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Effect of silver nanoparticles on marine organisms belonging to different trophic levels

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Abstract

Silver nanoparticles (Ag-NPs) are increasingly used in a wide range of consumer products and such an extensive use raises questions about their safety and environmental toxicity. We investigated the potential toxicity of Ag-NPs in the marine ecosystem by analyzing the effects on several organisms belonging to different trophic levels. Algae (*Dunaliella tertiolecta*, *Skeletonema costatum*), cnidaria (*Aurelia aurita* jellyfish), crustaceans (*Amphibalanus amphitrite* and *Artemia salina*) and echinoderms (*Paracentrotus lividus*) were exposed to Ag-NPs and different end-points were evaluated: algal growth, ephyra jellyfish immobilization and frequency of pulsations, crustaceans mortality and swimming behaviour, and sea urchin sperm motility. Results showed that all the end-points were able to underline a dose-dependent effect. Jellyfish were the most sensitive species, followed by barnacles, sea urchins, green algae, diatoms and brine shrimps.

In conclusion, Ag-NPs exposure can influence different trophic levels within the marine ecosystem.

Keywords: Ag, bioassay, crustaceans, jellyfish, microalgae, nanoparticles, sea urchin, toxicity.

1. Introduction

Nanotechnology is rapidly expanding with applications in different fields, from electronics to medicine, from remediation to engineering and food industry (Oberdörster et al., 2007; Das et al., 2013; Massarsky et al., 2013). Nowadays the products containing silver nanoparticles (Ag-NPs) are increasing, as well as their worldwide diffusion for industrial processes and treatments (Myrzkhanova et al., 2013), due to their importance as antimicrobial agents (Mohan et al., 2007; Zheng et al., 2008) and their particular magnetic, optical, electronic and catalytic properties, that make Ag-NPs suitable for applications in a wide range of fields (Johari et al., 2013). The Woodrow Wilson Database (2011) has listed about 1,317 NP-based consumer products currently on the market, 311 of which contain Ag-NPs. Nanotechnology enables the incorporation of these NPs into many daily personal care products, wound dressings, kitchen-ware, children toys, washing machine coatings, wall paints, food packaging and many more (Kim et al., 2007; Sotiriou and Pratsinis, 2010). Moreover 53% of the EPA (Environmental Protection Agency) -registered biocidal silver products likely contain Ag-NPs (Nowack et al., 2011). Such an extensive use and growing production raises questions about Ag-NP safety and environmental toxicity. To date the predicted environmental concentrations (PECs) for Ag-NPs in the environment are at the range of ng L^{-1} to mg kg^{-1} (Fabrega et al., 2011a; Reidy et al., 2013) and this value is estimated to be 0.03-0.08 μg L^{-1} in the water compartment, representing a high potential risk induced by Ag-NPs in the aquatic ecosystem (Mueller and Nowack, 2008). The investigation of Ag-NPs effects in the aquatic ecosystem is very important, since the wide variety of the applications containing Ag-NPs can potentially end up in the aquatic environment and reach the sea during waste disposal (Asharani al., 2008) as most of NPs do. Ag-NPs may aggregate and/or dissolve in the aquatic environment (Baun et al., 2008), so these processes may alter the fate, transport and toxicity of such NPs (Lowry et al., 2012).

Most of the currently available ecotoxicological data regarding Ag-NPs are limited to freshwater species used in regulatory testing (i.e. OECD, ISO), that represent key environmental organisms, such as algae, crustaceans and fish (Miao et al., 2010; Hoeheisel et al., 2012; Kashiwada et al., 2012; Wu and Zhou, 2013). The toxicity of Ag-NPs measured in freshwater depends on the test species (Blinova et al., 2013). For example, Ag-NPs are reported to be toxic for crustaceans at very low concentration (EC_{50} <0.1 mg L^{-1}), followed by algae (EC_{50} = 0.23 mg L^{-1}) , but the toxicity to fish is relatively low (EC_{50} = 7.1 mg L^{-1}, Kahru and Dubourguier, 2010; Ashgari et al., 2012).

On the contrary, the current knowledge on the fate, behaviour and ecotoxicity of Ag-NPs in the marine ecosystem is scarce. Recent findings indicate that salinity influences the stability and aggregation of Ag-NPs (Wang et al., 2014), therefore the fate of such NPs is primary to aggregate in the water column, precipitate and accumulate in sediments following release into the marine environment (Keller et al., 2010; Buffet et al., 2013). To date only sparse data on the potential toxicity of Ag-NPs to marine species (e.g. their effects on sea urchin and oyster development, fish and oyster physiology and blue mussel accumulation, Chae et al., 2009; Ringwood et al., 2010; Zuykov et al., 2011; Gambardella et al., 2013; McCarthy et al., 2013) are available.

Ag-NPs cause a significant decrease in marine biofilm volume and biomass (Fabrega et al., 2011b), inhibit the photosynthetic performance of green algae (Oukarroum et al., 2012) and induce mortality and a cyst hatching decrease in brine shrimp (Aruvalsu et al., 2014). As a contribution to this field, the effects of Ag-NPs on environmental relevant marine test species belonging to different trophic levels have been examined in the present paper. In order to obtain a comprehensive assessment of Ag-NP effects on seawater column organisms, toxicity testing was carried out across a battery of six species belonging to different trophic levels (primary producers and consumers), including algae (*Skeletonema costatum* and *Dunaliella tertiolecta*), cnidaria (*Aurelia aurita*), crustaceans (*Artemia salina* and *Amphibaanus amphitrite*) and echinoderms (*Paracentrotus lividus*). The diatom *S. costatum*, the green alga *D. tertiolecta*, the sea urchin *P. lividus*, the brine shrimp *A. salina* and the barnacle *A. amphitrite* were selected because they are established model species in standardized toxicity tests, ecotoxicological studies and in ecological risk assessment (Wong et al., 1995; UNI EN ISO, 2000; ASTM, 2004; Faimali et al., 2006; Losso et al., 2007; Pane et al., 2008; Dineshram et al., 2009; Pétinai et al., 2009; Garaventa et al., 2010; Piazza et al., 2012).

In addition, the jellyfish *A. aurita* was used in this work since it has been recently proposed as a very new, sensitive and innovative model organism in ecotoxicological studies. Besides occupying a key evolutionary position as basal metazoan (Faimali et al., 2014; Costa et al., 2015), cnidarians are important components of marine food webs both as major consumers of zooplankton (Riisgard et al., 2007) and preys (Cardona et al., 2012; Titelman et al., 2006). Moreover, increasing evidence has shown that jellyfish have an influence on microbial food webs, through direct and indirect effects, and are important regulators of marine biogeochemical fluxes (Turk et al., 2008).

Therefore, the aim of this study was to expand knowledge on the effects of Ag-NPs on the marine ecosystem, by analyzing different end-points, such as algal growth, jellyfish immobility and frequency of pulsation, crustacean mortality and swimming behaviour, and sea urchin sperm motility.

2. Materials and methods

2.1. Ag NPs characterization

Ag NPs were obtained from Polytech Inc. (Germany) as a 1000 ppm suspension of metallic silver in deionized water, with a nominal particle size provided by the producer in the range of 1-10 nm. Ag-NPs were suspended in 0.22 µm filtered natural seawater (FNSW, supplied from the

Aquarium of Genova (Italy, pH 8.27; Salinity 36.9‰) and sampled at few miles from the Ligurian Sea coast) to obtain a concentration of 1 mg mL^{-1} according to Gambardella et al. (2013), before bringing them to the different concentrations used in the tests (Tab. 1). After NP suspension preparation the toxicity tests were immediately performed. The testing concentrations were chosen on the basis of the results of a preliminary screening test using an order-of-magnitude dilution series (0-0.1-1-10-100 mg L^{-1}), with which we assessed the ecotoxicological end-points.

Ag-NPs were diluted 1:100 in deionized water in order to determine Ag concentration by Plasma Emission Spectrometry (ICP-OES). The ICP-OES instrument was an axially-viewed Varian (Springvale, Australia) Vista PRO with the following main operating conditions: RF Power: 1100 W; Plasma gas flow rate: 15.0 L min^{-1}. Sample uptake rate: 0.8 mL min^{-1}. To compensate for nonspectral interferences, the on-line internal standardization (4 g mL^{-1} Lutetium standard solution) was applied. Ag NP size (determined by Dynamic Light Scattering) and effective surface charge (ζ-potential) characterization, available in Gambardella et al. (2015), were 990 nm (diameter) and -3 ± 2 mV, respectively.

2.2. Toxicity tests

2.2.1. Algae

The potential of Ag-NPs to inhibit algal growth was evaluated using the green alga *D. tertiolecta* and the diatom *S. costatum*. Algae were obtained from culture collection of CNR ISMAR (Genova, Italy). Algal cells were cultured in artificial sea water Instant Ocean® with complete F2 culture medium (Guillard and Ryter, 1962) at 20 ± 0.5 °C with a 12–12 h light dark period and light intensity of 6,000–10,000 lux (Sbrilli et al., 1998). Toxicity tests were performed according to the method ISO 10253, 2006. Three replicates for each Ag-NP suspension, including the control, were prepared. After 72 hours, culture growth was stopped by using Lugol’s solution (Throndsen, 1978; ICRAM, 2001) and the algal growth inhibition was evaluated by counting cells with a haemocytometer Thoma, using a Leitz Diavert inverted microscope (Leitz, Germany). The reliability of the test was verified using cadmium as reference toxicant, according to UNI EN ISO 10253 method.

2.2.2. Cnidarians

Colonies of *A. aurita* polyps attached on PVC tubes were supplied by the “Acquario di Genova, Costa Edutainment S.p.A.”; once in the CNR – ISMAR laboratories, they were kept at 20 ± 0.5 °C in 1.5 L dark plastic tanks, covered with a lid in order to keep polyps in dark conditions. Tanks were filled with FNSW (37 ‰) and gently aerated. *A. aurita* ephyra were obtained from polyps as described by

Faimali et al. (2014). Once released by strobilation, ephyrae were transferred into a beaker and immediately used for the toxicity tests.

Ephyrae were placed into multi-well plates, one individual for each well containing 2 mL of Ag-NP solution (Tab. 1). For each NP suspension, three replicate plates were prepared, each replicate consisting of 8 wells containing one ephyra in order to avoid interactions among organisms. Plates were then sealed and kept at 20 ± 0.5 °C in dark conditions. A toxicity test using cadmium nitrate as reference toxic compound was also performed, according to Faimali et al. (2014).

After 24 and 48 hours, the acute (immobility) and sub-lethal end-point (frequency of pulsations) were evaluated. The acute end-point consisted in organism inability to perform any kind of movement (without changing their own barycentre position) for five seconds, measured as percentage of immobility compared to the Control. The sub-lethal end-point was the number of pulsations made by the ephyra within one minute (Fp), expressed as the measured as percentage of pulsation alteration compared to the control. Both end-points have been evaluated using the Swimming Behavioral Recorder (SBR). This system, developed at ISMAR-CNR, is a video camera based system, coupled with an image analysis software, specifically designed to track and analyze linear swimming speed of aquatic invertebrates (Faimali et al., 2006; Garaventa et al., 2010). SBR has been adapted to be used for measuring the frequency of pulsation alteration (Fp) of ephyrae (Faimali et al., 2014); organisms pulsations have been recorded in dark condition for 1 minute.

2.2.3. Crustaceans

II stage nauplii of the barnacle *A. amphitrite* and Instar I larvae of the brine shrimp *A. salina* were exposed to Ag-NPs. Nauplii were obtained from laboratory cultures of adult brood stock at CNR ISMAR (Genova, Italy) according to the method described by Faimali and Garaventa (2010) and Piazza et al. (2012). Twenty to thirty adult barnacles were reared in 700 ml beakers containing aerated 0.45 µm natural seawater at 20±1°C, with a 16:8 h light : dark cycle. They were fed every other day with 50–100 ml of *A. salina* at a density of 20 larvae mL^{1}, and 200–400 mL of *Tetraselmis suecica* at a concentration of 2·106 cells mL^{-1}. The seawater was changed three times a week, and barnacles were periodically rinsed with clean water to remove epibionts or debris. Nauplii were collected and maintained in 500 mL gently aerated beakers with 0.22 µm natural seawater until their use for the toxicity tests.

Instar I larvae were obtained as reported by and Garaventa et al. (2010): 500 mg of commercially available dehydrated cysts were incubated for 24 h at 28 °C under 16 h light, 8 h dark conditions and continuous aeration of the cyst suspension in seawater (37‰ salinity). After this period, newly hatched larvae were separated from non-hatched cysts based on their positive phototaxis and transferred by Pasteur pipette into a beaker containing filtered natural seawater in a final concentration of 10–15 larvae mL^{-1}.

Briefly, 10-15 organisms were placed into each well of multi-well plates, containing 1 mL of different concentrations of Ag-NPs (Tab. 1) and incubated, for 24 and 48 h, at 20 ± 0.5 °C for nauplii and 25 ± 0.5 °C for brine shrimps in dark conditions. Cadmium nitrate and potassium dichromate were selected as reference toxicants for barnacle nauplii and brine shrimp larvae respectively, according to Piazza et al. (2012) and APAT IRSA CNR 8070 (2003) protocols. All tests were performed in triplicates. After 24 and 48 hours, the acute (mortality) and sub-lethal endpoint (Swimming Speed Alteration, SSA) were evaluated. The number of dead organisms was counted under a stereomicroscope: larvae that were completely motionless were counted as dead organisms and the percentage of mortality was calculated compared to control. SSA was evaluated using the SBR (Faimali et al., 2006; Garaventa et al., 2010) set to record organisms movement for about three seconds in dark condition. Data were referred as swimming alteration, normalized to the average swimming speed of the control (S = average swimming speed):

Alteration (%) = [(S Treated – S Control) / S Control) x 100].

2.2.4. Echinoderms

Adult sea urchins were collected from an artificial rock ledge near Termoli, on the Southern Adriatic coast of Italy, and reared for about 14 weeks in a 200 L recirculating aquarium (temperature 18 °C, salinity 35‰, pH 8.00–8.20, natural photoperiod), fed ad libitum on NIFA feed (NOFIMA, Tromsø, Norway), a pelletized feed specifically formulated for sea urchins, in order to induce gonad maturation as described in Fabbrocini and D'Adamo (2011). Sea urchin semen samples were dry collected, after KCl injection. The sperm from three different specimens were mixed for the sperm motility test (MOT-test). This test was performed exposing sea urchin *P. lividus* sperm to different concentrations of Ag-NPs (Tab. 1) for one hour at 18° C. Cadmium as Cd(II) solution (Baker Italy) was used as positive control, as described in Fabbrocini et al. (2012). For each concentration and control, four replicates were prepared. Three independent trials were performed.

At the end of the incubation time, sperm motility was analyzed by a computerised motion analysis system, the Sperm Class Analyzer® (SCA, Microptic, s.l., Spain), as described in Fabbrocini et al. (2010); the curvilinear velocity (VCL) and the percentage of rapid sperm RAP; spermatozoa having VCL > 100 µm sec^{-1}) were evaluated and normalized to the relative control values in order to obtain the effect percentages.

2.3. Data analysis

Mortality and immobility tests were considered valid if the control mortality/immobility was less than 10% (US EPA, 2002). The algal growth inhibition tests was considered valid if the control cell density increased by a factor of more than 16 in 72 h (ISO 10253:2006).

For each test the median end-point values (LC_{50}, EC_{50} and IC_{50}) and related 95% Confidence Limits (CL) were calculated using Spearman-Karber analysis (Finney, 1978). The median values were expressed as IC_{50} for algal growth inhibition test (concentration able to inhibit the growth by 50% as compared to control, Finney, 1971), LC_{50} for crustacean mortality (the concentration able to cause the mortality of the 50% of the tested population), EC_{50} for cnidaria immobility and frequency of pulsations alteration, crustacean swimming speed alteration and sea urchin sperm motility (concentration required for obtaining 50% of a maximum effect, Finney, 1978).

One-way analysis of variance (ANOVA), followed by Student Newman–Keuls (SNK) pairwise comparison of the average values of each end-point for each treatment level (toxic concentrations) relative to the control, was performed. Prior to analysis, the assumption of the homogeneity of variances was tested by Cochran’s test (Underwood, 1997).

3. Results

3.1. Ag-NP characterization

The mean average of Ag concentration as determined by ICP-OES was 974 ± 4 mg L^{-1}, and therefore it substantially confirmed the nominal concentration reported by the commercial company (100 mg L^{-1}).

3.2. Toxicity tests

IC_{50}, LC_{50} and EC_{50} values are reported in Tab. 2. It was not possible to calculate the median concentration for both *A. aurita* ephyra investigated end-points and crustacean swimming speed alteration (with the exception of 2 4h SSA of *A. salina*) because they resulted to be lower than the lowest tested concentration.

3.2.1. Algae

Algal growth inhibition is shown in Fig. 1. A significant ($p<0.01$) growth inhibition activity of Ag NPs on *D. tertiolecta* was observed already at the lowest tested concentration with an IC_{50} of 0.9 mg L^{-1}, whereas *S. costatum* growth rate gradually decreased with the increase of Ag-NP concentration, being significantly different from the control at concentrations from 1.6 mg L^{-1} onwards and showing a 50% inhibition at 3.1 mg L^{-1}.

3.2.2. Cnidarians

The results obtained exposing ephyrae to different concentrations of Ag-NPs are reported in Fig. 2. A significant effect on both the evaluated end-points was observed at the lowest Ag-NP concentration, 0.1 mg L^{-1}, after the two exposures (24 h and 48 h, $p< 0.01$); the effect then increases in a dose-dependent manner till the concentration of 0.4 mg L^{-1} when it reaches the 100%.

3.2.3. Crustaceans

Results obtained exposing crustacean to Ag-NPs are reported in Fig. 3 and 4. The behavioral endpoint (swimming speed alteration) was more sensitive than mortality independently form the AgNPs concentration (Tab. 2). For example, about 80% swimming inhibition was observed in nauplii of *A. amphitrite* exposed to 0.1 mg L^{-1} of Ag-NPs after 24 h of exposure, where no mortality occurred (Fig. 3). Starting from 0.8 and 0.4 mg L^{-1} the acute effect prevails after 24 and 48 h of exposure, respectively.

Acute and behavioral end-points showed the same trend in larvae of *A. salina* exposed to Ag-NPs, although the swimming speed alteration was always more sensitive than the acute end-point (Fig. 4).

3.2.4. Echinoderms

Sea urchin sperm motility results showed a gradual decrease of the two investigated end-points with the increase in NP concentrations (Fig. 5). No differences in sensitivity were observed between the percentage of rapid spermatozoa (RAP) and curvilinear velocity (VCL); in fact, even if there is a little difference in the magnitude of the end-point, they both start to significantly differ from the

Control when sperm cells were exposed to 0.02 mg L^{-1} of Ag-NPs.

4. Discussion

The purpose of this study was to investigate the potential toxicity of Ag-NPs in the marine ecosystem by analyzing effects on invertebrates belonging to different trophic chain levels. The ecotoxicological bioassays performed and the evaluated end-points gave substantially similar results namely that a toxic effect of such NPs is evident. The potential risk of Ag-NP exposure to the selected taxonomic groups is added to that already reported for the exposure to other NPs across primary producers, primary and secondary consumers (Blaise et al., 2008; Cerdevall et al., 2012). Although we observed divergences in sensitivity among different organisms and end-points, all results indicate a dose-dependent effect of Ag-NPs towards all the selected species. These findings are in accordance with literature, where Ag-NPs were found to cause morphological and acute (i.e., mortality) responses in a dose-dependent manner in both freshwater and marine organisms (Asharani et al., 2008; Browning et al., 2013; Arulvasu et al., 2014).

Jellyfish, barnacles, sea urchins and green algae resulted to be more sensitive to Ag-NPs than diatoms and brine shrimps whose median effect concentrations ($IC_{50,}$ LC_{50} and EC_{50}) are one order of magnitude higher than other organisms tested. The comparison of LC_{50}/EC_{50} obtained with the selected species (Tab. 2) highlights that jellyfish appear to be the most sensitive model organisms among the investigated ones. Cnidarians are considered ecologically relevant due to their abundance and their role in the trophic chain (Gadelha et al., 2012), and for this reason they have been recently proposed as new model organisms in ecotoxicology, as reported by Faimali et al (2014) and Jovanovic and Guzman (2014) for the use of jellyfish and reef building corals. Furthermore, the sensitivity of species belonging to this phylum have been reported exposing them to reference toxic compounds and to several NPs, including Ag-NPs (Malvindi et al., 2008; Suwa et al., 2014). Such NPs were able to induce significant responses in the behaviour of scleractinian corals, up to inhibit the swimming in the larvae exposed to 0.05 mg L^{-1} of Ag-NPs after 48 h (Suwa et al., 2014). Likewise, we observed a significant effect in jellyfish ephyrae in both immobility and frequency of pulsation at the lowest Ag-NP concentration tested (0.1 mg L^{-1}). Although the trend of the two end-points is almost similar and overlapping, the alteration of the frequency of pulsation was affected by Ag-NPs much more than immobility, showing about 80% of effect already at 0.1 mg L^{-1} after 24 h of exposure (Fig. 2). Therefore, this behavioural end-point confirm to be more sensitive than immobility as already reported exposing *A. aurita* ephyrae to different toxic compounds (Faimali et al., 2014).

Mortality and swimming speed alteration in crustaceans showed a different responsiveness to Ag-NP exposure; in fact *A. amphitrite* nauplii seem to be more sensitive than *A. salina* larvae, with LC_{50} values of 0.55 and 9.96 mg L^{-1} at 24 and 0.27 and 3.79 mg L^{-1} at 48 h respectively. In addition $EC_{50\,24h}$ values were even lower than the lowest tested concentration in barnacle (Tab. 2). Due to their sensitivity to reference toxic compounds, pesticides and environmental matrices, larval stages of barnacles are currently used for studies dedicated to the standardization of new marine ecotoxicological tests (Faimali et al., 2006; Piazza et al., 2012) and have been recently proposed for nano-ecotoxicology bioassays (Mesarič et al., 2013).

Our observations confirm that barnacle larvae may represent working model organisms for nanoecotoxicological studies. In fact, the EC_{50} values measured after Ag-NP exposure are of the same order of magnitude of those found for freshwater crustaceans exposed to 10 nm Ag-NPs (0.1 mg L^{-1}, Ivask et al., 2014). In *Daphnia magna*, these authors investigated the toxicity of Ag-NPs with different size, ranging from 10 to 80 nm, by evaluating immobilization after 48 h exposure. EC_{50} values ranged from 0.01 to 0.2 mg L^{-1} Ag-NPs depending on NP size. After the same exposure time *A. amphitrite* nauplii seem to be less affected by Ag-NPs than *D. magna* with EC_{50} values of 0.27 and 0.01 mg L^{-1}, respectively even if the two tests differed for the media where NPs were dissolved (salt water in our study and freshwater in Ivask et al., 2014). The differences in the toxic effect may be due to the different physicochemical properties of the investigated media (freshwater *vs* seawater) and to the stability of these NPs in each media. In fact, the physicochemical characterization in seawater that we performed showed that Ag-NP size is higher than the nominal one provided by the commercial company, suggesting a different behaviour of such NPs in seawater (Gambardella et al., 2015). Moreover, in the same work the authors reported an increase of Ag-NP size after a short exposure time (one hour) suggesting a time-related agglomeration process that may explain the higher LC_{50} values found for marine crustaceans than freshwater ones.

This study together with others on the effect of carbon-based NPs and Ag-NPs on barnacles (Falugi et al., 2012; Mesarič et al., 2013), support the hypothesis that barnacle may be a suitable model organism for NP effect evaluation. Here, we registered a significant dose-dependent increase in barnacle naupliar mortality after 24 and 48 h of exposure with LC_{50} value similar to that reported by Falugi et al. (2012) that found a medial lethal concentration of 0.3 mg L^{-1} of Ag-NPs. Furthermore, in this paper, the sensitivity of *A. amphitrite* nauplii is indicated also by the behavioural end-point that highlights a dose-dependent decrease in the swimming speed of nauplii exposed to Ag-NPs. This end-point was more effective in pointing out Ag-NPs toxicity than the acute one (i.e., mortality), confirming the higher sensitivity of the behavioural end-point than the acute one as already observed exposing organisms to pesticides and neurotoxic compounds (Faimali et al., 2006).

The other crustaceans used in this work (the brine shrimps *A. salina*) are commonly used as model organisms in ecotoxicological assays because of their ecological relevance (since they play a key role in the food chain energy flow in marine environment) and because they are easy to maintain under laboratory conditions (Sanchez-Fortun et al., 1997; Nunes et al., 2006). *A. salina* larvae showed to be the most resistant to Ag-NPs among the organisms used in the tests we performed. Brine shrimps are known to be very resistant to toxic compounds and NPs (Varò et al., 2002; Garaventa et al., 2010; Gaiser et al., 2011; Libralato, 2014); in addition, recently *A. salina* larvae exposed to metal NPs and carbon NPs for 48 h showed no mortality (Cornejo-Garrido et al., 2011;

Ates et al., 2013; Gambardella et al., 2014; Mesarič et al., 2015).

Arulvasu et al. (2014) found that Ag-NPs have significant effect on *A. salina* considering different end-points such as mortality, hatching percentage and apoptosis; however it is difficult to clearly relate the reported effect to Ag-NPs rather than other stresses, considering that authors hypothesize that the acute effect (i.e. mortality) was most likely due to NP aggregates into the gut. In this regard we did not observe any aggregates in the larvae after 24 and 48 h of exposure (data not shown). Therefore, the different results obtained in these studies may be due to the different physicochemical properties of the Ag-NPs.

Our results pointed out lethal and behavioural responses, quantified by means of both LC_{50} and EC_{50} values; LC_{50} values are in agreement with those reported by Falugi et al. (2012), that investigated the toxicity of the same Ag-NPs against brine shrimp after 24 h and 48 h of exposure obtaining LC_{50} values at 24 and 48 h of 5 and 7 mg L^{-1} respectively.

The LC_{50}/EC_{50} values for *A. salina* reported in the present study appear to be higher than those recently found by Becaro et al. (2014); in fact, the authors obtained an EC_{5048h} value for *A. salina* immobility of 0.05 mg L^{-1} of polyvinyl alcohol - stabilized Ag-NPs. These findings highlight that Ag-NP toxicity can be ascribed to the nature and the characteristics of the NP used, indicating that such a kind of coating (i.e., polyvinyl alcohol as stabilizing agent) may decrease the toxicity of AgNPs.

As previously observed for barnacle nauplii, the results obtained exposing brine shrimps to Ag-NPs highlight that the behavioral end-point (swimming speed alteration) was more affected than the acute end-point (Tab. 2). These results reiterate that behavioural responses, like swimming, represent sensitive end-points to assess the impact of contaminants (e.g. pesticides, insecticides, Venkateswara Rao et al., 2007; Garaventa et al., 2010; Alyuruk et al., 2013) and NPs (Gambardella et al., 2014) in *A. salina* larvae at concentrations far below those able to cause lethal effects (Amiard-Triquet, 2009).

Ag-NPs exert a toxic effect on sea urchin sperm cells after 1 h of exposure, which was quantified by means of EC_{50s} in both the investigated end-points (VCL and RAP). Sea urchin sperm motility is currently used in biomonitoring programs for marine coastal environments (Fabbrocini et al., 2010; D'Adamo et al., 2014). Sperm velocity parameters have been found to be significantly correlated with fertilization rate in sea urchins (Levitan, 2000; Au et al., 2002), but no evidence has been reported on NPs. On the basis of these results, it may be supposed that Ag-NPs affected sperm cell integrity, reflected in decrease sperm motility in a dose-dependent manner. Although fertilization capability of sea urchin sperm is not directly influenced by Ag-NPs, some cellular alterations are transmitted from sperm to offspring, since we previously found morphological anomalies in sea urchin embryos and larvae from sperms exposed to Ag-NPs (Gambardella et al., 2013). Similarly, in mammalian gametes after exposure to Ag-NP motility but not viability was affected in human sperm (Moretti et al., 2013), no effect on cell morphology was observed in porcine sperm (Tiedermann et al., 2014) and an increase in ROS (reactive oxygen species) was recorded in mice spermatogonia (Braydich-Stolle et al., 2005); moreover, a decrease in motility percentage and sperm velocity and an increase in ROS levels was found after long-term exposure in rabbit semen (Castellini et al., 2014). Therefore, more studies are needed to understand the mechanisms by which Ag-NPs may affect the sperm motility and the offspring from sperms exposed to such NPs. The MOT-test on sea urchin sperm cells reveals to be a rapid, simple and striking method, able to detect sensitive responses if compared with others (e.g. fertilization rate). We investigated two endpoints after 1 h of exposure that did not significantly differ each other in terms of EC_{50s} values, in agreement with previous studies (Fabbrocini et al., 2010, 2012). However, both end-points highlight that Ag-NPs was able to affect sperm motility speed and percentage (VCL and RAP) in a dose-dependent manner. In this regard, these results are consistent with those obtained from rainbow trout sperm cells exposed to the same NPs (Johari, 2014).

Dose-dependent developmental anomalies and behavioural changes in swimming pattern have been also observed in *P. lividus* larvae at Ag-NP concentrations of the same order of magnitude we found (Siller et al., 2013). Moreover, the EC_{50s} measured by investigated VCL and RAP are comparable with the LC_{50} found in barnacle nauplii after 24 h of exposure, suggesting the high sensitivity of sea urchin gametes to NPs.

Considering results obtained exposing algae, a different response was observed in the investigated algae: the green alga *D. tertiolecta* was more sensitive to Ag-NPs than the diatom *S. costatum.* This result is in contrast with the common view that consider diatoms as more sensitive and reliable test species than the flagellate algae, when monitoring the toxicity associated to marine sediments and pesticides (Walsh, 1983; Wong et al., 1995). Nevertheless, our observations are in agreement with those reported for flagellate algae and diatoms exposed to heavy metals and other NPs (Wikfors et al., 1991; Nassiri et al., 1997; Miller et al., 2012). Regarding NPs, little is known on their effects in these two marine algae. To our knowledge, the only study that compares the effects due to NPs in *D. tertiolecta* and *S. costatum* is provided by Miller and colleagues (2012), that highlight a significant toxicity induced by TiO_2 NPs in the growth rate of flagellate algae but not on that of the diatom. Here, we reported a similar trend for both species, since a significant inhibition of *D. tertiolecta* growth compared to the control, was observed at the lowest tested concentration of Ag-NPs, while for *S. costatum* this effect was registered only if exposed to higher concentrations of Ag-NPs (0. 4 mg L^{-1} *vs* 1.6 mg L^{-1}; Fig. 1). Taking into account the similar results reported for the two species exposed to heavy metals, and the size (990 nm) of the Ag-NPs used in this study as well, it can be hypothesized that Ag-NPs act mostly by releasing Ag ions in the medium and the diatom, more sensitive to Ag than the green alga, shows a more detrimental effect.

5. Conclusions

Our results showed that Ag-NPs exposure resulted to be toxic to all the tested organisms in a dosedependent manner suggesting that this kind of metal NPs may affect different trophic levels within the marine ecosystem. Furthermore, selected organisms showed different level of sensitivity to AgNPs and *A. aurita* ephyrae and *A. amphitrite* nauplii proved to be the most sensitive ones. On the basis of these results it is possible to provide the following species-sensitivity increasing sequence: brine shrimps (*A. salina*), diatoms (*S. costatum*), green algae (*D. tertiolecta*), sea urchins (*P. lividus*), barnacles (*A. amphitrite*) and jellyfish (*A. aurita*). Further studies based on testing concurrently sensitivity to Ag-NPs and Ag ions could provide new insights, allowing to better define Ag-NP toxic impact and mode of action in the selected model species.

In conclusion these results contribute to increase knowledge about the possible toxic effects related to NPs but further investigations on the trophic interactions within the food web are needed to confirm and increase the ecological relevance of these findings.

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Legend to Tables and Figures

Tab. 1. Ag-NPs suspensions and the end-points evaluated for each model species.

Tab. 2. Median effect concentration values with 95% Confidence Limits (CL) from the different tests with Ag-NPs. nc = not calculable

Fig. 1. Growth inhibition of *D. tertiolecta* and *S. costatum* after 72 h of exposure to Ag-NPs (Mean ±SE, n=3). $^{**}p<0.01$ (one-way ANOVA and SNK Test).

Fig. 2. Alteration of Frequency of pulsation (% Fp) and immobility (% I) of ephyrae of *A. aurita* after 24 h and 48 h of exposure at increasing concentrations of Ag-NPs (Mean ±SE, n=3). * * = $p< 0.01$ (one-way ANOVA and SNK Test).

Fig. 3. Mortality (% M) and Swimming Speed Alteration (% SSA) of nauplii *A. amphitrite* after 24 h and 48 h of exposure at increasing concentrations of Ag-NPs (Mean ±SE, n=3). ns = not significant. * * = $p< 0.01$ (one-way ANOVA and SNK Test).

Fig. 4. Mortality (% M) and Swimming speed alteration (% SSA) of larvae of *A. salina* after 24 h and 48 h of exposure at increasing concentrations of Ag-NPs (Mean ±SE, n=3). $^{*}p<0.05$, ** = $p< 0.01$ (one-way ANOVA and SNK Test).

Fig. 5. Motility parameters (RAP, % rapid sperm; VCL, curvilinear velocity) of sea urchin after 1 h of exposure at increasing concentrations of Ag-NPs (Mean ±SE, n=3). $^{**}p<0.05$ (one-way ANOVA).

Table

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Tab. 1. Ag-NPs suspensions and the end-points evaluated for each model species.

Species	Life-Stage	Tested concentrations (mg L^{-1})	End-points	Protocol
D. tertiolecta *S. costatum*	Actively growing cells	0 -0.4-0.8 -1.6 -3.2 -6.4	Growth inhibition	ISO 10253, (2006)
A. aurita	Ephyrae	0 -0.1 -0.2 -0.4 -0.8 -1.6	Immobility Frequency of pulsations	Faimali et al. (2014)
A. amphitrite	II stage nauplii	0 -0.1 -0.2 -0.4 -0.8 -1.6	Mortality speed Swimming alteration	Faimali et al. (2006), Piazza et al. (2012), M.U. 2245:12
A. salina	Instar I larvae	0 -1 -5 -10 -50	Mortality Swimming speed alteration	Garaventa et al. (2010)
P. lividus	Sperm cells	0-0.02 -0.2 -0.5 -0.6 -0.7 -1	Sperm motility	Fabbrocini et

	al. (2012)

Tab. 2. Median effect concentration values with 95% Confidence Limits (CL) from the different tests with Ag-NPs and with the reference toxic compounds. nc = not calculable

Species	End-point	LC_{50}, EC_{50}, IC_{50} (mg L^{-1}, CL 95%)	
		Ag-NPs	Reference toxicants
			$K_2Cr_2O_7$
D. tertiolecta	Growth Inhibition	72h-IC_{50}=0.9 (0.4-1.5)	72h-IC_{50}=34.42 (26.9-47.5)
S. costatum		72h-IC_{50}=3.1 (2.7-3.5)	72h-IC_{50}=1.9 (1.5-2.3)
			$Cd(NO_3)_2$
A. aurita	Immobility	24h-EC_{50}=<0.1 (nc)	24h-EC_{50}=0.09(0.07-0.1)
		48h-EC_{50}=<0.1 (nc)	48h-EC_{50}=0.15(0.12-0.19)
	Frequency of Pulsations	24h-EC_{50} =<0.1(nc)	
		48h-EC_{50} = <0.1 (nc)	
			$Cd(NO_3)_2$
A. amphitrite	Mortality	24h-LC_{50}=0.55(0.51-0.59)	24h-LC_{50}=0.91(0.85-1.07)
		48h-LC_{50}=0.27(0.26-0.28)	48h-LC_{50}=0.53(0.50-0.56)
	Swimming Speed	24h-EC_{50}=<0.1 (nc)	
		48h-EC_{50}=<0.1 (nc)	
			$K_2Cr_2O_7$
A. salina	Mortality	24h-LC_{50}=9.96(6.64-14.94)	24h-LC_{50}=18.62(16.97-20.43)
		48h-LC_{50}=3.79(2.28-6.29)	48h-LC_{50}=18.86(16.8221.16)
	Swimming Speed	24h-EC_{50}=3.56(1.99-6.35)	
		48 h-EC_{50}=<1(nc)	
			Cd(II)
P. lividus	Sperm motility	1h-VCL EC_{50}=0.55(0.53-0.57)	1h-VCL EC_{50}=2.90 (2.75-3.05)
		1h-rap EC_{50}=0.56(0.53-0.60)	1h-rap EC_{50}=2.71 (2.12-3.47)

Figure
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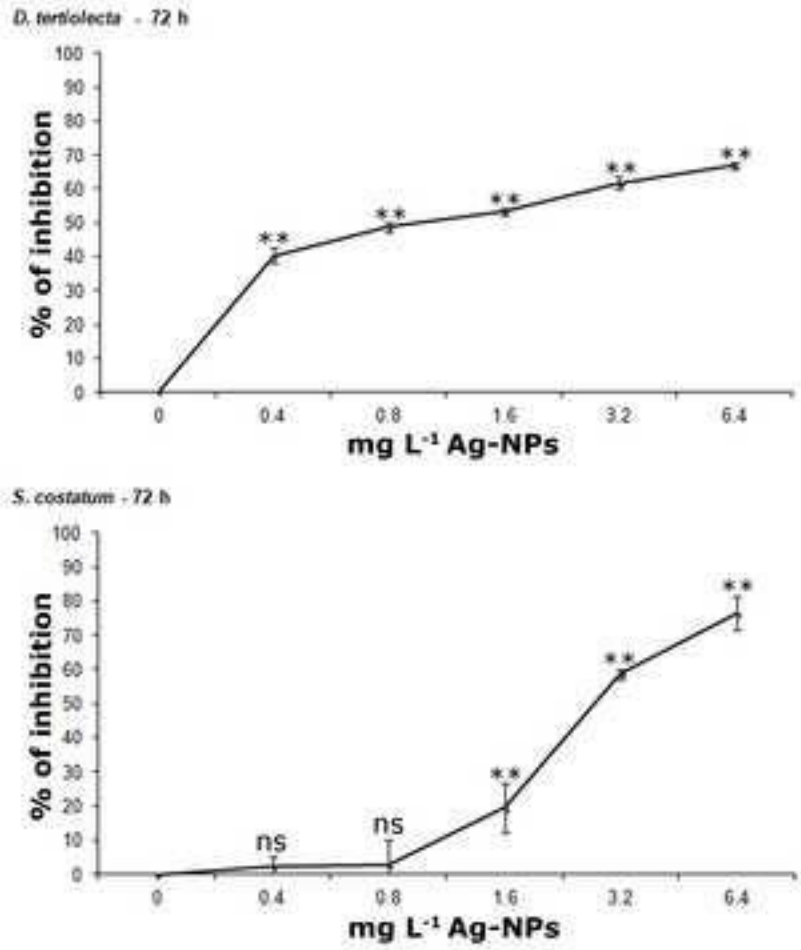

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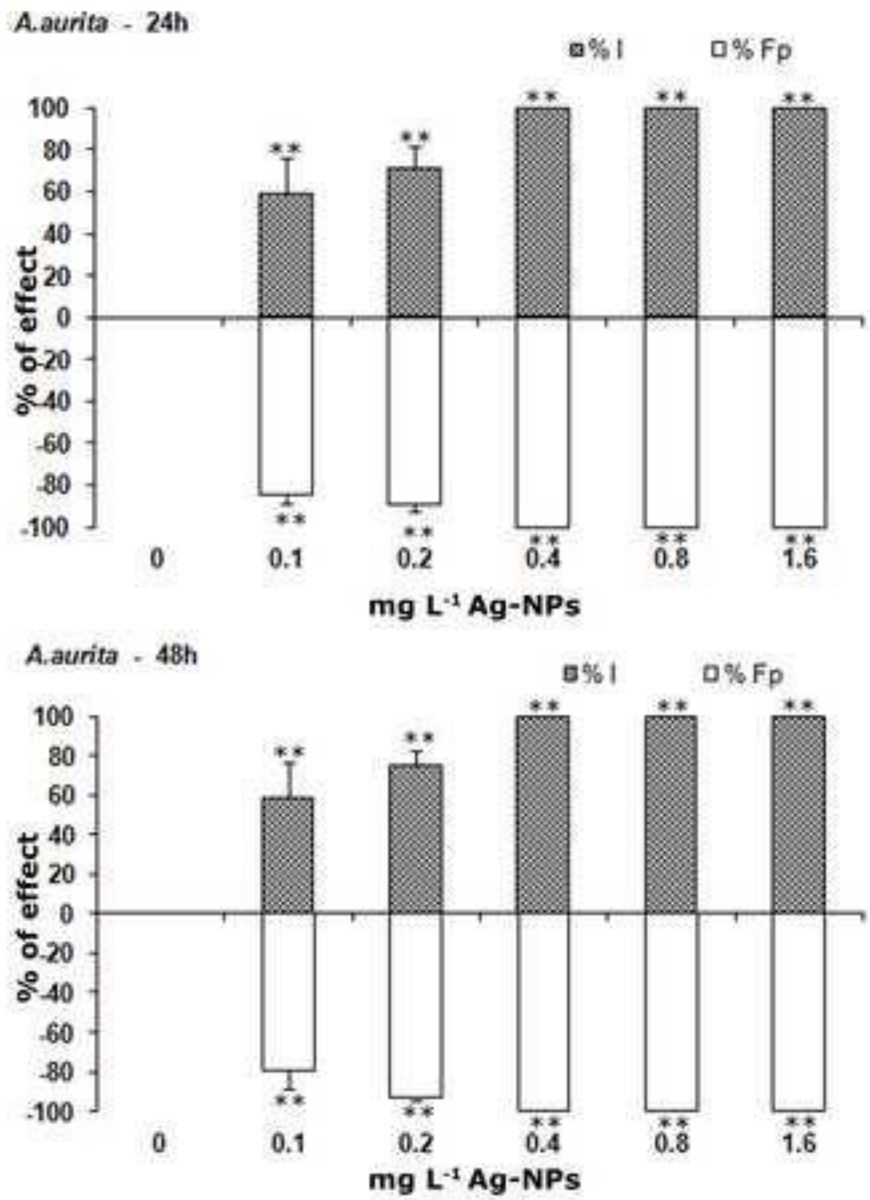

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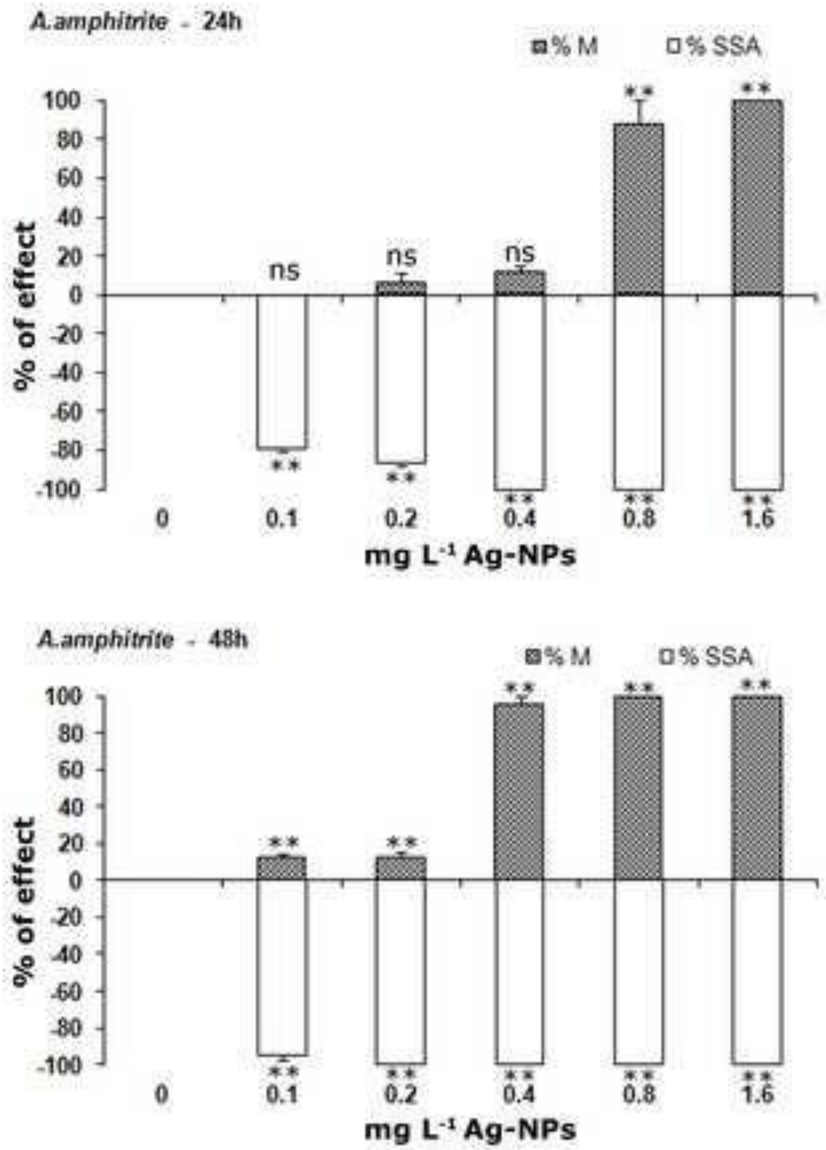

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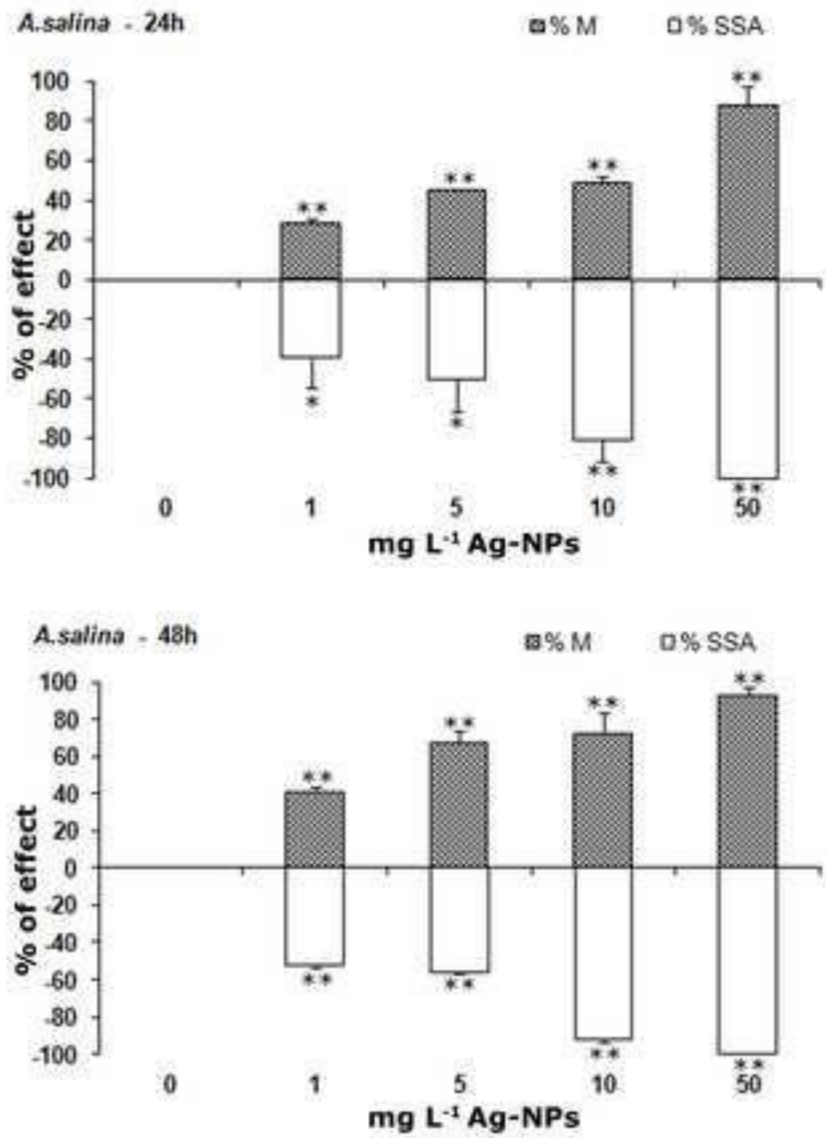

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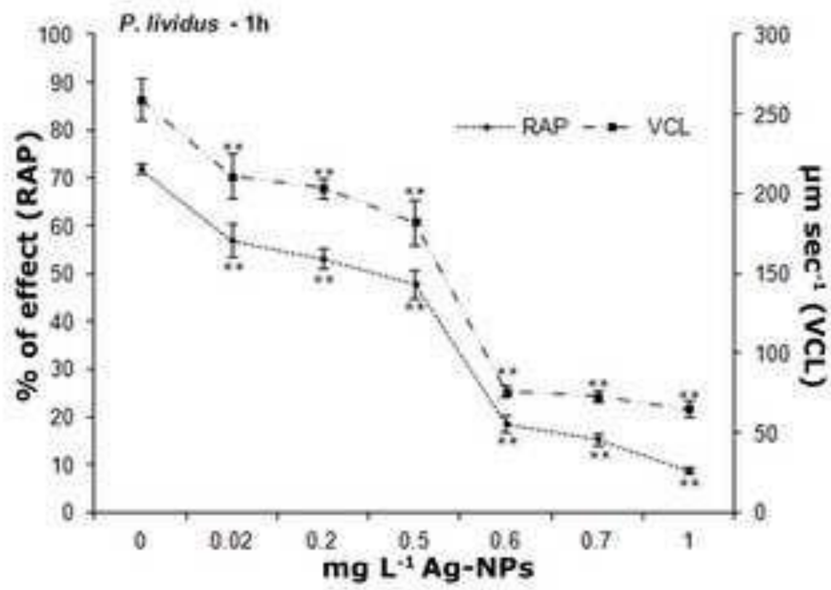

Gambardella et al. Highlights

- Ag-NP effects were investigated in marine species at different trophic levels
- Algae, cnidarians, crustaceans and echinoderms were exposed to Ag-NP suspensions
- Acute and behavioural end-points were evaluated to detect Ag-NP toxicity
- All end-points underlined a dose-dependent effect at any level of the trophic chain
- Ag-NPs exposure influenced different trophic levels within the marine ecosystem