CHAPTER 5 – Silver ENPs high order level effect prediction: a Dynamic Energy Budget Theory approach

INTRODUCTION

The scientific community is trying to identify and study the effects of nanomaterials in the marine environment, but the majority of these surveys are still focused on sub-lethal and or organismal responses. These parameters are very important, but do not always allow a broader indication about the ecosystem health status

Therefore, it is important to add an ecological approach in order to understand actual hazards and risks associated with long-term exposure to ENPs in the ecosystems. Only few studies have adopted this strategy (Ward and Kach, 2009; Klaine et al., 2008; Bernhardt et al. 2010; Holden et al., 2013).

As we know environmental conditions are able to influence nanoparticle behaviour in the water (e.g., salinity, pH, temperature and composition; Fabrega et al., 2011) so AgNP effects on marine organisms are not easy to investigate (Stensberg et al., 2011). Moreover, NPs toxicity is influenced by the exposure duration (Zhu et al., 2010), that may also contribute to increase the metal speciation activity and the bioavailability to bivalves (Navarro et al., 2008; Zuycov et al. 2011a, b; Guo et al., 2002; Reeder et al., 2006). In this context, functional traits such as assimilation, respiration and heart beat rate (Burnett et al. 2013) may represent an useful approach to investigate how AgNPs affect the principal metabolic processes of aquatic organisms (See Chp3).

Energy is essential for all living systems and its allocation is a dynamic process that may vary with ecological fluctuations, such as food availability, temperature, pollutants, and the

population per se. The presence of ENPs are likely to change environmental settings, which can in turn affect the physiological and physical fitness of testing organisms. Hence, the adverse effects observed in vivo should be partially attributed to the disruption of the energy budget. The energy budget model that correlates the energy essential for life cycle activities such as growth, development and reproduction with environmental variables can be a useful tool to quantify the energy uptake and allocation in biological systems in the presence of nanomaterials (Holden et al., 2013).

Dynamic Energy Budget (DEB; Kooijman, 2010) enables prediction of how energy is assimilated and assigned to the different needs for life – growth, development, and reproduction – under fluctuating environmental conditions, assuming ambient food and temperature are known. This theory is based on Kooijman's (2010) κ-rule which states that a fixed fraction κ of energy/matter mobilised from the reserve, is allocated to growth and somatic maintenance whilst the rest is devoted to maturity maintenance, maturation and reproduction (Fig. 43).

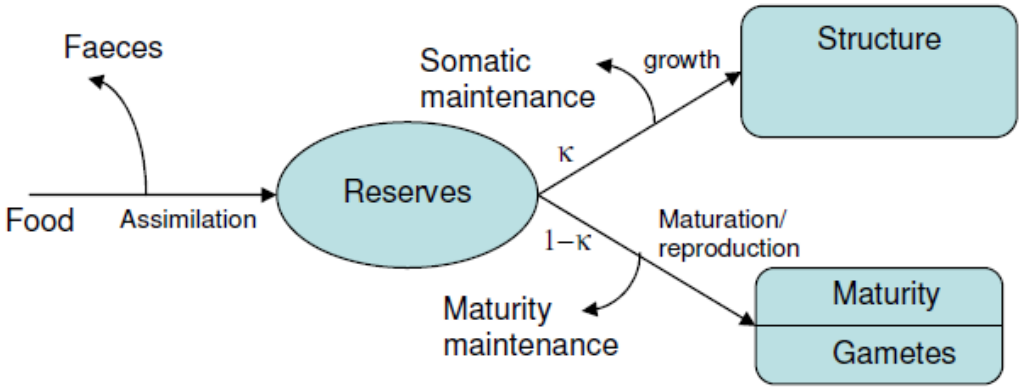

Fig 43 - Representation of the energy fluxes following the DEB approach (Kooijman, 2000)

The use of DEB model in a context of pollution research needs a number of steps as follows:

Phase I — first we need to get DEB parameters of target species and this phase (hereafter called DEB model parameters phase) includes both experimental (Sarà et al. 2013) and mathematical and modellistic procedures as the covariation method (Kooijman 2010; Lika et al. 2011);

Phase II — the second step involves a number of experiments in lab mesocosms (hereafter called experimental phase with contaminant) to estimate how functional traits (e.g. feeding, respiration, assimilation rates etc.) change under the contaminant treatment;

Phase III — once investigated at which level of the energy budget the target contaminant exerts an effect (e.g. reduction of assimilation or increase the maintenance costs; the third step (hereafter called Obtaining life history traits from DEB) involves the DEB simulation to predict the potential effect of the target contaminant on two important life history traits as body size and fecundity (Sarà G, personal communication).

RATIONAL OF DEB APPROACH AND EXPERIMENTAL DESIGN

Phase I: DEB model parameters. The standard DEB aims to capture how isomorphic organisms (i.e., organisms that don't change in shape during growth) acquire energy and matter from their surroundings and allocate it to growth, maintenance, development and reproduction (Sarà et al. in press; Sarà G. personal communication). A few pillars are needed to explain the DEB mechanistic nature (Kooijman, 2010):

1) energy and matter assimilated from food are assumed to be proportional to the organisms' surface area following the type II functional response (Holling, 1959);

2) they are directed to the reserve (e.g., fat, protein);

3) the reserve flux is mobilised according to the κ-rule (i.e., a fixed fraction (κ) is allocated to growth and somatic maintenance while the remaining 1-κ is allocated to maturity maintenance, maturation or reproduction;

4) maintenance has priority over growth and growth ceases when all reserves are required for somatic maintenance.

DEB parameters of Mytilus galloprovincialis Lam are usually considered similar to those of the congener species the blue mussel Mytilus edulis as recently done in other recent studies (e.g. Sarà et al. 2011; 2012; Saraiva et al. 2011). We know indeed that physiological rates all depend on body temperature, and in the same species all rates should change as a function of temperature in the same way. For a species-specific range of temperatures, the description proposed by S. Arrhenius (1889) usually fits well (Kooijman 2010; Sarà et al. 2013 in press). Estimates of Arrhenius temperature (TA) and of the lower and upper boundaries of the tolerance range are typically based on experiments of oxygen consumption carried out at different acclimation temperatures. While most authors extrapolate the thermal tolerance range from published data (Cardoso et al. 2006, Pouvreau et al. 2006), the most reliable approach is a direct calculation of physiological rates (e.g. feeding, excretion, heartbeat, respiration) obtained from representative samples of the population through laboratory experiments. Since the thermal optimum is species-specific and often linked to the geographical area in which the species lives (e.g. ~17−20°C for Mediterranean or 13−15°C or less for North Atlantic species), it is suggested the use of the calculated species-specific value. A typical approach depicts the entire thermal window of organismal functioning (Angilletta et al. 2010, Kearney et al. 2010). Once the optimal thermal window (e.g. 0−40°C) of the species has been established and physiological rates obtained at different steps (e.g. every 5°C step), it is possible to calculate the Arrhenius temperatures (Sarà et al. 2013; Sarà et al., submitted).

Here below, we report the list of DEB parameters adopted in this study, by means we run DEB model simulations (see Phase III).

Parameter	Unit	*M. galloprovincialis*
{J˙Xm}, Maximum surface area-specific ingestion rate	J cm^{-2} h^{-1}	8.2
[p˙M], Volume-specific maintenance cost	J cm^{-3} h^{-1}	1
[Em], Maximum storage density	J cm^3	2190
[EG], Volume-specific cost of growth	J cm^3	5993
κ, Fraction of mobilised reserve spent on soma	-	0.8
δm, Shape coefficient	-	0.2254
Vb, Structural volume at birth	cm^3	0.0000013
Vp, Structural volume at puberty	cm^3	0.06
Ae, Assimilation efficiency	-	0.75
XK, Saturation coefficient	µg chl-a l^{-1}	2.1
kR, Fraction reproductive energy fixed	-	0.8
TA, Arrhenius Temperature	°K	3243 (*)
TL, Lower boundary of tolerance range	°K	275 (*)
TH, Upper boundary of tolerance range	°K	308 (*)
TAL, Rate of decrease at lower boundary	°K	4139 (*)
TAH, Rate of decrease at upper boundary	°K	1739 (*)

***Tab. 4*-Parameters used for the DEB models (Sarà et al. 2011; 2012; Saraiva et al. 2011; Sarà et al. submitted; Montalto et al. submitted); (*) estimated in this study**

Phase II: experimental phase with contaminant. This phase involves experiments carried out in mesocosms treated with nanoparticles to estimate the effect of those contaminants on functional traits of Mytils galloprovincialis (See Chp3 for details).Briefly, we collected in July 2012 or 2013 500 specimens of Mytilus galloprovincialis Lam. (length 4-6 cm) from a local farm

(Lake Faro, Messina, Italy). Once brought back to the Laboratory of Experimental Ecology at University of Palermo, all animals were acclimatized in aquaria for 6 days using recirculating filtered natural sea water (35‰, pH 8.2) at 20°C, fed ad libitum with a monoculture of unicellular algae (Isochrysis galbana). After acclimation, mesocosms with contaminants (nanocosms) were prepared. To do it, a stable suspension of Ag ENPs (5nm, Amepox) was prepared in ultrapure water in a stock solution at 1 g/L. A stable solution of Ag^{+} as nitrate salt was also prepared in ultrapure water in a stock solution at 1 g Ag/L. Actual concentrations in stock solutions were checked and further adjusted after ICP-MS measurements. Three exposure concentrations were used including an environmentally relevant concentration of 0.2 µg/L and two higher levels (2.0 µg/L and 20 µg/L). Mussels were maintained in nanocosms for 28 days (Sarà et al., 2013). Silver in either forms was added to each experimental group daily. Under every treatment, we estimated the effect of every contaminant concentration on feeding acquisition traits as expressed by the clearance rate in mussels (Widdows and Staff 2006) and assimilation through the Conover ratio and lastly the respiration rate as a possible estimate of the effect on somatic maintenance.

Phase III. Obtaining life history traits from DEB. Once obtained all functional traits under all treatment and translated into DEB parameters of Mytilus galloprovincialis, we run DEB model using both Matlab and a MS Excel routine provides by Mike Kearney (Kearney 2012) and already successfully used in other companion papers (e.g. Sarà et al. 2012). In this paper, we run DEB models under subtidal conditions (Sarà et al. 2011; 2012; in press) with a fixed functional response (F) of 0.322 corresponding to about 1 µg l^{-1} of local food availability as expressed by chlorophyll-a and under two different constant body temperatures, 15 and 17°C. Such simulations assume as in most DEB and DEBtox simulations that body temperature was constant (fixed at 15 and 17°C) throughout the entire life span cycle of mussels (24 months).

Per every of these scenarios, we run DEB models using the combination of functional traits as estimated under different conditions of silver treatments and reported in Tab. 5 (see below). The simulation was run for 2 years, and outputs were (Sarà et al. in press) the maximum theoretical total shell length (TL, cm) reached by mussels under silver vs. CONTROL conditions at 24 months from birth and the total reproductive outputs (called Darwinian fitness; Bozinovic et al. 2011) as number of eggs produced per biomass unit per reproductive period. Model results were compared with all available data published in the current literature on maximum size (shell length) reached throughout the study area and growth rate was validated through experiments carried in lab (see above).

Clearance rates (CR, l h^{-1}) is defined as the volume of water cleared of suspended particles per hour. CR was calculated from the exponential decline in cell concentration in a closed system (beaker) over a period of 1.5 to 2 h from 16 individuals. Measurements were repeated twice to enable accounting for the variability of individual clearance response.

Food absorption efficiency was measured by comparing the proportion of organic matter in the algal cells and the mussels faeces according to the 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 faeces were filtered on pre-ashed and weighed GFC filters (Whatman GF/C, 0.45 µm). After collecting mussel faeces on the filters, washing with ammonium formate was performed for each sample to remove salts. Then, the samples were dried at 100 °C for 48 h and the dry weights were recorded as soon as possible after cooling in a desiccator. Then samples were ashed in a furnace at 450 °C for 2 h and subsequently re-weighted to obtain the ash-free dry weight of the faeces.

Measurements of *respiration rate* (RR, O2 µmol/h/g) were carried out every 2 days on randomly selected specimens per treatment using a portable dissolved oxygen meter (Hanna Instruments HI9143M). For each measurement, a 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 oxygen concentration was measured and from that moment onwards every 10 minutes the oxygen consumption was recorded, for a total of 30 minutes. Respiration rate was then calculated according to Sarà et al. (2013).

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Biometrical, gravimetrical and mussel dry weight measurements. As immediate validation of treatment's effects we measured length, width and weight (wet weight, dry weight and ash-free dry weight) of all experimental mussels for each treatment (digital calliper DIGI-Kanon; ± 0.001 mm and balance Sartorius BL 120s; ± 0.0001 gr). After completing the ecophysiological measurements, bivalve tissues and shells were removed and stored in pre-weighed aluminium foils for the dry weight measurements. The samples were weighted and

dried to constant weight at 100 °C for 24 h and. After recording the dry tissue and shell weight, the samples were ashed at 450 °C for 24 h and weighed again to obtain the ash-free dry weight. In Table 5 we report how each functional trait, experimentally estimated, was converted as the corresponding DEB parameter. While about AE and JXm the translation from simple functional trait to DEB parameters may be intuitive, less immediate is to use the experimental respiration rates estimated under each treatment to extrapolate a possible effect on somatic maintenance DEB parameters as expressed by pM. In this case, we added the energetic value due to the treatment to the standard pM value in order to obtain a possible role of the effect.

Functional parameter	**Equation**	**Parameter**	**Unit**	**Formula with experimental rate**
Respiration rate, RR	RR = [C(t0)-C(t1)]*(Vr)*60/(t1- t0)	[$\dot{p}$M], J d-1 Volume- cm-3 specific maintenance costs	J d-1 cm-3	[$\dot{p}$M]=RR *0.456J/V
Respiration rate, RR	RR = [C(t0)-C(t1)]*(Vr)*60/(t1- t0)	TA, Arrhenius K temperature	K	TA=ln|RR(T)/RR(1)|*(T1*T)/(T- T1)
Clearance rate, CR	CR = (Vol)*(lnC1-lnC2)/Δt	{$\dot{J}$Xm}, J d-1 Maximum cm-2 surface area-specific ingestion rate	J d-1 cm-2	{$\dot{J}$Xm}=CR*mgPOM*18.5J/ f V2/3
Conover ratio, AE	AE = (F - E) / [(1 - E) F]	AE, - Assimilation efficiency		AE = (µx $\dot{J}$X)/$\dot{p}$A

Tab. 5-How we translated functional traits into DEB parameters (Sarà et al. 2013; in press)

Statistical analysis. An analysis of variance (ANOVA) was performed using R software (version 2.15.1) to test the effect of nanoparticles on mussel's response variables using Ag ENP 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).

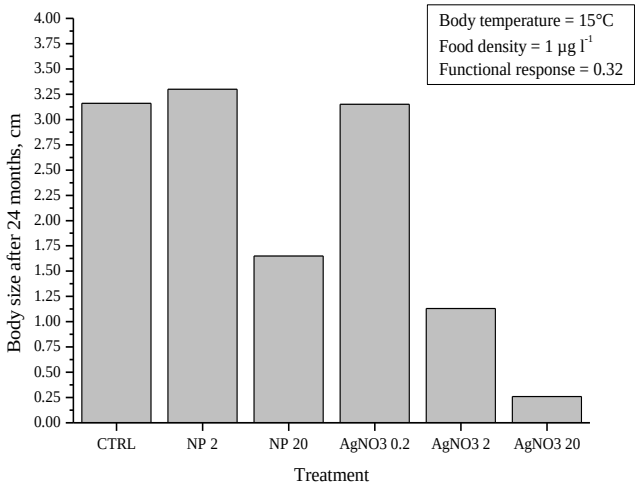
RESULTS

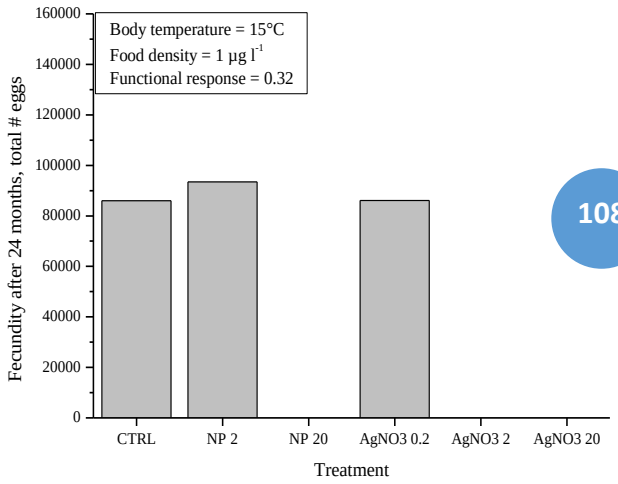
Present data (Tab. 6) indicate a clear impairment of feeding acquisition rate and the assimilation efficiency that was dose dependently affected. Also energy consumption as expressed by the respiration was significantly affected. The percent difference estimated for every parameter shows AE that was on average reduced of over 70% under contaminant treatments, clearance rates as an expression of JXm generally increased under the treatments, while pM decreased under silver-nano and increased under silver nitrate treatment.

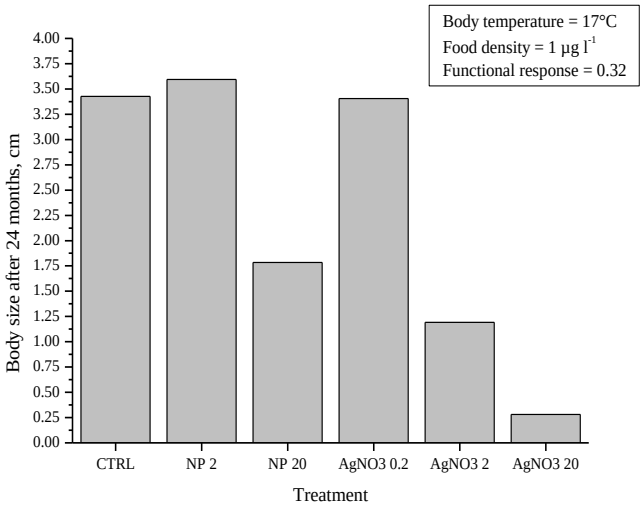
Treatment	**AE**	**% CTRL**	**Jxm (J h^{-1} cm^{-2})**	**% CTRL**	**pM**	**% CTRL**
Control (CTRL)	0.93	-	8.2	-	0.84	-
Ag ENPs 2 µg/L	0.22	-76	43.0	424	0.53	-37
Ag ENPs 20 µg/L	0.14	-85	34.3	318	0.79	-6
AgNO3 0.2 µg/L	0.27	-71	36.1	340	1.36	61
AgNO3 2 µg/L	0.09	-90	36.6	346	1.12	33
AgNO3 20 µg/L	0.06	-94	11.4	39	0.90	8

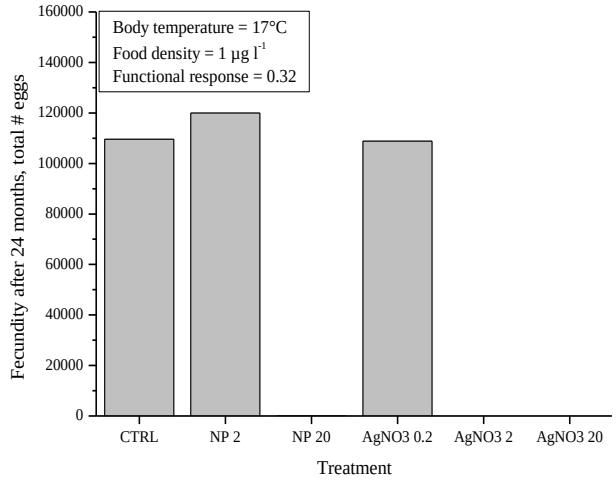
Table 6-DEB parameters for the effect of Ag ENPs and AgNO3 on the ecophysiological performance of Mytilus galloprovincialis. Shown are treatment and values for assimilation efficiency (AE), area specific maximum ingestion rate (Jxm), and volume-specific maintenance costs (pM). These parameters were derived from phase II as described above (see Table 6). We indicate also the percentage change respect to the control.

Our analysis of M. galloprovincialis data showed that ecophysiological effects on respiration, food assimilation efficiency and growth, measured during a 28-day exposure period (Table 6), would cause effects upon life-long exposure at concentrations as low as 2 and 20 µg/L for ionic Ag and Ag ENPs, respectively (see also Chapter 3). This means corresponding NOECs for life-long exposure are 0.2 and 2 µg/L, respectively.









Translating functional traits into life history effects through the DEB simulations shows that significant effects and rendered putative LOEC values for either mussel traits (growth or fecundity) of 20 µg/L and 2 µg/L, respectively for 5 nm Ag ENPs and ionic silver. Furthermore, NOEC values were calculated as 2 µg/L and 0.2 µg/L, respectively. However, it should be

pointed out that since 5 nm Ag ENPs are unstable in seawater, ENP doses appeared to be comparable with the ionic Ag ones. In fact, NOEC AgENP, 2 µg/L, in the context of the silver persistence model developed (Chapter 2), corresponded to a much lower level estimated as 0.1 µg/L/h). LOECs for AgENP, instead, corresponded to an estimated amount of 1 µg/L, therefore, in the same magnitude order of ionic silver.

The entire data set of silver tissue concentrations is not yet available for these specific experiments and therefore at this stage it is not possible to validate the DEB responses with internal silver doses measured in the mussels.