentration of the tested concentration at which a significant ($p \leq 0.05$) effect was detected
817