CHAPTER 2 - Ecotoxicological effects of silver nanoparticles in marine mussels

INTRODUCTION

Silver nanoparticles are widely used in various industrial fields due to their physical and chemical properties. In particular, their use is taking hold in medical applications and their bactericidal and disinfectant properties make them protagonists in food and personal care industry, as well as in water remediation (Sánchez et al., 2011).

The massive use of Engineered Nanoparticles (ENPs) however leads to a consequent release of these in the environment, which is still difficult to limit or control with post-production systems and it is quite impossible for example for a range of products related to consumer care.

From an ecotoxicological point of view, the scientific community is trying to determine if relase of engineered nanoparticles into the environment may pose an ecological risk greater than that determined by bulk materials (non-nano) and/or ionic forms (in the case of metallic and metal oxide based nanomaterials) (Moore, 2006, Ju-Nam and Lead, 2008, Shaw and Handy, 2011).

To date, however, there is a knowledge gap for what concerns the speciation of these products in the environment -either terrestrial or aquatic-; the retention capacity by the water treatments plants, as well as the effects at biological (uptake, internal distribution, mechanisms of toxicity, excretion) and ecotoxicological level (Reidy et al., 2013).

Concerning the marine environment, indeed, the number of studies is still limited. The behavior of ENPs in the sea is in fact very different from that in freshwater, due to phenomena

of aggregation, agglomeration and precipitation, mainly caused by the interference of ions with the surface potential, an intrinsic characteristic of the nanoparticles.

Recently, the scientific community has been focusing its attention towards the study of nanomaterial bioavailability and toxicity using ectothermal species as bioindicators. Marine invertebrate filter feeders are indeed widely recognized as sensitive species with a great ecological importance (Baun et al., 2008, Canesi et al., 2008, Moore, 2006, Canesi et al., 2010a, Canesi et al., 2010b, Canesi et al., 2012, Barmo et al., 2013).

Some authors have already performed nanopartcicle toxicity studies using the ectothermal bivalve Mytilus galloprovincialis, focusing essentially on sublethal effetcs. Some studies highlight cellular stress and toxicity of $nTiO_2$ on hemocytes of Mytilus (Canesi et al., 2010a, b), histomorphological alterations in gill and digestive gland as well as increasing of transcriptional of metallothionein (mt) genes and oxidative DNA damage in the haemocytes (D'Agata et al.,2013). Accumulation of nano-CuO_2 is higher in digestive gland than in gills, causing oxidative stress in the form of activated antioxidant enzymatic activities and lipid peroxidation of membrane lipids, as well as MT induction (Gomes et al., 2012). DNA damage on mussel's haemocytes is the most common damage caused by metal nanoparticles (Gomes et al., 2013a). Recently, some studies validate the analysis "omics" as reliable biomarkers to observe the change in protein expression in organisms exposed to nanoparticles (eg, AgNPs), indicating the presence of stress in various tissues and giving an indication of their mechanism of action (Gomes et al., 2013b)

To our knowledge, however, no ENPs ecotoxicological survey was yet carried out in the Mytilus complement. The aim of this work, therefore, was to provide the full range of acute and chronic toxicity for the effects of two nano-metallic silver commercial preparations

showing nominal size of 50 and 5 nm (the latter are also referred as 8 nm ENP in body text). Effects of ionic silver were also considered for comparison with the colloidal forms.

Moreover, In order to characterize the effects of three different types of silver forms, i.e. the ionic form (from $AgNO_3$) and two metallic nanoparticles of 5 nm and 50 nm, was constructed a model of speciation of silver, based essentially on the persistence of the metal in the water column .

This model, generated according to the log-logistic function and the least square method from chemical data observed in the time, has allowed us to express the toxicity data according to what has been called "standardized integrated dose" of Ag.

MATERIALS AND METHODS

Preparation of artificial seawater (salinity 35‰)

Artificial sea water (ASW) was prepared in large tanks with a capacity of 200 L each, equipped with a system for constant recirculation and filtration of the water. Seawater was prepared using deionized water further purified with coal filters for retaining the organic matter and chlorine

ASW was prepared using the formula published by Marchi et al. (2000): NaF 0.045 mM, $SrCl_2 \cdot 6H_2O$ 0.049 mM, H_3BO_3 0.32 mM, KBr 0.56 mM, KCl 6.3 mM, $CaCl_2 \cdot 2H_2O$ 5 mM, Na_2SO_4 22 mM, $MgCl_2 \cdot 6H_2O$ 16 mM, $NaHCO_3$ 2.3 mM, NaCl 473 mM. To prepare water properly and avoid the formation (and thus precipitation) of insoluble salts, method described by Kester et al. (1967) was used: gravimetric salts were combined in water in one container, while $CaCl_2$, $MgCl_2$ and $SrCl_2$ were dissolved in another container. After, the two solutions were thoroughly mixed and combined while stirring. All reagents were of analytical grade and purchased from “Sigma-Aldrich”. Once prepared, ASW has been left for a few days in continuous agitation and

constant temperature at 16°C before use. Water was ready to use when dissolved oxygen and pH values reached respectively > 8 mg/L and 8.1 ± 0.5.

Animals collection and acclimatization

Adult mussels (*Mytilus galloprovincialis* Lam.) were sampled from a natural population in front of Gabicce Mare (Italy, North Adriatic Sea) and transported to laboratory in a cool box under controlled condition (0-4°C). Animals (n= 600-900) were than selected to form homogeneous groups in size (5-6 cm), separated from one another by carefully cutting off the byssal threads and washed with seawater. Then, mussels were acclimatized for 30 days in static tanks containing filtered 35‰ aerated ASW. Temperature was kept constant at 16±1°C and animals fed daily with a commercial nutritive solution for marine invertebrates (Marine Liquifry, INTERPET).

Chemicals

Two types of silver engineered nanoparticles (AgENPs) were used in this study, having nominal diameter of 2-8 nm (average 5 nm) and 50 nm. These NMs were provided respectively by the Polish industry "Amepox" (provided in stabilized aqueous solution, concentration 1 ppm) and by Czech industry "Nanotrade " (supplied in powder form). Ionic silver (Ag^+) in the form of $AgNO_3$ was obtained from Sigma Aldrich.

Experimental design

Mussels were randomly assigned to experimental (Ag treated) and reference non exposed groups. Five different nominal exposure levels –from 10 mg/L to 0.001 mg/L Ag with a Log10 series- and 1/2 reference (not-exposed) control groups were set-up for each replicated experiment (n=4). Exposed mussels were treated for four days with each silver form (either 5 nm or 50 nm or Ag^+). Silver was added daily -along with water renewal- from freshly prepared

stock water-suspension/solution. In general, at least fifteen mussels per replicate were tested. Animals were not fed during these exposures.

After acclimation fifteen mussels were randomly placed into 21 L polypropilene plastic vessels with 35‰ aerated (110 L/h) ASW, pH 8.1 ± 0.5, at a density of 1 animal/L at 16°C.

The 50 nm AgENPs stock solution was prepared according to the "PROSPECT 2010: Protocol for Nanoparticle Dispersion" (www.nanotechia-prospect.org). Briefly, nanoparticles were weight and placed in a glass vial; a few drops of MilliQ water were added to create a concentrated nanoparticle paste and then more water was added to make up the desired concentration (1 g/L). After sonication for 30 sec with an ultrasound probe at middle power intensity the suspension was stirred by hand for 10 sec, and only the clear supernatant was further withdrawn for the exposure. Actual silver concentration was evaluated –ex post- in all stock solution.

The ecotoxicological parameters -mussel mortality, production of byssus- along with physical/chemical parameters (pH, T, O_2) were monitored at least daily.

96-h acute toxicity test – mortality in water

Mortality was evaluated daily before the water change and at the end of the 4th exposure day. Mussels were considered dead (and thus withdraw from the experiment) when the lack of adductor muscle activity was recorded.

Semi-chronic toxicity test – mortality under aerial exposure

This test was carried out essentially as described by Viarengo et al. (1995). Briefly, after the exposure period in ASW, surviving specimens were emerged to aerial conditions and kept at 16°C/90% humidity. Mortality was evaluated daily as aforementioned described. The experiment was terminated when 100% mortality in each group was recorded.

96-h chronic toxicity test – byssogenesis

A simple binomial procedure was set up to test byssus synthesis/functionality in immersed mussels. Only mussels with functional byssus threads attached to the tank or to another individual were considered positive for byssogenesis. Byssal threads were gently cut with a scissor from every mussel daily to allow further measurements during a 4 day exposure period.

Determination of silver concentration in water

Actual silver concentration was determined in the water column during the 4 day exposure period. 50 ml water samples were withdrawn from each tank at regular intervals of 0, 1, 4, 24 h from water/chemical renewal. 10 ml aliquots were immediately subjected to ultracentrifugation at 100,000 g for 2 h at 4°C to collect the dissolved (ionic) silver fraction. Silver content analysis was performed by Inductively-Coupled Plasma Mass-Spectrometry (ICP-MS). The sensitivity limit (LOD) for silver was 0.5 µg/L in the acid matrix. Samples were delivered for analysis to an external laboratory, which analyzed the samples according to a standard referenced procedures (UNI EN ISO 17294-2:2005).

Determination of silver concentration in mussel tissues

Total silver burdens were evaluated in whole soft tissues from mussels exposed for 4 days to the different silver forms. Frozen tissues were washed extensively in seawater to normalize samples and under cold tap water to get rid of weakly bound particles adhering to tissues. For the metal analysis content, 1 g of tissue from single individuals were thawed and homogenized with the addition of 5 ml deionized water. Samples were digested acid in a microwave oven with 50 ml of a 3:1 mixture of concentrated HCl:HNO_3 (aqua regia) and further analysed by ICP-MS analysis according to the referenced procedure EPA 3051A. LOD was 0.5 µg/L in the acid matrix

Statistical analysis

Differences in acute toxicity levels (survival of submerged animals) and byssus frequencies were evaluated using one way and two way analysis of variance by means of the Kruscall-Wallis non parametric test statistics ($p < 0.05$).

Survival probability for mussels kept under aerial exposure after silver exposure were computed according to the Kaplan–Meier survival function probability (Kaplan and Meier, 1958; Uno et al., 2009). Differences between survival probabilities were analysed using the non-parametric test Kolmogorov-Smirnov with a confidence greater than 95% (Lilliefors, 1967; Massey, 1951).

RESULTS AND DISCUSSION

96-h acute toxicity test – mortality in water

M. galloprovincialis exposure to 50 nm and 5 nm silver ENPs showed a significant effect on survival only at the highest level tested, 10 mg/L (t-student, $p<0.05$ $n=4$). Ionic silver was effective also at 1 mg/L (Fig. 1) and, in general, it appeared more toxic than NP forms. Nominal silver concentrations were considered for the analysis.

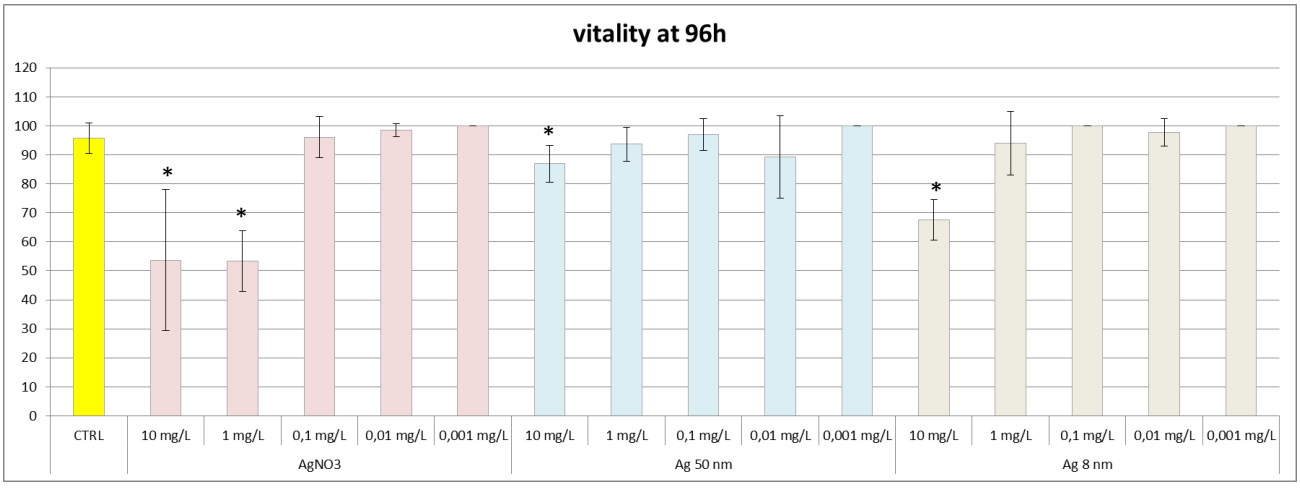


***Fig 1* - 96 h *M. galloprovincialis* acute toxicity test for silver**
**** P<0.05 Kuskall –Wallis Test Statistics***

Sub-chronic toxicity test - mortality under aerial conditions

Fig. 2 shows survival under aerial conditions as a function of time in either control or silver exposed specimens. This test allowed to unveil a dose dependent pattern for all silver forms.

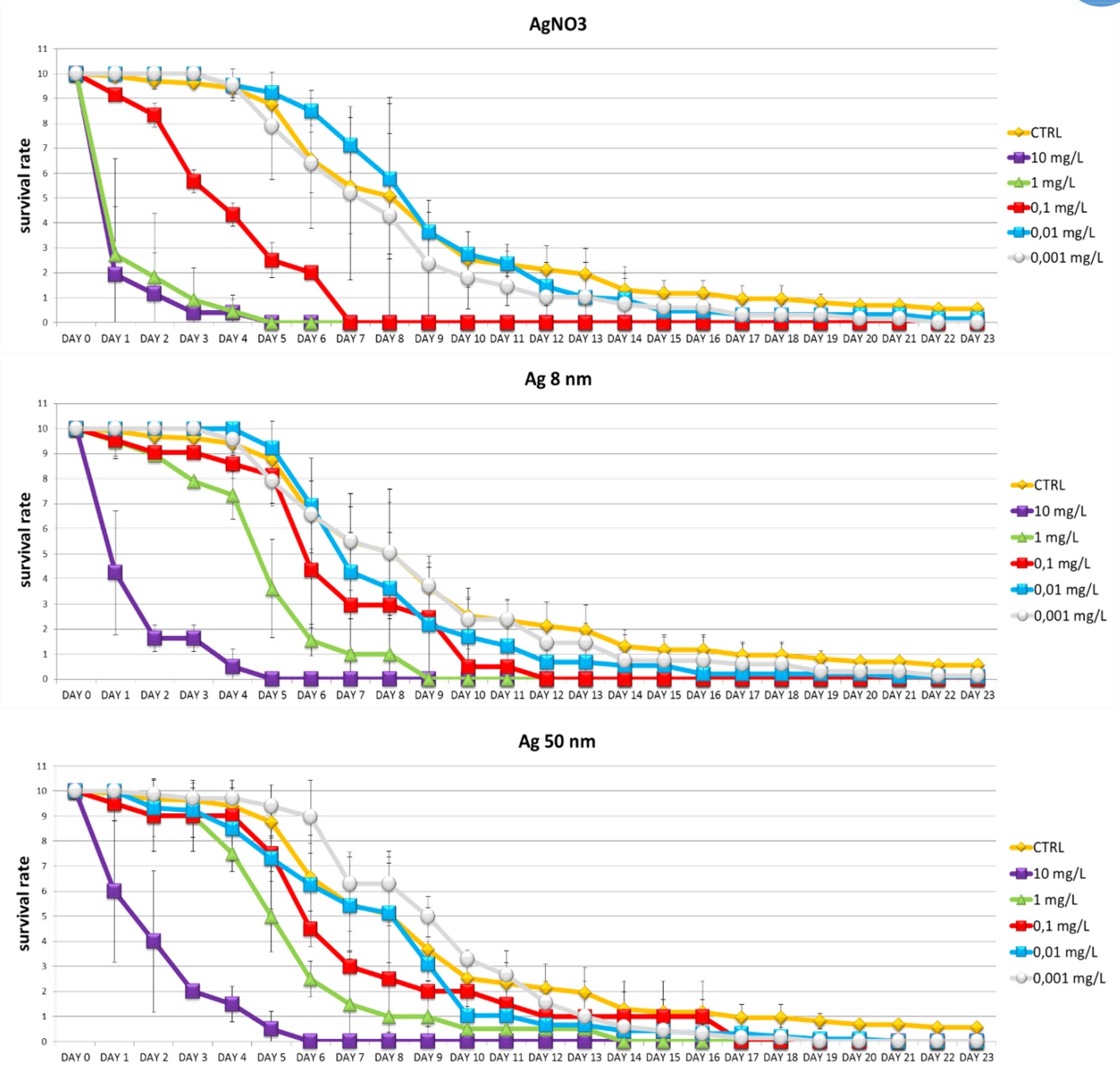


***Fig 2* - Survival plot under aerial conditions.**

Data were further analyzed by plotting the survival probability according to Kaplan-Meyer (Fig. 3). This procedure allowed to assess statistical differences among samples within the same group and among different treatments. The Kolmogorov-Smirnov distribution test was used to compute the Lowest Observed Effect Concentration (LOEC) and then assess differences

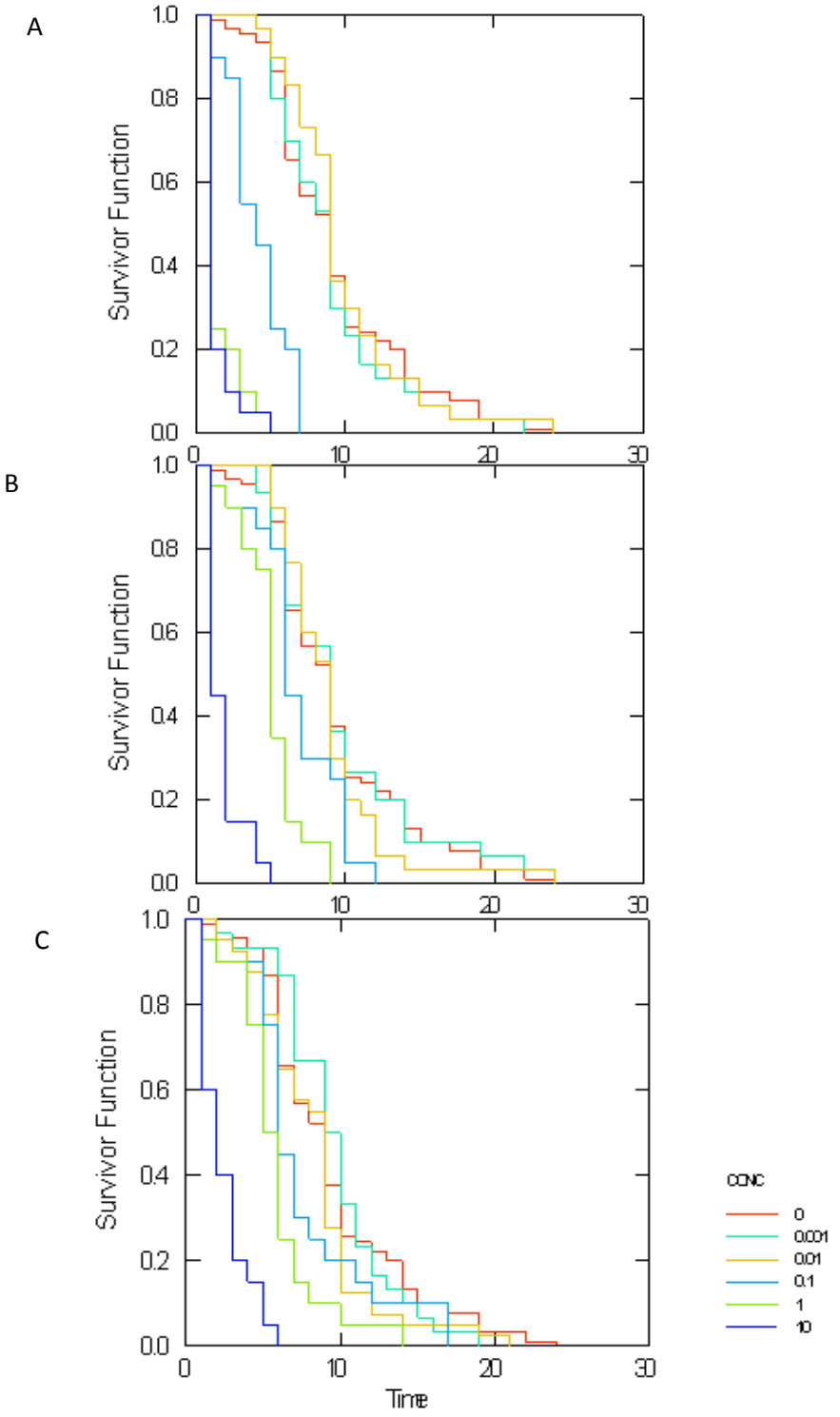
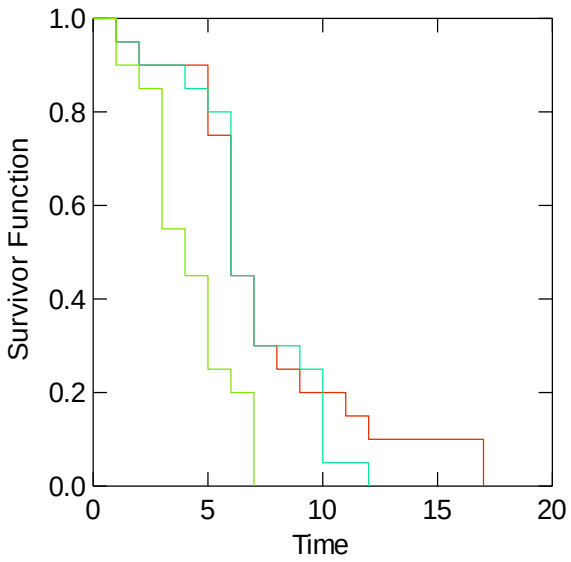


***Fig 3** - Kaplan-Meyer probability for survival in aerial exposure. A. Ionic silver; B, 5 nm AgENPs; C, 50 nm AgENPs*

among pair of groups (Fig. 4). Even if the LOEC level turned out 0.1 mg/L for the three different silver forms, ionic silver resulted the most toxic agent (Fig. 4).

Values obtained for the Not Effective Observed Concentration (NOEC) were 0.01 mg/L in all tested condition (Fig. 3) (Kolmogorov-Smirnov test, $p<0.05$).

A



B

Kolmogorov-Smirnov Two Sample Test results
Two-sided probabilities

	Ag 50nm	**Ag 8nm**	**$AgNO_3$**
Ag 50nm	1.000		
Ag 8nm	0.954	1.000	
$AgNO_3$	0.008	0.002	1.000

PART
Ag 50nm
Ag 8nm
AgNO3

***Fig 4* - Comparison of survival probability at LOEC level for ionic and ENP silver forms**
A. Kaplan-Meyer survival probability.
B. Output of the Kolmogorov-Smirnov test for difference for pairs of groups

96-h chronic toxicity test – byssogenesis

Byssus is the silky filament generated by mussel foot and used to attach animals (as well larvae at the end of development) to the rocky substrate. A binomial test based on the presence/absence of functional byssus threads was used as a proxy for chronic toxicity of silver. Fig. 5 shows byssogenesis frequencies found across the 4 day exposure period for the various treatments.

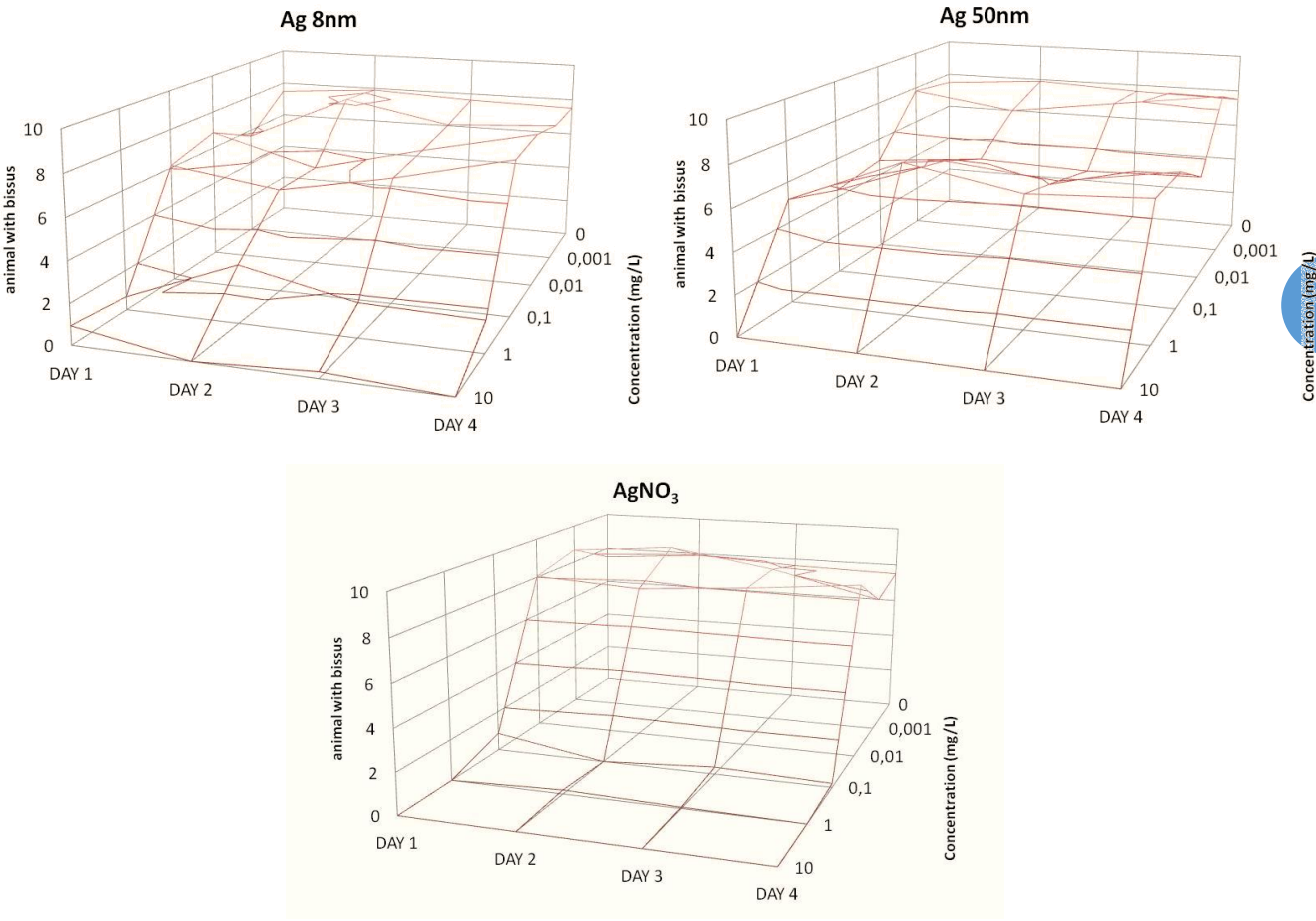


***Fig 5** - Functional byssus frequency in silver exposed mussels*

One way analysis of variance (non parametric Kruskall-Wallis non parametric test, $p < 0.05$, n=26) showed significant effects for all silver forms. LOEC values were 0.1 mg/L, 1 mg/L and 10 mg/L respectively for the ionic form, 5 nm and 50 nm particles. Thus, the byssus test ranked again the ionic form as the most toxic type of silver, while 5 nm ENPs turned out more effective than the 50 nm ones.

Bioaccumulation of silver in mussel body burden

To explain differences in toxicity among exposure to different silver forms, Ag body burdens were evaluated in mussel soft tissues. From Fig. 6, showing data distribution found, it appears clear that metal loads were higher for the exposure to ionic silver > 5nm ENPs >>50 nm ENPs.

These results, therefore, are consistent with a direct relationship between bioaccumulation and the toxicity observed for acute (mortality) and chronic toxicity (Fig. 1; Fig. 5).

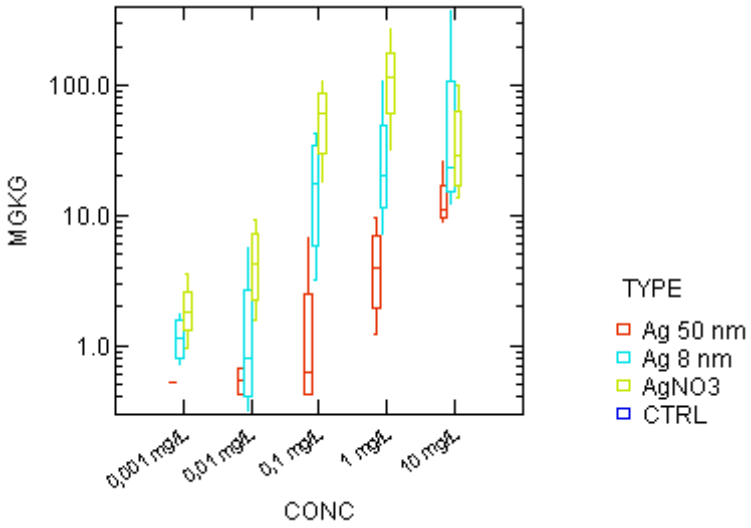


Fig 6 - Ag in soft tissues (mg/kg w.w.)

Persistance of AgENPs in seawater: development of a logistic model to compute the actual Ag dose

To explain differences in Ag loads into mussel soft tissues there are two possible explanations: either toxicokinetic parameters or the persistence of silver forms in the seawater column are different. In every case, the determination of external Ag concentrations is required to test such hypotheses, as uptake and elimination kinetics does depend on the environmental concentration.

The sudden precipitation of silver aggregates, clearly visible at the highest tested concentrations (data not shown), in particular for both ENPs, is, however, consistent with a modification in time of the actual silver title. Salinity, in fact, is the major factor affecting zeta-potential, the electrostatic potential near the particle surface that is a measure of the magnitude of repulsion or attraction between particles (Hochella et al., 2012). In case of ionic

silver (provided to the system in form of nitrate) the concentration is expected to remain longer constant due to the formation of complex ions with chloride and hydroxide (Santore and Driscoll, 1995).

To this aim a kinetic model based on the logistic function was used to fit empirical data (Fig. 7-10), i.e. seawater silver concentration as a function of time (0-24 h, since 24 h was the renewal time in semi-static exposures). Then, this equation was used to determine the actual silver dose (mg/L/d or h) which mussels were exposed to. The least squares method was used to fit data into the model. The method of least squares is an optimization technique that allows to find a function, regression curve, which is as similar as possible to an empyrical dataset. The best function is the one that minimizes the sum of squares of the distances between observed and modeled data.

The general equation of the logistic model was:

$$y = max/(1+(x/\tau))^{b} \qquad (1)$$

where **y** represents the silver concentration (mg/L), **x** is time (h), **τ** is the half life (h) of silver in the water column, **b** is the slope of the log phase, **max** is silver concentration at time zero. Indeed, our results showed that ENPs are unstable in seawater. As a rule of thumb, half-lives (τ) were often inversely correlated with the initial silver concentration, suggesting the occurrence of concentration dependent aggregation and precipitation processes. Moreover, the stability of 5 nm ENPs was higher than that of larger particles. In fact, their τ values, typically, displayed differences higher than one magnitude order (Fig. 9-10)). As an example, at 0.1 mg/L (nominal) level, τ_{5nm} and t_{50nm}were 1.3 h vs 0.09 h, respectively. At a higher level, 1 mg/L, τ_{5nm} and t_{50nm}were 0.47 h vs 0.02 h, respectively. At 10 mg/L level, precipitation processes were almost instantaneous as argued by the τ values, respectively of 0.01 h and 0.001 h. Half life for the 5 nm particles could be also computed at 0.01 mg/L level, rendering

a τ equal to 0.85 h. At lower concentration levels, data could not be fitted, since environmental Ag was below the detection limit of the technique (LOD, 0.0005 mg/L).

As expected, ionic silver showed the highest persistence in seawater, however, at relative low Ag^+ levels (0.01 and 0.001 mg/L) there was a relevant titre drop, most likely due to sulfur speciation and uptake in tissues.

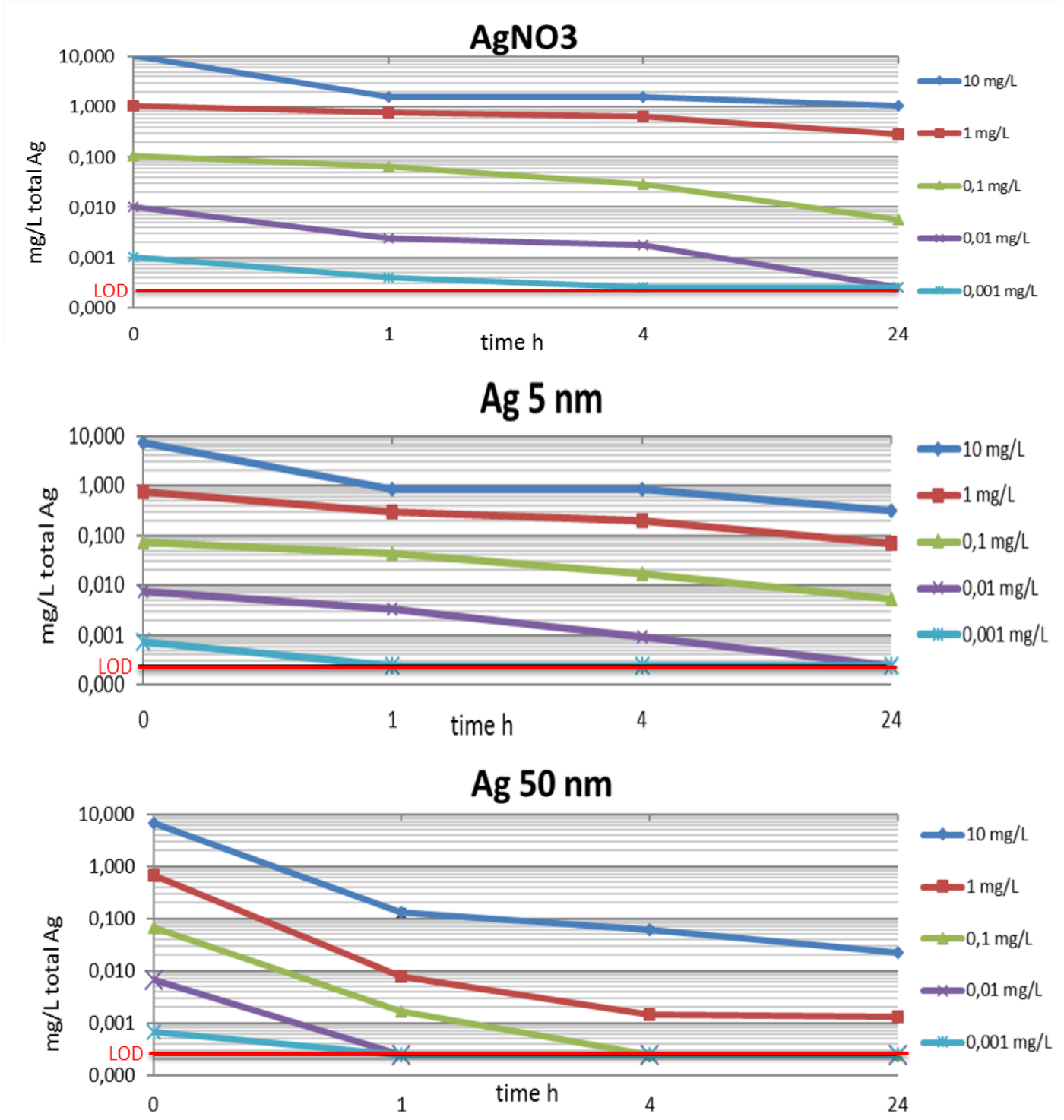


***Fig 7** - Total Ag concentration in seawater. Raw data used to derive the logistic model. Median values are shown (n=4). In legend is reported the color Id for each nominal exposure level*

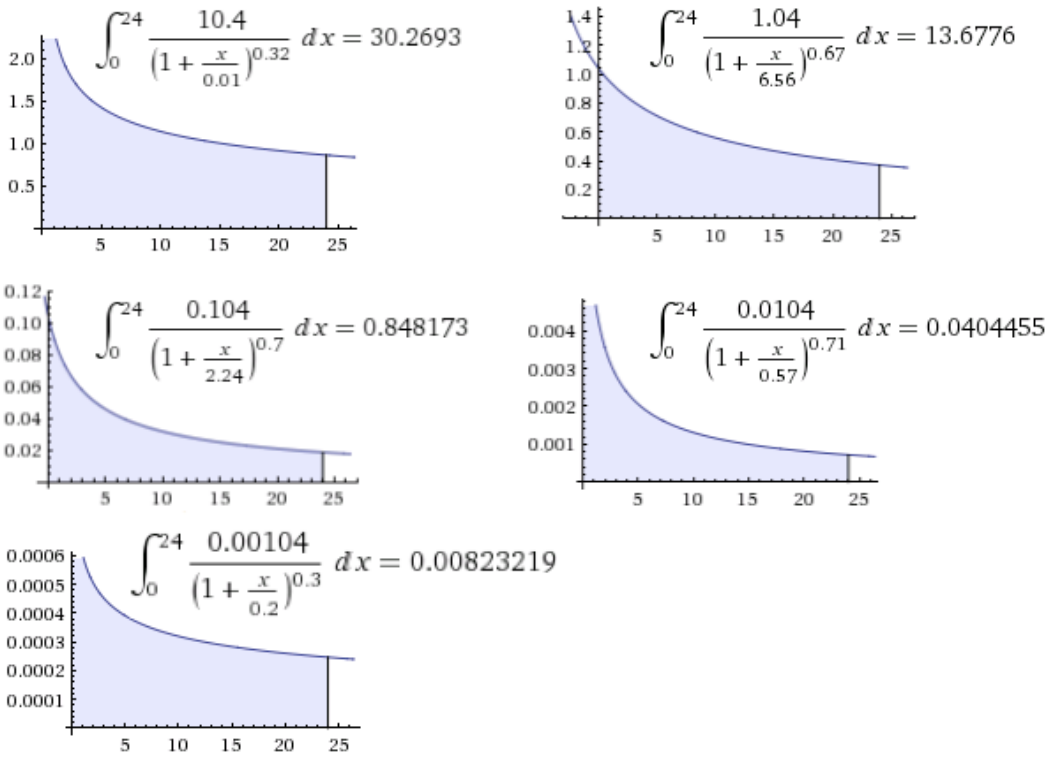


***Fig 8** - Kinetic model for $AgNO_3$ persistence in seawater.*
Shown are curves and integrals obtained from fitting chemical data obtained at each exposure level (from 10 mg/L to 0.001 mg/L, left to right, top to bottom) into the logistic model. Each integral was resolved between 0 and 24 (h) to calculate the actual silver dose (mg/L/d) available to mussels during each exposure day.

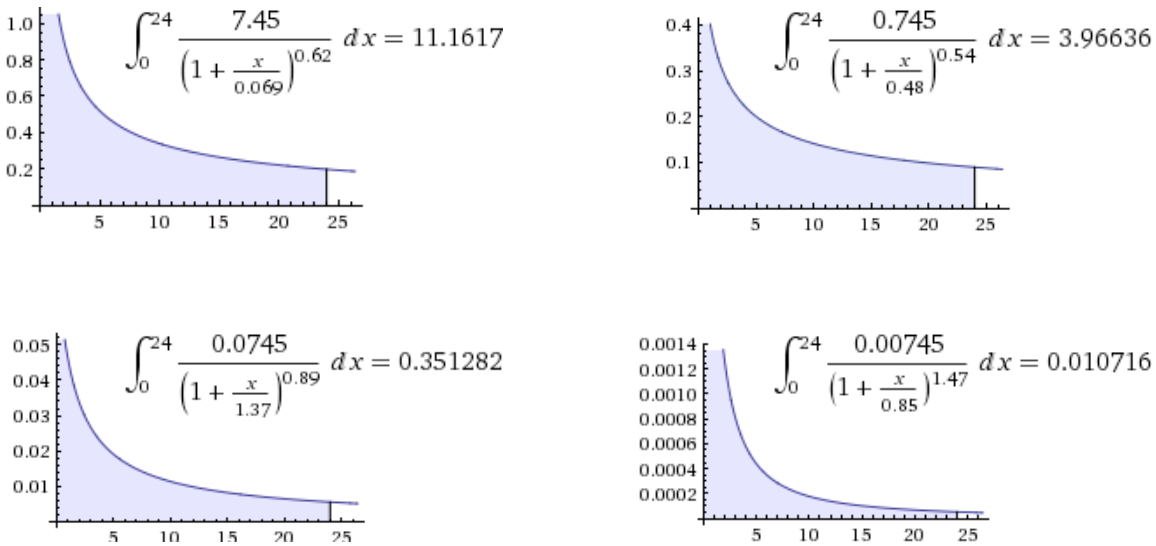


***Fig 9** - Kinetic model for AgNP 5nm persistence in seawater.*
Shown are curves and integrals obtained from fitting chemical data obtained at each exposure level (from 10 mg/L to 0.01 mg/L, left to right, top to bottom) into the logistic model. Each integral was resolved between 0 and 24 (h) to calculate the actual silver dose (mg/L/d) available to mussels during each exposure day.

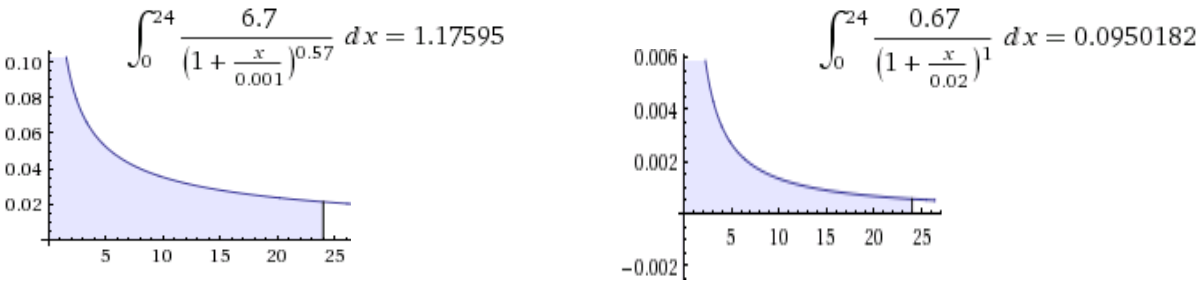


***Fig 10* - Kinetic model for AgNP 50nm persistence in seawater.**
Shown are curves and integrals obtained from fitting chemical data obtained at each exposure level (10 mg/L and 1 mg/L) into the logistic model. Each integral was resolved between 0 and 24 (h) to calculate the actual silver dose (mg/L/d) available to mussels during each exposure day.

Toxicity as a function of actual silver dose

Mortality was fitted into a non linear model as dependent variable of the actual silver dose with no assumption about the form of silver used. About 90% of observed variance could be explained by the model and analysis of variance indicated a significant relationship with the independent variable (F test, $p < 0.001$; $r=0.88$) (Fig. 11).

Similar resuls were obtaied for the effects on byssus (Fig. 12)

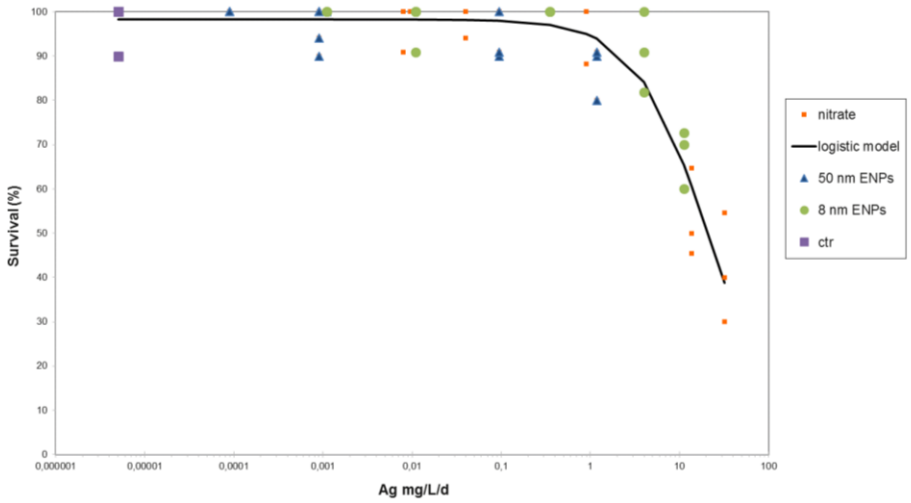


***Fig 11* - Dependence of mortality on actual silver dose (Logistic model)**
Data are depicted with different colors for the reader's convenience. Trimmed data (0.5 -0.95 percentile were used. r= 0.88

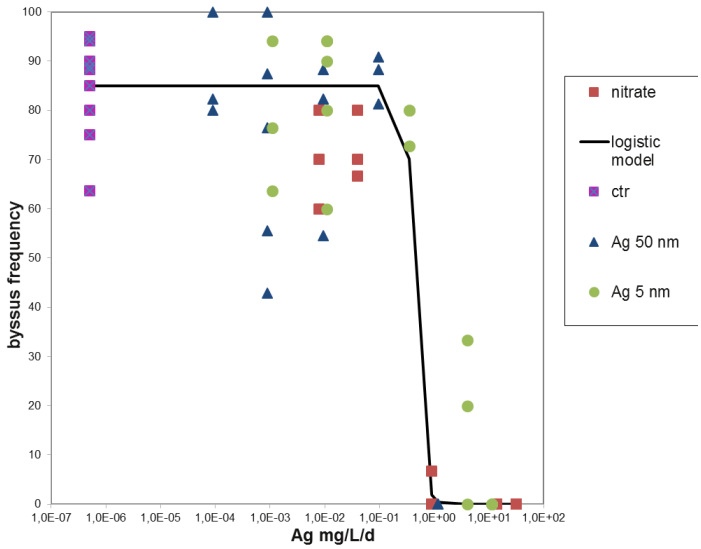


***Fig 12** - Dependence of functional byssus frequency on actual silver dose (Logistic model)*
Data are depicted with different colors for the reader's convenience. r= 0.79%, p < 0.0001, F test statistics.

Also differences observed in bioaccumulation patters (Fig. 6) could be fairly explained through the actual silver dose. Fig. 13 in fact shows a direct, yet non-linear, relationship between total Ag levels and metal burdens in mussel soft tissues.

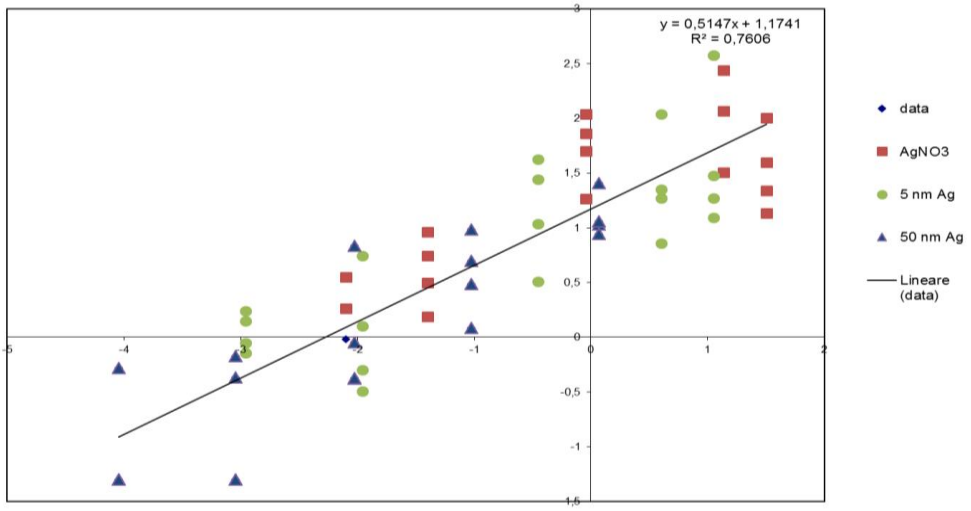


***Fig 13** - Dependence of Ag burdens on actual dose (log-log plot)*

Indeed, bioaccumulation parameters for the two AgENP and nitrate appears do be were very similar, as judget by the slope of the log-log plots (Fig. 14).

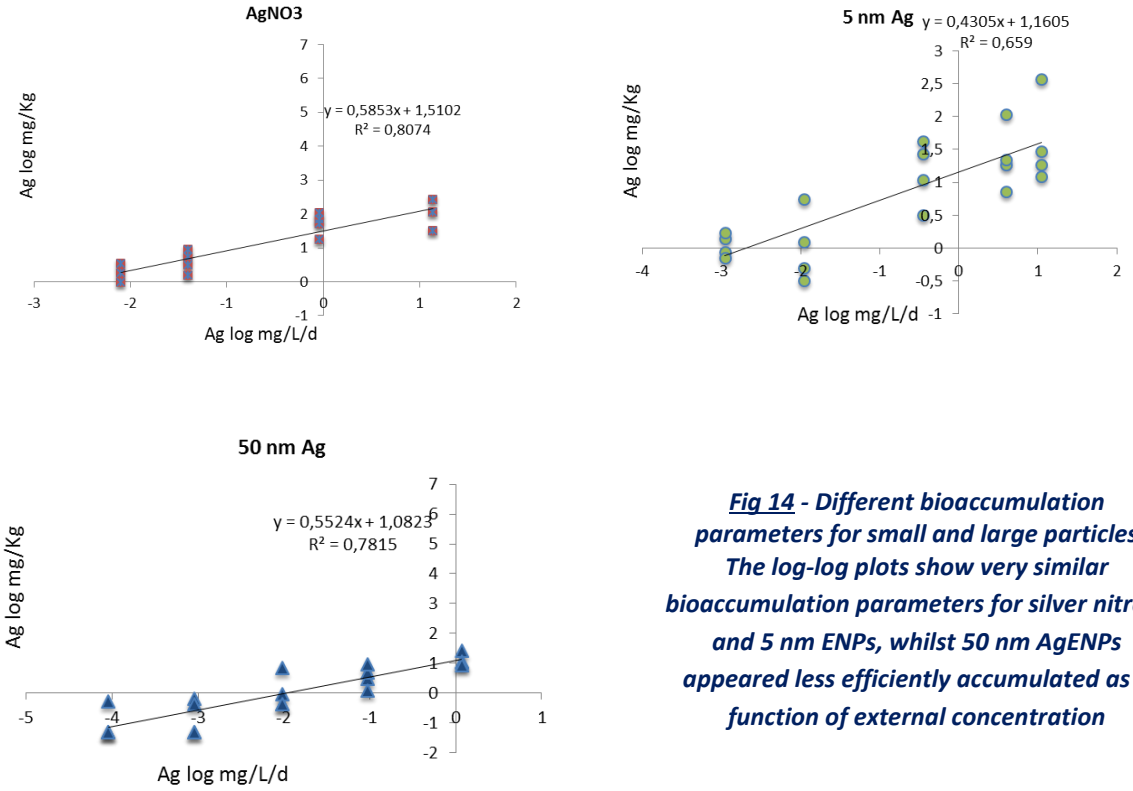


Fig 14 - Different bioaccumulation parameters for small and large particles The log-log plots show very similar bioaccumulation parameters for silver nitrate and 5 nm ENPs, whilst 50 nm AgENPs appeared less efficiently accumulated as a function of external concentration

Computation of various ecotoxicological endpoint

Ecotoxicological endpoints were finally calculated for the whole battery of test carried out in marine mussels. When possible a regression curve was generated from cumulative toxicity data and ecotox endpoints derived mathematically. Table 3 shows the full endpoint list using the measured integral silver dose expressed as mg/L/h.

According to the results so far presented, the three different silver forms appeared to exert similar ecotoxicological potential when considered merely on a mass scale basis. This indication is then converted in similar EC values, in particular $EC_{50/20}$ which are less prone to uncertainty.

In general, a good sensitivity was obtained from the use of selected tests. Of course best results were for non acute tests, such as byssus synthesis but even better survival probability

in aerial exposure. The latter showed NOEC values in the ng/L range that is consistent with worse case scenarios predicted for surface waters and Ag(NP) contamination (Gottschalk et al, 2010).

particle size/other characteristic	endpoint	NOEC	LOEC	EC1	EC5	EC10	EC20	EC50	units
$AgNO_3$	96h mortality	0,02083	0,56958	0,00176	0,01565	0,04203	0,12279	0,76764	mg/L/h
amepox 3-8nm	96h mortality	0,16625	0,40667	0,00802	0,04089	0,08546	0,19022	0,74697	mg/L/h
nanotrade 50 nm	96h mortality	0,00445	0,04454	0,00035	0,00618	0,02271	0,09332	1,04492	mg/L/h
$AgNO_3$	survival time probability after emersion	0,00156	0,02083	na		na	na	na	mg/L/h
amepox 3-8nm	survival time probability after emersion	0,00046	0,00596	na		na	na	na	mg/L/h
nanotrade 50 nm	survival time probability after emersion	0,00004	0,00045	na		na	na	na	mg/L/h
$AgNO_3$	bissus synthesis	0,00156	0,02083	0,00039	0,00090	0,00130	0,00195	0,00388	mg/L/h
amepox 3-8nm	bissus synthesis	0,00596	0,16625	0,00007	0,00056	0,00146	0,00415	0,02464	mg/L/h
nanotrade 50 nm	bissus synthesis	0,00445	0,04454	na	na	na	na	na	mg/L/h

particle size/other characteristic	endpoint	comments	comments	regression (R^2)
$AgNO_3$	96h mortality	acute	EC values from logistic regression, NOEC & LOEC from ANOVA	0,83
amepox 3-8nm	96h mortality	acute	EC values from logistic regression, NOEC & LOEC from ANOVA	0,92
nanotrade 50 nm	96h mortality	acute	EC values from logistic regression, NOEC & LOEC from ANOVA	0,73
$AgNO_3$	survival time probability after emersion	sub-chronic	LT50, 3 days	na
amepox 3-8nm	survival time probability after emersion	sub-chronic	LT50, 6 days	na
nanotrade 50 nm	survival time probability after emersion	sub-chronic	LT50, 6 days	na
$AgNO_3$	bissus synthesis	chronic	EC values from logistic regression, NOEC & LOEC from ANOVA	0,9
amepox 3-8nm	bissus synthesis	chronic	EC values from logistic regression, NOEC & LOEC from ANOVA	0,81
nanotrade 50 nm	bissus synthesis	chronic	EC values from logistic regression, NOEC & LOEC from ANOVA	0.53, suboptimal

***Tab. 3** – Full ecotoxicological endpoint table for Mytilus galloprovincialis exposed to ionic silver and silver nanoparticle*