CHAPTER 3 –Ecophysiological effects. Bio-energetics measurements in long-term exposure

INTRODUCTION

It is our current inability to accurately predict the environmental concentration of ENPs in the future, as well as the difficulty to detect very low concentrations. Despite these uncertainties, the patterns of environmental burden suggest that in light of current scientific discoveries most ENPs are not currently substantial environmental risk factors (Gottschalk et al., 2009). However, numerous studies show that ENPs are potentially hazardous for organisms, but most of them used short-term laboratory exposure tests with relatively short-lived species, such as daphnids and bacteria. This approach is limited, as it hinders the use of the results in making ecologically relevant predictions.

An important role of ecotoxicology is to assess the hazard potential of anthropogenic contaminants before they become real environmental problems. One way forward is to test the influence of ENPs on ecologically-relevant organisms, such as keystone or ecological engineer species in life-cycle experiments or in long-term study (Byers et al., 2006). However, many of these species are long-lived, so it will be possible to determine ENPs impacts on the individual performance in relatively small portions of the life history. This information however can be used to model the outcome on the whole life duration, if one accepts the caveat that different life stages may have different susceptibility (Muller et al., 2010).

To better understand the potential impacts of ENPs on populations of long-lived organisms, we can couple laboratory-based studies with modeling, thereby allowing us to simulate the effects of ENPs on populations based on results from individuals.

To reach more realistic environmental conditions, experiments need to be conducted in complex natural systems such as mesocosms or microcosms, i.e. “experimental systems that simulate real-life conditions as closely as possible, while allowing the manipulation of environmental factors” (Food and Agriculture Organization, 2009). To date, another challenge is to work with environmentally relevant concentrations of ENPs, or at least close to those used in studies examining chronic risks (Buffet et al., 2014).

In the case of metal-based ENPs, their (eco)toxicity can be due either to their physico-chemical surface characteristics (i.e. reactive oxygen species generation, adsorption of biomolecules, redox reaction) or to the toxicity of dissolved metals released in the environment or the tissues of organisms.

The following experiments were conducted using chronic ecotoxicological test as a starting point to investigate long-term effects of silver nanoparticles by recreating an environment able to better represent the real scenario of ENPs contamination. To this end, we looked at AgENPs impacts on physiological performance of *Mytilus galloprovincialis* Lam, a coastal marine ecosystem engineer that helps maintaining seawater quality through filter-feeding, builds biogenic reefs supporting biodiversity, and with representing a relevant prey-species to many coastal marine predators (Haven et al., 1966, Suchanek, 1986).

Animals were kept in mesocosms (microcosms) for 4 weeks. Vital parameters such as feeding acquisition traits (expressed by clearance rate), hearth beat rate, assimilation efficiency and the respiration rate as a possible estimate of the effect on somatic maintenance. (Widdows and Staff 2006) were monitored at regular intervals.

It was chosen to test only 5 nm AgENPs since they were found to be more stable than 50 nm particles in seawater column (see Chapter 2).

Exposure concentrations were selected in the nominal range of 2-20 µg/L that according to the Ag persistence model developed (Chapter2) correspond to a dose range of 10-1000 ng/L/h. Indeed, 10 ng/L/h represents nano-Ag predicted environmental concentration (PEC) for superficial water (Gottschalk et al 2010) and 5 nm AgENP EC_1 for short term chronic toxicity test (synthesis of byssus, Chp), while 1000 ng/L/h represents EC10 for same test.

Two experiments were conducted:

1. the first to test the three AgENP effects across time
2. the second performing exposures in parallel with AgENPs and ionic silver ($AgNO_3$) to assess differences on the physiological impact of ionic and colloidal form

MATERIAL AND METHODS

Chemicals

The AgENPs used in this study were those with nominal diameter of 2-8nm and provided by the Polish industry "Amepox" (provided in stabilized aqueous solution, concentration 1 ppm). Ionic Ag^+ was in the form of silver nitrate and purchased from Sigma Aldrich.

Primary and secondary characterization of 5 nm Ag-ENP was carried out as described in Chapter2 and in Appendix.

Animals collection and acclimatization

Mytilus galloprovincialis were collected in the Lake of Ganzirri and Faro (Messina, eastern Sicily, 38° 15' North , 15° 36' East), transported to the laboratory under temperature/humidity controlled conditions, cleaned from epibionts and allowed to acclimate for 15 days at a temperature of 22 °C (~ 37 ‰ salinity; pH 8.0-8.1). Temperature was maintained constantly throughout acclimatization by means of a thermostatic chamber. Organisms were fed daily

fresh cultures of *Nannochloropsis spp.* or *Isochrysis galbana* using adjustable drip. Animals were divided into 50 L tanks and immersed in natural filtered seawater constantly aerated (60 L/h).

Experimental design

The first exposure experiment was carried out in July 2012. Organisms were treated for 28 days with 5 nm AgENPs. Three nominal exposure concentrations were considered, 0.2 µg/L 2.0 µg /L and 20 µg Ag/L as well as not exposed reference controls. Two mesocosms with 50 L capacity per treatment were prepared, so as to have two biological replicates. AgNPs were added daily to experimental samples from a 1 ppm stable suspension prepared in deionized water by the manufacturer. During the exposure, natural filtered seawater (1 L/animal) constantly aerated at 60 L/h was used. About 10% seawater was changed daily, then a full change was carried out at day 15 from start. Organisms were fed ad libitum with fresh algal cells of *Nannochloropsis spp* or *Isochrysis galbana.*

During the first exposure, eco-physiological parameters included: filtration rate (clearance rate, CR), respiration rate, absorption efficiency and heart beat rate (HBR). The physiological rates such as CR and HBR were measured at intervals of 24 hours, 7 days, 14 days and 28 days. Mortality was checked daily.

Mussel soft bodies (whole body, digestive gland and gills) were regularly sampled for subsequent cytochemical or molecular/biochemical analysis (Chapter 4).

For the second experiment, Ag^+ as the nitrate salt was also included along with 5 nm AgENPs from a stock solution made of 1 g Ag^+/L prepared in acidulated ultrapure water. The exposure concentrations were same of those used in the first experiment, also for Ag^+ (0.2-20 µg/L). Mussels were maintained in nanocosms for 28 days. Silver in either forms was added to each experimental group daily.

In each experiment parameters like temperature, pH and [0_2] were daily monitored and maintained at constant level (25°C and pH value of 8.0 ± 0.2)

Mortality in water

Mortality was evaluated daily throughout all the 28 days. Mussels were considered dead and excluded from the experiment when they were open and squeezing did not lead to immediate closing of shells (because of lack of abductor muscle activity).

Clearance rate (CR)

For measurement of CR, 14 mussels were transferred individually into a beaker containing 1 liter of filtered sea water. Two beakers without shellfish were used as controls in order to assess sedimentation of algae. Seawater was mixed by a magnetic stirrer. To avoid any physical disturbance, each mussel was positioned to one side of the beaker away from the stirrer bar

After an acclimatization period of approximately 10 min, fresh algae were added to an initial concentration of approximately 25000 cells/ml. After about 1 min an aliquot of 20 ml was removed from the center of the beaker. Each sample was counted (average of three measures) for the number of particles present by means of a particle counter (Coulter Counter, Z2 version). From this moment onwards, samples were taken from 20 ml every 30 min for 2 hours. The CR by individual mussels is then calculated from the linear decline in log cell concentration over time, using the following equation (Coughlan, 1969; Widdows and Staff, 2006):

$$CR\ (l\ h^{-1}) = (Vol) \times (\log_e C_1 - \log_e C_2) / \text{time interval in h}$$

where Vol is the volume of water (1L), and C_1 and C_2 are the cell concentrations at the beginning and end of each time increment. The control beaker should not show a significant change in cell concentration.

Respiration rate (RR)

Measurements of respiration rate (RR, O_2 µmol/h g) were carried out every 2 days, on 3 randomly selected specimens per treatment, using a portable dissolved oxygen meter (Hanna Instruments HI9143M). For each measurement, single mussel was placed in a respirometry chamber containing 0.5 L of 0.45 µm filtered seawater (Whatman GF/C), which was stirred by a magnetic stirrer bar. The oxygen probe was put into the respirometry chamber and after a period of 20 minutes, once bivalves started to filter, the initial value of oxygen concentration was measured and, every 10 minutes the final value of the oxygen consumption was taken, for a total of 30 minutes. The rate of oxygen consumption is then calculated as follows (Widdows and Staff, 2006).

$$\text{Rate of } O_2 \text{ uptake } (\mu\text{moles } O_2\ h^{-1}) = [C(t_0) - (C(t_1)] \cdot (Vr) \cdot 60/(t_1 - t_0)$$

where t_0, t_1 = start and finish times (min) of the measurement period; C(t) = concentration of oxygen in the water (µmoles $O_2\ l^{-1}$) at time t; Vr = volume of respirometer minus the animal

Heart beat rate (HBR)

Heart beat rate (HBR, beat/min) measurements were made by means a non invasive cardioplethysmographich technique (Sarà and De Pirro 2011; Burnett et al. 2013) at 1-7-14-28 days (n = 8 per treatment). To record the mussel heart beat, infra-red sensors were glued on the left size of the mussel shell, just below the umbone. To avoid causing excessive stress to the organism, sensors were positioned the day before the measurement. The heart beat signals obtained were amplified and filtered using a special amplifier card and then detected

by means of a portable oscilloscope (Pico Scope 236) connected to a laptop computer equipped with the PicoScope 6.0 software. During the experimental session, HBR was recorded at intervals of 10 minutes per mussel. HBR values for each animal were obtained as the average of 3 randomly selected views of the total 10 min measurements, while the HBR values for treatment were the average of 15 randomly selected specimens.

Absorption efficiency (AE)

Food absorption efficiency was measured by comparing the proportion of organic matter in the algal cells and the mussels faeces according to Conover equation (1966):

$$AE = (F-E)/[(1-E)\ F]$$

where F is a relationship between dry weight and ash free dry weight of algal food while E is a relationship between dry weight and ash free dry weight of faecal pellets.

Faecal pellets from each treatment were collected daily and placed in separate vials. Collected feaces were filtered on pre-ashed and weighed GFC filters (Whatman GF/C, 0.45 µm). After collecting mussel feaces on the filters, washing process with ammonium formate was made for each samples to remove salts. Then, samples were dried at 100° C for 48 h and the dry weights were recorded as soon as possible after cooling in a desiccators. Then samples were ashed in a furnace at 450° C for 2 h and then re-weighted to obtain the ash free dry weight of the faeces.

Biometrical, gravimetrical and mussel dry weight measurements

Length, width and weight (wet weight, dry weight and ash free dry weight) of all experimental mussels were measured for each treatment (digital calliper DIGI-Kanon; ± 0.001 mm and balance Sartorius BL 120s; ± 0.0001 gr). After completing the eco-physiological measurements, bivalve tissues and shells were removed and stored in pre-weighed aluminum foils to carry

out the dry weight measurements. Then samples were weighted and dried to constant weight at 100 °C for 24 h and. After recording the dry tissue and shell weight, samples were ashed at 450 °C for 24 h and weighted again to obtain the ash free dry weight.

Statistical analysis

An analysis of variance (ANOVA, Underwood, 1997) was performed using R software (version 2.15.1) to test the effect of nanoparticles on mussel's response variables using AgNPs concentration (CONC; fixed, 4 levels) and time (TIME; fixed, 5 levels). The assumption of homoscedasticity was tested using Cochran's C test. Post hoc comparisons were made using the Student Newman Keuls test (SNK-test).

RESULTS AND DISCUSSION

Mortality

During the 28 days of exposure were not observed significant decreases in mortality of organisms for each condition. Unfortunately, treatment with nanoparticles 0.2 µg/L was interrupted due to an incident happened to aquariums

Ecophysiological parameters

The determination of the clearance rate at 28 days of exposure show a significant effect at all concentrations. The increase in the filtration rate may indicate an adaptation of the organisms to the condition of stress, while the decrease (for $AgNO_3$ 20 µg/L) can mean a stress situation that organisms are not able to counteract (Figure 15)

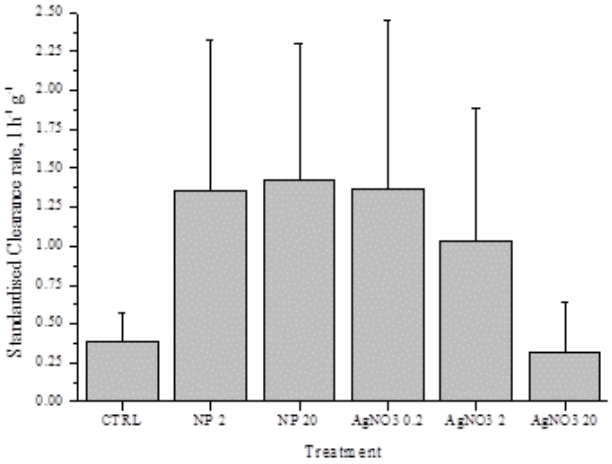

Fig. 15 – Comparison of Clearance rate for Mytilus for all the conditions at 28 days

The values of absorption efficiency show a strong effect of the various treatments at 28 days on the ability to absorb the nutrition (Figure 16). This finding is in agreement with that of CR, as an increase in the animal's pumping activity is usually coupled to a decrease in the efficiency at which a metal was absorbed across the biological membrane (Wang, 2001).

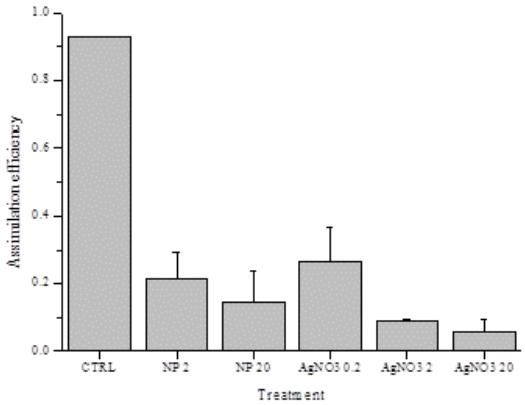

Fig. 16 – Comparison of AE for Mytilus for all the conditions at 28 days

These data allow us to calculate the Consumption Rate (C), which was assumed equivalent to the ingestion rate since no pseudofeces were observed to be produced. Ingestion of organic matter (OM) was calculated by multiplying the filtration rate (L h^{-1} g^{-1} d.w., calculated with allometric scaling for body size) by the concentration of OM in the diet (mg OM L^{-1}). The standard convention of 50% carbon content in natural particulate organic matter and 19.43 Joules of energy per milligram carbon was then used to calculate consumption rate as Joules h^{-1} g^{-1} dry tissue weight of mussel.

$$C\ (J\ h^{-1}\ g^{-1}\ DW) = FR\ (L\ h^{-1}\ g^{-1}\ DW) \times OM\ (mg\ L^{-1}) \times 0.5\ (mg\ C\ mg^{-1}\ OM) \times 18.43\ J\ mg^{-1}$$

Results showed a pattern very similar to the clearance rate, that demonstrates a metabolic commitment of organisms to cope with the treatment (Figure 17).

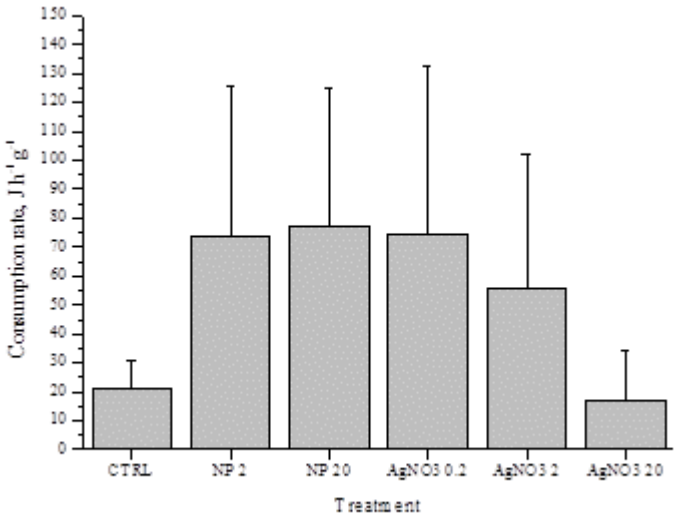

Fig. 17 – Comparison of Consumption rate for Mytilus for all the conditions at 28 days

By observing the results of the respiration rate, we can see an increase in the level of oxygen consumed in some concentrations ($AgNO_3$ 0.2 µg/L) and a decrease in the median concentration of nanoparticles (AgNP 2 µg/L). An increase of this value may signify the

presence of metabolic stress, while a lowering means a situation of metabolic depression (Figure 18).

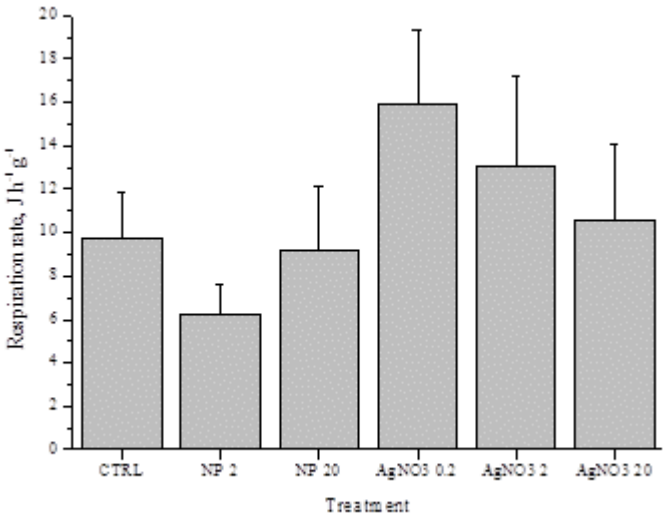

***Fig. 18** – Comparison of Respiration rate for Mytilus for all the conditions at 28 days*

Silver effects on respiration rate of mussels are not well known, but many other studies were carried out by studying the response of this parameter as a result of the presence of other types of nanoparticles. Regarding zinc nanoparticles (ZnO), for example, it has been observed that induce a significant reduction in breathing capacity in *M. galloprovincialis* bodies after 12 weeks of exposure, although the effects are much more pronounced in younger bodies and therefore smaller (Hanna et al., 2013).

By means of data obtained it was possible to calculate the scope for growth. The scope-for-growth (SFG) is a measure of an animal's actual physiological growth potential. SFG was developed as a health assessment metric for aquatic organisms, but it also represents a potentially useful way to examine how rates and fates of material processing can vary spatially

and temporally. SFG summarizes the information on various physiological rate functions and is a closer approximation of an animal's actual growth rate, closely correlating with long-term growth performance (Beiras et al. 1994). Unlike traditional growth measurement, SFG is a near instantaneous measure, thereby avoiding need to resample and measure the body size of individuals over protracted time periods (Bayne et al. 1976).

SFG is calculated from the results of component measures of feeding rate, oxygen consumption rate, ammonia excretion rate, and food absorption efficiency. SFG is best-assessed using units of energy as per Widdows and Donkin (1992). The goal is to construct a mass balance for energy usage:

$$C = P + R + E + F$$

where C = energy consumed, P = energy used for animal productivity, R = energy lost in respiratory processes, E = energy excreted in dissolved by products, and F = energy lost in defecation.

C was calculated by converting filtration rates to energy Units. Respiration was measured directly as the oxygen consumption rate, which was converted to units of energy using standard oxycaloric quotients. The excretion term (E) was calculated similarly, by relating measured ammonia excretion rates. Defecated biomass was measured and converted to the energy loss using established organic matter (OM): energy conversion factors. By difference, P was estimated as the energy available for growth and reproduction; i.e., the scope-for-growth.

$$SFG = P = C - (R + E + F)$$

It was calculated the SFG with the results obtained from the experiment. Results are shown in figure 19. A high value indicates energy available for growth and reproduction. Conversely negative values indicate that animals are using all the energy to counter the stress situation.

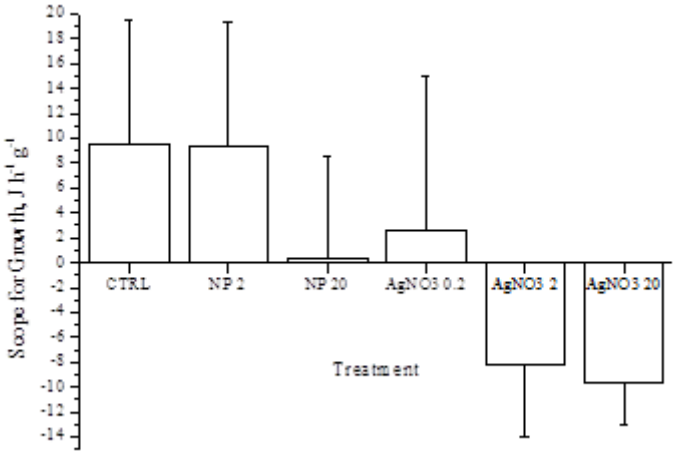

***Fig. 19** – Scope for growth for Mytilus at 28 days of exposure*

It is important to remember that the value of scope for growth indicates only a snapshot of the energy status of these animals, does not give any indication about the dynamic evolution of the health status of the population.

To date there are few studies that have examined the ecological effects of silver nanoparticles on aquatic organisms (Baun et al., 2008; Handy et al., 2008); and is always greater than the need to implement the research in this field and especially in the marine environment. Although some studies show the toxicity of metal nanoparticles on marine organisms, few of these adopt an eco-physiological approach (Ward and Kach, 2009). The aim of this work was to analyze whether there are obvious effects of these toxic compounds on some eco-physiological basic parameters such as breathing, heart activity, filtration and absorption. These results are critical to future study on the effects of nanoparticles silver and, more

generally, on the ability of energy resource management of marine organisms. (Sarà et al., 2012).

ECOPHYSIOLOGICAL EFFECTS OF AgENP IN THE ALLOCHTONOUS MUSSEL *Brachidontes pharaonis*

Like *Mytilus galloprovincialis*, *Brachidontes pharaonis* (Bivalvia, Fischer P., 1870) is an organism widely diffused in the Mediterranean Sea but, while the former is a native species of great economic interest, the second one is an invasive species that is colonizing the coasts of southern Italy.

Brachidontes is a mytilid native of the Indo-Pacific area, mainly south-eastern Asia, that it has colonised hard substrata as far away as the Red Sea, where it has established dense mussel mats (Oliver, 1992). In the last few decades this small mytilid has been able to colonize many intertidal habitats from Israel to the western Mediterranean coasts via the Suez Canal, spreading west to Sicily (Gianguzza et al., 1996). To date, in the western Mediterranean, *B. pharaonis* is confined to a few high temperature, high salinity habitats where it has established dense beds on hard substrata.

In the last ten years, *B. pharaonis* has been more frequently found in many marine intertidal habitats of the Tyrrhenian Sea (Sarà et al., 2007) where it is establishing dense beds on natural and artificial hard substrata, out competing indigenous bivalves like *Mytilaster minimus* and *Mytilus galloprovincialis* (Safriel and Sasson- frostig, 1988; Sarà et al., 2007). This may reflect that B. pharaonis is spreading throughout the western Mediterranean colonising not only small patches of con fined hyper-salinity habitats, but also wider zones of marine intertidal environments with more oceanic features.

It seemed therefore interesting to test the effects of silver nanoparticles also on this species, as it is now widespread in the Mediterranean Sea.

Brachidontes pharaonis specimens were collected in the Ettore Pond of Stagnone of Marsala (Trapani, Western Sicily, 37° 52´ North; 12° 28´ East). Once collected, mussels were brought back to the laboratory in an ice cooler, were cleaned of epibionts and kept into 12 tanks each with 5 L of filtered running seawater. Before to start experiments, organisms were acclimated in laboratory for 15 days at the same temperature (20° C), salinity (~ 36 ‰) and pH (8.2 ±0.1). During the acclimation and experimental periods, organisms were fed ad libitum with fresh algal cells of *Isochrysis galbana*.

Animals were treated to three different exposure levels, selected according to the developed kinetic model of silver persistence in seawater (Chapter 2). Nominal concentrations were 2 - 20 - 40 µg/L 5 nm AgENP, corresponding to actual doses of 100 -2000 ng/L/h. Animals were pooled in 4 experimental groups, each corresponding to one level of AgNPs, each of 90 individuals (divided into 3 tanks each of 30 animals) and exposed for 10 days during which AgNPs were added to tanks daily. During exposure organisms were fed ad libitum with fresh algae *Isochrysis galbana*. The eco-physiological rates tested during experiments were respiration rate (RR) and heart beat rate (HBR) were carried out every 2 days. Mortality was measured daily.

The test of mortality showed no significant effects until about a week of treatment, after which there is a continuous loss and dose-dependent of organisms up to values of about 40% for organisms exposed to the highest concentration (Figure 20)

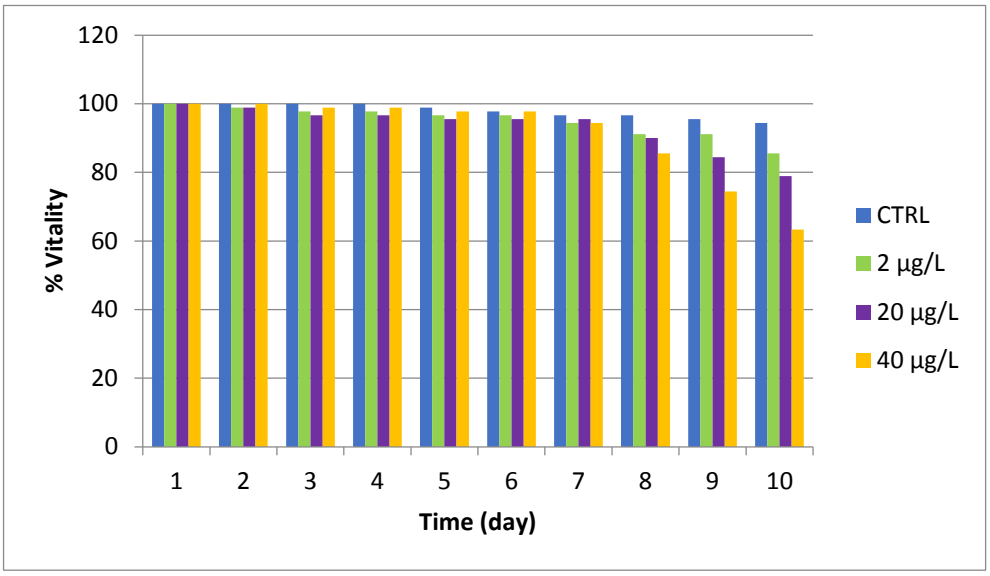

***Fig 20** - Mortality of Brachidontes pharaonis after 10 days of exposure*

Heart Beat Rate (HBR) measurement of *B. pharaonis* was significantly affected by concentration and exposure time. Significant differences were detected in organisms exposed to 20 and 40 µg/L of AgNPs, while no differences were observed under control and 2 µg/L AgNPs conditions (Figure 21). At day 8 mussels exposed to highest concentrations displayed a significant increase of the number of heart beats per minute respect to the control and to specimens exposed to 2 µg/L of AgNPs. In organisms exposed to 40 µg/L of AgNPs there was a significant increase of HBR at day 8 respect to days 2 ($P = 0.004$) and 4 ($P = 0.002$) but no significant differences with days 0 ($P = 0.07$) and 6 ($P = 0.8$). The only significant difference within mussels exposed to 20 µg/L of AgNPs rised between days 0 and 8 ($P = 0.01$). It is noteworthy to underline that heartbeat rate did not change for Mytilus spp during even a longer exposure (Figure 22).

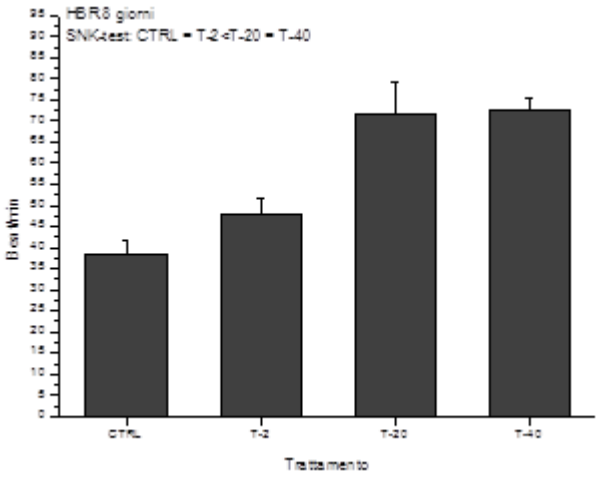

Fig 21 - Comparison of the average values of HBR of the organisms of B. pharaonis at each treatment

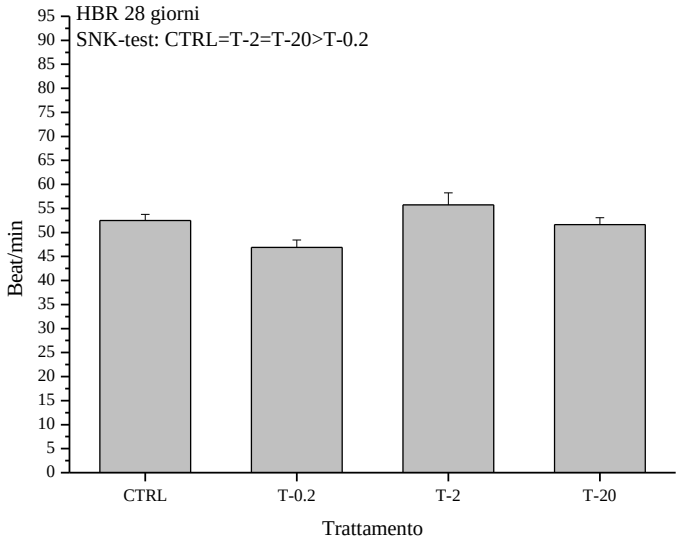

Fig 22 - Comparison of the average values of HBR of the organisms of M. galloprovincialis at each treatment

The increase in cardiac activity in organisms of *B. pharaonis* subjected to higher concentrations (20µg/L and 40 µg/L) show an effect of the AgNPs on the individual that attempts to compensate for a stress condition by increasing the number of heartbeats (Sarà and De Pirro, 2011).

The results on Respiration rate (RR) show statistically significant difference for organisms exposed to 2 and 40 µg/L AgNPs, when compared to the control (Figure 23). If we observe the pattern of each treatment as a function of time, it does not show any significant difference

during treatment with 20 and 40 µg/L, while in organisms treated with 2 µg/L AgNPs there is a significant increase in the respiration rate (188 µmol l^{-1} g^{-1}) at the end of the test phase.

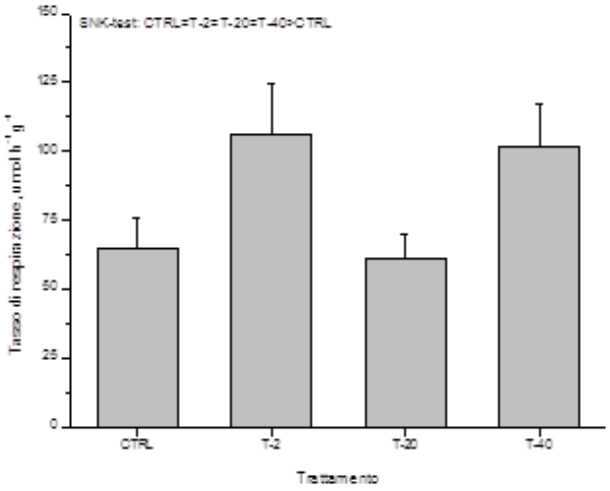

***Fig 23** - Comparison of the average values of RR of the organisms of B. pharaonis at each treatment*

It should be said that this experiment was not exhaustive as the one carried out in Mytilus spp since we considered it as a preliminary exposure for dose range finding. In fact, only a few parameters were collected and it was not possible to evaluate high order level effects, such as the scope for growth. However, there were clear differences between the two mussel. Indeed, the heart beat rate was hyper-stimulated in Brachidontes sp, while not affected at all in Mytilus spp. Furthermore, AgENP elicited acute toxicity in allochthonous mussels. It can be argued that the different responses obtained might be explained with the size of the organism that is much smaller than Mytilus spp. From previous studies in trace metal it was observed that larger mussels (Mytilus spp) are less sensitive than smaller ones, in terms of changes to physiological parameters (Richir and Gobert, 2014). In a recent research, also, Hanna et al. (2013) demonstrated that ZnO NPs acute toxicity is dependent on the specimen (Mytilus spp) size. In the same study it was demonstrated that respiration rate was consistently higher for small mussels than large mussels.

Further experimentations will be required to better define the overall impacts of AgENP on bio-energetic patterns in Brachidontes sp