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Ciclo XXVIII

Cultivation of the marine calanoid copepod *Acartia tonsa* (Dana) as alternative feed in aquaculture: evaluation of reproductive performance (fecundity and larval survival) and gene expression in different stress conditions

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Summary

Abstract

I Copepodi sono una sottoclasse di crostacei di piccole dimensioni presenti sia nella acque marine che negli habitat dulciacquicoli. Quali costituenti del plancton, sono l'alimento principale di molti pesci e di altri organismi acquatici. Nel seguente lavoro di tesi, è stata focalizzata l'attenzione sul copepode calanoide *Acartia tonsa*, che è stato proposto come organismo modello in acquacoltura ed ecotossicologia in virtù della sua ampia distribuzione, di un breve ciclo vitale ed una elevata produttività. L'approfondimento scientifico delle conoscenze sulla fisiologia di tale specie, anche a livello genico, può fornire validi strumenti per ottimizzarne la produzione al fine di utilizazre questa specie sia in acquacoltura, come alimento base per le larve di pesci, sia come organismo modello in studi ecotossicologici. Questo lavoro è stato sviluppato seguendo tre tematiche principali: 1) allevamento del copepode *A. tonsa* in laboratorio e sperimentazione della conservazione a freddo delle uova embrionate in modo da ottenere la schiusa delle larve al momento del bisogno, da utilizzare in acquacoltura; 2) analisi dell'effetto di sostanze tossiche (nichel solubile e nanoparticelle) sulla riproduzione di *A. tonsa* quali possibili fattori di stress ambientali che potrebbero ridurre la fecondità; 3) sequenziamento del trascrittoma ed analisi dell'espressione genica di esemplari di *A. tonsa* esposti ai differenti composti tossici. L'allevamento degli stadi larvali di pesci richiede l'utilizzo di elevate quantità di prede vive per le prime fasi dell'alimentazione. Una strategia per avere quantità sufficienti di prede vive per l'utilizzo in acquacoltura può essere la conservazione a freddo. Le uova embrionate di *A. tonsa* ritardano in effetti la schiusa quando mantenute a freddo (4 °C). Le prove di conservazione a freddo delle uova (fino ad un tempo massimo di 240 giorni) e di verifica della capacità di schiusa e della sensibilità delle uova mantenute a 4 ° hanno evidenziato che la conservazione fino ad un massimo di 4 mesi permette di limitare la perdita di materiale biologico (perdite inferiori al 60%) rendendo tali uova valide per l'utilizzo in acquacoltura. Uova stoccate per 30 giorni hanno mostrato inoltre una sensibilità non differente rispetto alle uova non stoccate a freddo (controllo) aprendo quindi nuove prospettive per l'utilizzo di *A. tonsa* sia in acquacoltura che in ecotossicologia. La sperimentazione con i nano materiali è stata condotta utilizzando nanoparticelle di nichel (NiNPs) e nanoparticelle Quantum dot (QDs) e conducendo differenti tipologie di test: test acuto cronico (48h) e semicronico (5 giorni) a partire dalle uova e valutandone la percentuale di schiusa e di mortalità e test cronico (4 giorni) con adulti, valutando la produzione di uova e pellet fecali e la percentuale di schiusa delle uova. Nel complesso, i risultati suggeriscono che i primi stadi vitali (nauplii) potrebbero essere più sensibili agli effetti tossici delle nanoparticelle, mentre non risultano significativi effetti a livello di produzione di uova e schiusa, se non ad elevate concentrazioni. Per quanto a nostra conoscenza, questo è il primo studio che ha valutato la tossicità di due diverse forme di Ni e QD sulla fisiologia

riproduttiva del copepodi. Sia la fecondità che la schiusa delle uova risulta invece essere ridotta quando gli organismi sono esposti al sale del Nichel disciolto ($NiCl_2$). Il sequenziamento del trascrittoma e l'analisi di espressione genica aprono nuove prospettive per lo studio della fisiologia dei copepodi e permette di selezionare gli organismi con una buona fitness riproduttiva. Il sequenziamento comparativo tra gruppi di adulti di controllo e gruppi di adulti esposti ai differenti composti tossici ha permesso di creare una banca dati sulle variazioni di geni up e down-regolati in risposta allo stress. Tra le indagini effettuate, quelle relative alle analisi dei pathway di biosintesi lipidica hanno mostrato che non vi sono alterazioni geniche significative in seguito alla esposizione ai composti tossici testati. Ulteriori, approfondite indagini saranno effettuate per comprendere se i segnali di regolazione genica possono essere utilizzati per comprendere i meccanismi riproduttivi e fisiologici, in generale, del copepode *A. tonsa*.

Abstract

The calanoid copepod *Acartia tonsa* has been recommended as a marine organism for aquaculture and ecotoxicological tests due to its wide distribution, short life cycle and high productivity. Marine fish larvae in cultures require a large number of organisms as the first-feeding larvae. Eggs stored under cold conditions that delay hatching could ensure sufficient quantities of biological materials for aquaculture. In the current study early-spawned eggs were stored at 3 ℃ (±1) up to 240 days and their hatching success was evaluated on a monthly basis. Our results showed that the percentage of hatching success for eggs stored for 30 days was >80% and decreased by about 8% for every 20 days of storage, up to 120 days. A further increase of time in cold storage brought about a significant reduction, in statistical term, of hatching success compared with the control group (43.69 ± 22.19%). Almost 50% of eggs hatched or died during the cold storage period, with more than 80% lost after periods longer than 150 days.

Nanoparticles compounds are widely used in industries and have been massively introduced in the environment. Here we report the effect of three different chemical forms of $NiCl_2$ (as reference) and nickel nanoparticles (NiNPs) and Quantum dot nanoparticles (QDs), on the reproduction of *A. tonsa*. The behavior of NiNPs was analyzed with different techniques and with two protocols. Acute and semichronic tests, conducted exposing *A. tonsa* eggs to $NiCl_2$ concentrations from 0.025 to 0.63 mg L^{-1}, showed EC50 of 0.164 and 0.039 mg L^{-1}, respectively. Acute tests with QDs (concentration tested from 0.15 to 1.5 nM) showed an increased naupliar mortality in response to QD treatment, exhibiting an EC_{50} of 0.7 nM. Overall, *A. tonsa* is more sensitive to $NiCl_2$ than NiNPs with EC_{50} being one order of magnitude higher for NiNPs. Finally, we exposed adult copepods for chronic test (4 days) to $NiCl_2$ and NiNPs (chronic exposure) to study the effect on

fecundity in terms of daily egg production and naupliar viability. Egg production is not affected by either form of nickel, whereas egg viability is significantly reduced by 0.025 mg L^{-1} $NiCl_2$ and by 8.5 mg L^{-1} NiNPs. At NiNP concentration below the acute EC50 (17 mg L^{-1}) only 9% of eggs hatched after 4 days. Chronic test showed no negative effect on egg production, except on the last two days at the highest QD concentration (2.5 nM). No significant reduction of the percentage of egg hatching success was recorded during the exposure.

Interestingly, the percentage of naupliar mortality (>82%) observed in the semichronic test at the nominal concentration of 10 mg L^{-1} NiNPs corresponding to almost 0.10 mg L^{-1} of dissolved Ni, was similar to that recorded at the same Ni salt concentration. Electron microscopicalanalyses revealed that *A. tonsa* adults ingest NiNPs and excrete them through fecal pellets. Overall, these results suggest that species unable to swim along the water column, and early hatched copepods, could be more exposed to toxic effects of NPs which tend to aggregate and settle in seawater. To the best of our knowledge, this is the first study investigating the toxicity of two different forms of Ni and QDs on the reproductive physiology of the copepod.

Transcriptome sequencing and gene expression analysis open new perspective in studying copepod physiology and allow to select organisms with a good reproductive fitness. The comparative sequencing of *A. tonsa* between control and toxic groups has made a database about the gene variations involved with some pathways, which might potentially reflect stress response exposed to toxicants. In the Lipid biosynthesis pathway, there were no significant differentially gene regulation under slightly toxic level exposure. This conclusion was also supported by results of fecundity of *A. tonsa*, which were also not significantly effected by toxic exposure. This is the first study which investigate on the transcriptome and gene expression of *A. tonsa* copepod.

0 PREFACE

In recent decades, fish represented an important emerging protein source for human food, therefore marine fishery resources are going to be overexploited with large-scale capture systems. There are many factors that lead to dramatically boosting fish consumption, such as rapid human population growth, global urbanization, facilitated systematic shipment of fishery product and advanced fishing techniques. As the results of overfishing, biodiversity and function of marine ecosystems have been largely damaged (FAO, 2014). In order to reduce the capture stress, captive breeding of fish is suggested as effective way to maintain sustainability of fishery resources, and actually, products coming from aquaculture activities have exceeded those from fishery. From 1950 to 2012, the gap between aquaculture and capture production becomes greater (FAO, 2014). The FAO report (2014) showed that world overall production volume in aquaculture progressively increasing, at an average rate of 2.3 percent per year, from 1950 to 2012 (Fig. 0.1).

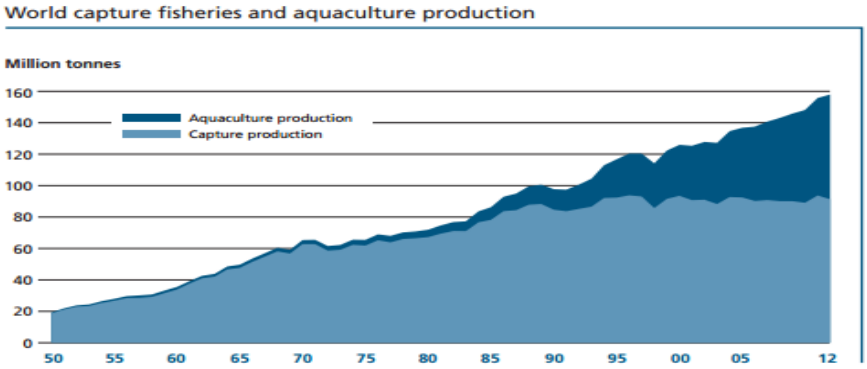


Figure 0.1 World capture fisheries and aquaculture production (FAO, 2014, modified)

Fish productions from aquaculture take up over one-quarter of all fish directly consumed by humans. As the human population continuously expanding, its reliance on aquaculture as an important protein source will also increase (Naylor et al., 2000). It is a common idea that aquaculture production will make up for the deficiency in ocean harvests as ocean fisheries decline, or that fish farming will supplement wild populations destroyed by capturing fisheries.

More than 220 species of fish and shellfish are cultured by human (FAO, 2012), and the demand for high-value species, is increasing steadily in emerging economies (FAO, 2014). However, aquaculture should pose some problems, if practiced in intensive way, such as a strong impact on the habitat; a reduction in the quality of reared fish, pollution and utilization of waste products; economic and social impacts. Therefore scientific research in these fields should be greatly improved (Idyll, 1973).

High mortality of marine fish larviculture during first feed is always a world-class problem, as the “bottle neck” to restrict the aquaculture activity. The early life stages of many emerging farm

fish species are controlled by the first feeding of young larvae. Mass mortality of marine fish larvae in cultures are linked with final yolk absorption when the larvae convert to the exogenous feeding. Low planktonic abundance and quality of appropriate prey organisms, or lack of suitable feed, is assumed to be responsible for that mortality (Hans Jörgen Fyhn, 1989). In aquaculture, formulated diets and live feed are two kinds of frequently-used feeds. Formulated diets consist of powdered feed (crude protein > 45%, lipid > 10%, crude fiber < 6%, ash < 14%, calcium < 2.5%, phosphorus > 1.0% and moisture < 13%) (Carneiro et al., 2003). Live feed is mainly composed by rotifers, brine shrimp *(Artemia)* and copepods (Conceição et al., 2010; Cheng et al., 2011; Kumar et al., 2012; Mahjoub et al., 2012; Olivotto et al., 2012). Compared with live feeds, larval fish fed with formulated diet do not growth efficiently (FAO, 1996). The biochemical composition, enzymatic content and behavioral attraction of live prey to fish larvae are the major components that rule in their favor and, in some cases, makes it impossible to substitute them (Drillet et al., 2006; Støttrup & McEvoy, 2003; Koedijk et al., 2010). Nutrition values of feeds affect on the growth, the survival, metamorphosis and pigmentation of larvae. As essential elements, highly unsaturated fatty acids (i.e. ω-3 HUFAs), are considered necessary for fish larval diet (Yone & Fuji, 1975; Næss & Lie, 1998; Sargent et al., 1999; Shields et al., 1999). Considering the response of feed in larval stage of most fish species, live feed has many advantages over formulated diets, i) live feed can swim in culture system; ii) live feed can be constantly available for larvae (Lee et al., 2010; Wu et al., 2011). Rotifers and *Artemia*, particularly easy to culture, are often used as two live preys in hatcheries even if they are originally poor of some essential nutrients required by marine fish larvae, and also need a series of enrichment (Yone & Fuji, 1975; Sargent et al., 1999;). Moreover, a comparison of fatty acid (FA) composition (highly unsaturated fatty acids (HUFA) and docosahexaenoic acid (DHA)/ Eicosapentaenoic acid (EPA)), certifies that copepods surpass rotifers and *Artemia* nutritionally, even after enrichment (Olivotto et al., 2012).

Also in terms of larval survival, copepods demonstrated superiority over rotifers and *Artemia*, as well as the formulated feeds used in hatcheries (Payne et al., 2001; Meeren et al., 2008; Wilcox et al., 2006; Olivotto et al., 2012). Growth rate and survival rate of fish larvae were found to be significantly influenced by copepods, which indicates their nutritional importance in such critical stage (Fox et al., 1999; Haslob et al., 2009; Möllmann et al., 2004). Therefore, copepods have recently been selected as potential candidates to use in aquaculture hatcheries, alternative to Artemia and rotifers, also because the size of their different larval stages (nauplii and copepodites) mapped to an high range of dimensional size, as requested by the different fish larval growth during their life cycle (Evjemo et al., 2003; Delbare et al., 1996; Olivotto et al., 2008a; Olivotto et al., 2008b). In fact, the mouth size of the first-feeding larvae are usually mechanically restricts the size

of the food particles which can be ingested. Various copepod sizes can be choosen for specific larviculture applications, favouring an efficient uptake by fish larvae at different developmental stage. Compared with rotifers and Artemia, copepod has a typical zigzag movement, then following a short gliding phase, which can make an important visual stimulus for many fish, this character letting copepod more favorable by fish larvae. Another superiority of copepods as living feeding is that copepods keep the walls of the fish larval rearing tanks clean by grazing on the algae and debris (FAO, 1996). These beneficial characteristics of copepods have attracted the attention of researchers, and studies have been conducted to improve the knowledge on massive cultivation of copepods (Conceição et al., 2010; Zhang et al., 2013).

0.1 References

Carneiro PCF, Mikos JD, Schorer M, Filho PRCO, Bendhack F (2003) Live and formulated diet evaluation through initial growth and survival of jundiá larvae, *Rhamdia quelen*. Scientia Agricola 60(4): 615-619.

Cheng SH, K â S, Kumar R, Kuo CS, Hwang JS (2011) Effects of salinity, food level, and the presence of microcrustacean zooplankters on the population dynamics of rotifer *Brachionus rotundiformis*. Hydrobiologia 666: 289-299.

Conceição LE, Y úfera M, Makridis P, Morais S, Dinis MT (2010) Live feeds for early stages of fish rearing. Aquaculture Research 41: 613-640.

Delbare D, Dhert P, Lavens P (1996) Zooplankton. Pp. 252-281 in Manual on the production and use of live food for aquaculture P. Lavens, & P. Sorgeloos, eds., FAO. Belgium, 295 pp.

Drillet G, Iversen MH, S ørensen TF, Ramløv H, Lund T, Hansen BW (2006) Effect of cold storage upon eggs of a calanoid copepod, *Acartia tonsa* (Dana) and their offspring. Aquaculture 254: 714-729.

Evjemo JO, Reitanb KI, Olsena Y (2003) Copepods as live food organisms in the larval rearing of halibut larvae (*Hippoglossus hippoglossus* L.) with special emphasis on the nutritional value. Aquaculture 227(1–4): 191-210.

FAO (1996) Manual on the production and use of live food for aquaculture. Roma.

FAO (2012) The State of World Fisheries and Aquaculture 2012. Rome. 209 pp.(also available at www.fao.org/docrep/016/i2727e/i2727e00.htm).

FAO (2014) The State of World Fisheries and Aquaculture 2014. Roma. (also available at http://www.fao.org/3/a-i3720e.pdf).

Fox CJ, Harrop R, Wimpenny A (1999) Feeding ecology of herring (Clupea harengus) larvae in the turbid Blackwater Estuary. Marine Biology 134: 353-365.

Hans Jörgen Fyhn (1989) First feeding of marine fish larvae: Are free amino acids the source of energy? Aquaculture, 80(1-2): 111-120.

Haslob H, Rohlf N, Schnack D (2009) Small scale distribution patterns and vertical migration of North Sea herring larvae (*Clupea harengus*, Teleostei: Clupeidea) in relation to abiotic and biotic factors. Sci. Mar. Scientia Marina 73: 13-22.

Idyll CP (1973) Marine Aquaculture: Problems and Prospects. Journal of the Fisheries Research Board of Canada 30(12): 2178-2183.

Koedijk RM, Folkvord A, Foss A, Pittman K, Stefansson SO, Handeland S, Imsland AK (2010) The influence of first-feeding diet on the Atlantic cod *Gadus morhua* phenotype: survival, development and long-term consequences for growth. Journal of Fish Biology 77(1): 1-19.

Kumar R, Souissi S, Hwang JS (2012) Vulnerability of carp larvae to copepod predation as a function of larval age and body length. Aquaculture 338: 274-283.

Lee CH, Dahms HU, Cheng SH, Souissi S, Schmitt FG, Kumar R, Hwang JS (2010) Predation of *Pseudodiaptomus annandalei* (Copepoda: Calanoida) by the grouper fish fry *Epinephelus coioides* under different hydrodynamic conditions. Journal of Experimental Marine Biology and Ecology 393: 17-22.

Mahjoub MS, Kumar R, Souissi S, Schmitt FG, Hwang JS (2012) Turbulence effects on the feeding dynamics in European sea bass (*Dicentrarchus labrax*) larvae. Journal of Experimental Marine Biology and Ecology 416: 61-67.

Möllmann C, Kornilovs G, Fetter M, Köster FW (2004) Feeding ecology of central Baltic Sea herring and sprat. Journal of Fish Biology 65: 1563-1581.

Naylor RL, Goldburg RJ, Primavera JH, Kautsky N, Beveridge MCM, Clay J, Folke C, Lubchenco J, Mooney H, Troell M (2000) Effect of aquaculture on world fish supplies. Nature 405:1017-1024.

Næss T, Lie (1998) A sensitive period during first feeding for the determination of pigmentation pattern in Atlantic halibut, *Hippoglossus hippoglossus* L., juveniles: the role of diet. Aquaculture Research 29(12): 925–934.

Olivotto I, Buttino I, Borroni M, Piccinetti CC, Malzone MG, Carnevali O (2008b) The use of the Mediterranean calanoid copepod Centropages typicus in Yellowtail clownfish (*Amphiprion clarkii*) larviculture. Aquaculture 284: 211-216.

Olivotto I, Capriotti F, Buttino I, Avella AM, Vitiello V, Maradonna F, Carnevali O (2008a) The use of harpacticoid copepods as live prey for *Amphiprion clarkia* larviculture: effects on larval survival and growth. Aquaculture 274: 347-352.

Olivotto I, Gaiot G, Holste L, Tulli F, Cardinaletti G, Piccinetti CC, Gioacchini G, Carnivali O (2012) Are *Acartia tonsa* cold-stored eggs a suitable food source for the marine ornamental species *Amphiprion polymnus*? A feeding study. Aquacult. Nutr 18: 685-696. DOI: 10.1111/j.1365-2095.2012.00970

Payne MF, Rippingale RJ, Cleary JJ (2001) Cultured copepods as food for West Australian dhufish (*Glaucosoma hebraicum*) and pink snapper (*Pagrus auratus*) larvae. Aquaculture 194: 137-150.

Sargent J, Bell G, McEvoy L, Tocher D, Estevez A (1999) Recent developments in the essential fatty acid nutrition of fish. Aquaculture 177(1-4): 191-199.

Shields RJ, Bell JG, Luizi FS, Gara B, Bromage NR, Sargent JR (1999) Natural Copepods Are Superior to Enriched Artemia Nauplii as Feed for Halibut Larvae (*Hippoglossus hippoglossus*) in Terms of Survival, Pigmentation and Retinal Morphology: Relation to Dietary Essential Fatty Acids. Journal of Nutrition 129(6): 1186-1194.

Støttrup JG, Mcevoy LA (2003) Live feeds in marine aquaculture, Blackwell Science Ltd., Oxford, pp.318, http://dx.doi.org/10. 1002/9780470995143

Van der Meeren T, Olsen RE, Hamre K, Fyhn HJ (2008) Biochemical composition of copepods for evaluation of feed quality in production of juvenile marine fish. Aquaculture 274: 375-397.

Wilcox JA, Tracy PL, Marcus NH (2006) Improving Live Feeds: Effect of a Mixed Diet of Copepod Nauplii (*Acartia tonsa*) and Rotifers on the Survival and Growth of First-Feeding Larvae

of the Southern Flounder, Paralichthys lethostigma. Journal of the World Aquaculture Society 37: 113-120.

Wu CH, Dahms HU, Cheng SH, Hwang JS (2011) Effects of food and light on naupliar swimming behavior of *Apocyclops royi* and *Pseudodiaptomus annandalei* (Crustacea, Copepoda). Hydrobiologia 666: 167-178.

Yone Y, Fuji M (1975) Studies on the nutrition of read sea bream-XI. Effect of ω3 fatty acid supplement in a corn oil diet on growth rate and feed efficiency. Bull Jpn Soc Fish Sci 41: 73-77.

Zhang J, Wu C, Pellegrini D, Romano G, Esposito F, Ianora A, Buttino I (2013) Effects of different monoalgal diets on egg production, hatching success and apoptosis induction in a Mediterranean population of the calanoid copepod *Acartia tonsa* (Dana). Aquaculture 400-401: 65-72.

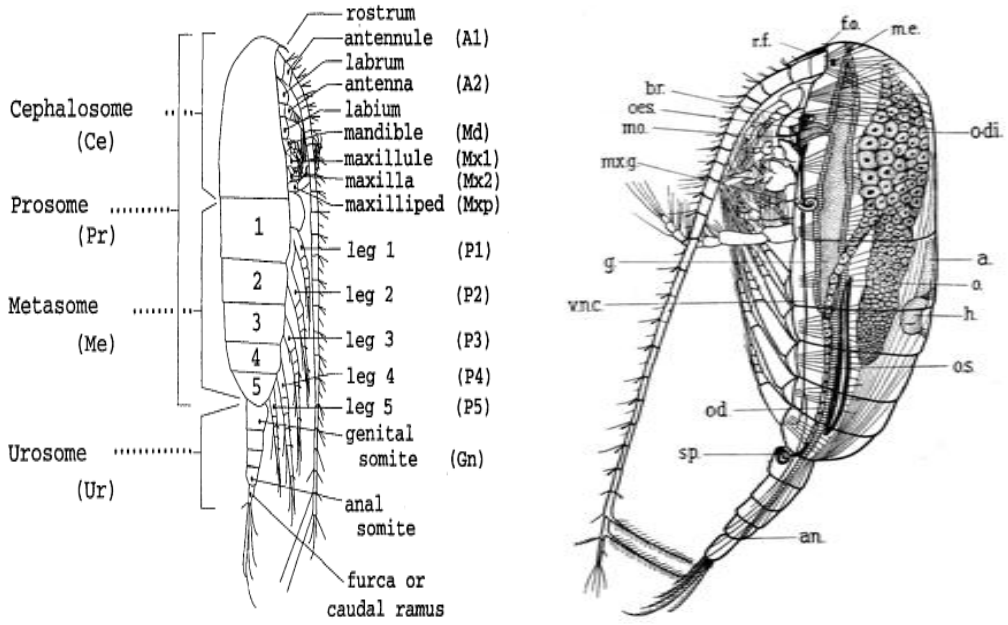
1 State of the art

1.1 Copepod anatomy and physiology

More than 80% of the zooplankton assemblages in aquatic environment are copepods (Crustacea) (Blaxter et al., 1998). There are almost 53,700 identified copepod species, mostly of them are free-living pelagic copepods(Walter & Boxshall, 2016). Copepods represent the link between the primary (phytoplankton) and the secondary producers (carnivores). Calanoida is the order of copepods that includes 40 families in both marine and freshwater copepods (Blaxter et al., 1998). In the ocean calanoids are dominant in the total plankton community, representing more than 55%–95% of plankton samples. They are therefore important in many food webs, taking in energy from phytoplankton and algae and "repackaging" it for consumption by higher trophic level predators.

Calanoid copepods′body is divided into three parts: prosome (head and sensory organs, with a pair of maxillipeds to catch algal food), metasome (with legs and swimmerets), and urosome (Fig 1.1). We usually differentiate related species by their long first antennae and biramous second antennae, as well as the presence of a joint between their fifth and sixth body segments and sexual appendages (Fig. 1.1 and 1.2). Compared with males, females have slightly larger shape, with longer and straighter antennae, nevertheless, male with curved antennae, used to grasp the female during reproduction. Males and females of calanoid can also be differentiated based on the morphology of their urosomes and swimmerets (pleopods) (Hubareva et al., 2008; Marcus & Wilcox, 2007; Blaxter et al., 1998; Thor, 2003).

Fig 1.1B shows that the short oesophagus connects mouth with gut. The labrum is in front of the mouth to form part of anterior wall and bears two rows of fine chitinous teeth as well as the eight openings of the labral glands. The oesophagus opens into the mid-gut, which is wide in the head region and has a wide diverticulum stretching forward almost to the front end of the head. At second free segment of the thorax, the gut suddenly becomes narrow, then continues backwards to the proctodaeum, to occupy little more than the last urosome segment.



A B

Figure 1.1 Schematic representation of copepod anatomy. A: external morphology and appendages of a female calanoid copepod. The metasome has five clearly defined segments, numbered 1-5; This species has five pairs of swimming legs and so these five metasome segments are synonymous with pedigerous segments 1-5. Legs 1-5 are the swimming legs (Blaxter et al., 1998, modified). B: Internal anatomy of a female *Calanus* from the side. a., aorta; an., anus; br., brain; f.o., frontal organ; g., gut; h., heart; m.e., median eye; mo., mouth; mx.g., maxillary gland; o., ovary; o.di., oviducal diverticula; od., oviduct; oes., oesophagus; o.s., oil sac; r.f., rostral filament; sp., spermathecalsac; v.n.c., ventral nerve cord (Heidelberg, 1972, modified).

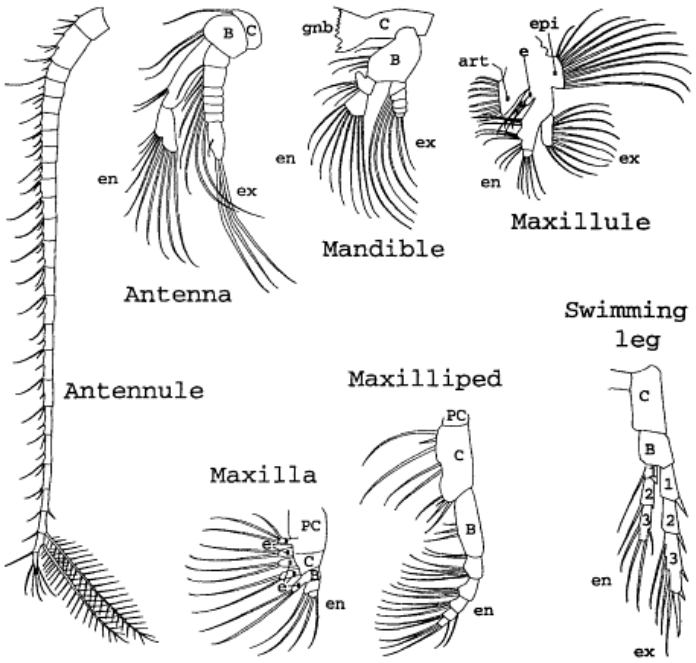


Figure 1.2 Diagrammatic representations of the appendages of a calanoid copepod. The swimming legs usually have developed endopods and expods with up to three segments, numbered 1-3 here. Terminology after Huys & Boxshall (1991). Art, arthrite; B, basis; C, coxa; e, endite; en, endopod; epi, epipodite; ex, exopod; gnb, gnathobase; PC, praecoxa. (Blaxter et al., 1998, modied).

Copepods feed on immotile and on motile prey, depending on what type of prey is available in the greatest numbers. To feed on immotile prey (diatoms), they produce a feeding current using their feeding appendages to draw in food, then filter the cells by using their second maxillae to squeeze water out. To feed on motile prey (ciliates), copepods sink in the water without moving their feeding appendages and sense prey using mechanoreceptors on their antennae (Jakobsen et al., 2005; Kiørboe et al., 1996; Blaxter et al., 1998; Roman et al., 2006; Saiz & Kiørboe, 1995; Saiz, 1994; Stoecker & Eglof, 1987).

High nutrient contents of copepods derive from phytoplankton diet, for instance, essential fatty acids (EFAs), eicosapentaenoic acid (EPA) 20: 5n-3 and docosahexaenoic acid (DHA) 22: 6n-3, being the high-quality larval stage feed of most marine organisms and fish (McKinnon et al., 2003; Norsker & Støttrup, 1994; Watanabe, 1993).

Free-swimming planktonic crustaceans, copepods can survive sudden changes in some environment conditions, such as tolerating from -1 to 32ºC (temperature) and from 1 to 40‰ salinity, but most of them live in depths from 0-50 meters,17-25 ℃ and 30-38‰ of salinity.

During the mating the male attach the spermatophores on the genital pore of the female (Fig. 1.3), the fertilized eggs are drived in the passage, finally through the genital pore to in vitro. The females can maintain the eggs in ovigerous sac attached to the body, until their hatching or can release them into the water where sink slowly (Fig.1.4). Eggs are spherical, approximately 70-90 µm in diameter, depending by the species, and hatch into nauplii within approximately 48 hours at 20-25 ℃, for species living in temperate areas. The larval phases follows VI naupliar stages, V copepodite stages similar to the adult stage, and the adult stage.

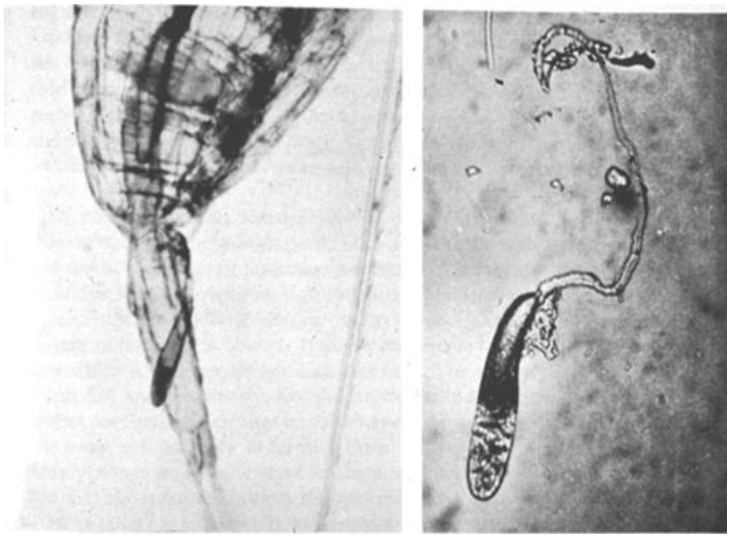


Figure 1.3 Spermatophore attached to female of *Acartia tonsa* (remating is necessary in many cases).

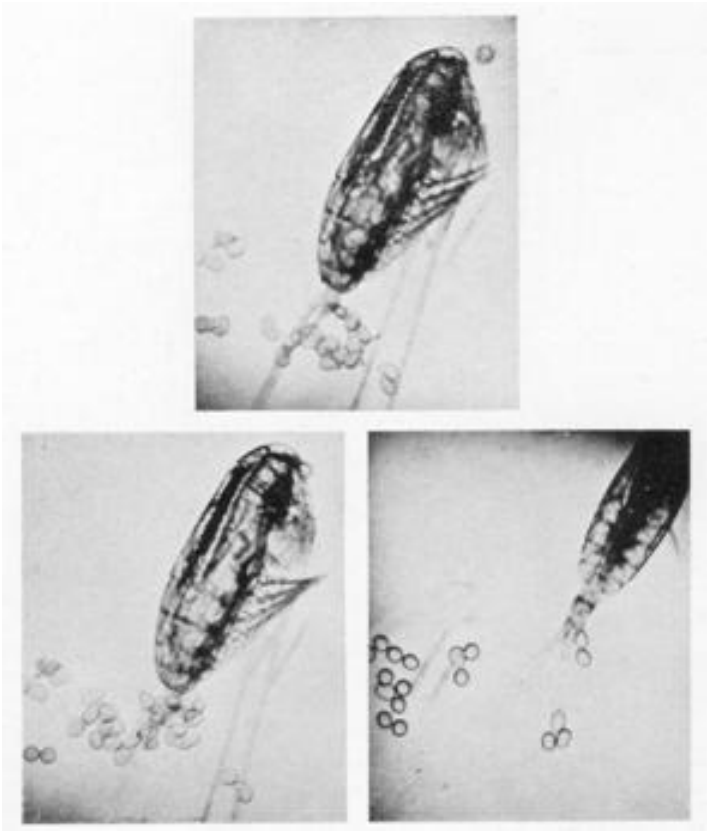


Figure 1.4 Successive stages in egg laying of female of *A. tonsa*.

In temperate areas copepods breed continuously and can release 10-80 eggs per day, therefore, multiple generations can be found in every season, depending by the species. In their life time, they spend most of time feeding or making the behaviors associated with feeding, which are affected by water turbulence and prey type. For most copepod species females usually survive longer than males, which 70-80 days versus 15 days. The longevity is often dependend by the type of available food, predation, salinity, and temperature (Danilo Calliari et al., 2008; Holste & Peck, 2005; Marcus & Wilcox, 2007; Blaxter et al., 1998; Miller & Roman, 2008; Richmond et al., 2006; Sei et al., 2006).

1.2 Research status on copepod rearing techniques

Innovative protocols for massive cultivation of copepods have been experimented by many authors with two different purposes: to use copepods in aquaculture, as first feed for larval fish (Zhang et al., 2013; Buttino et al., 2009; Camus & Zeng, 2009; McKinnon et al., 2003; Milione & Zeng, 2008; Ohs et al., 2010a; Ohs et al., 2010b; Drillet et al., 2011), and in ecotoxicological studies as a model organism to test water or sediment quality (Zhou et al., 2016a; Zhou et al., 2016b; Gorbi et al., 2012; Buttino et al., 1994). All these approaches require large amounts of adult organisms, eggs and an appropriate culture system, including the massive cultivation of monoalgal strains to be used as copepod feed. The cultivation of copepods is not easy, requiring in depth

knowledge of life traits and specific equipment with continuous, time-consuming laboratory activities. Furthermore, culturing copepods in a small-scale laboratory conditions was one important way to understand their ecology and physiology and is an effective approach to explore large-scale culture needs (Støttrup et al., 1986).

Moreover, the availability of copepods to be used for bioassays is of great concern in ecotoxicology and the possibility to obtain copepods in great amount could have several positive effects. One possibility is to collect and store eggs to be used for different purposes. Therefore, it becomes important to focus researches on the protocols to cold-storage copepod eggs and to study the effect of the low temperatures on egg viability, for aquaculture applications (Støttrup et al., 1999; Drillet et al., 2006a, 2006b, 2007, 2011a, 2011b; Peck & Holste, 2006; Peck et al., 2008; Olivotto et al., 2012).

1.3 Research status on copepod genome

The new scientific technology progress, such as the "genomic revolution" has let researchers to use more advanced methods and to explore the field of physiology and the fitness of the species in the environment. Genomic revolution can be used as sentinel method to assess the responses of organisms in different environmental conditions, and also to obtain a best traits in selected individuals. Molecular analysis could be also an effective early warning system to evaluate toxic effects on organisms in the marine environment, when no significant response in individual physiology occurs (Zhou et al., 2016b).

The hotspot of genomic is now focused on a few species of fish (Hook, 2010), and only few studies have been conducted on crustaceans even if the information on genomic DNA sequences is increasingly in many model organisms (Stillman et al., 2008). Several genome sequencers are available and enable us to obtain extensive DNA or cDNA sequence information in a short period of time (Margulies et al., 2005). Now next generation sequencing projects have been met the demands of crustaceans′ sequencing, also for copepod (Lee et al., 2010). To date, several research groups have used sequencers to obtain massive sequence information from zooplankton (Ning et al., 2013; Muñoz et al., 2014; Carotenuto et al., 2014; Kim et al., 2014; Lenz et al., 2014).

In the ecotoxicology, potential changes in some functions have frequently been attributed to changes in transcript levels, which used as an indication of potential changes by toxic genomic studies, caused by toxicants. For instance, to interpret endocrine disruption in oxidative stress responses or in immunological processes (Perez-Casanova et al., 2011; Wiseman et al., 2013). As cells have a whole lot of regulatory processes (Taylor et al., 2013), there are two kinds of main mechanisms: regulated at the level of the transcript and regulated via post-translational modification. So not all changes at the transcript level can be detected at the protein level, some need to be tested

at the differentially gene expression level (Taylor et al., 2013). In toxic risk assessment, the responses of transcriptome, as other assessment ways, just provide an indication to show the imperceptible changes in organisms, but from molecular level (Poynton & Vulpe 2009; Nikkinmaa & Rytkonen, 2011; Straalen & Feder, 2012). Actually very few studies are focused on the genome and gene expression of copepods (Ning et al., 2013; Carotenuto et al., 2014; Kim et al., 2014; Lenz et al., 2014).

1.4 The copepod *Acartia tonsa* (Dana) as a model species

The copepod species *A. tonsa* is a free-spawning calanoid species with a widespread distribution, being the dominant copepod in many subtropical and temperate coastal and estuarine areas (Blaxter et al., 1998). Originally living in the Indo-Pacific region, this species is now distributed in the Atlantic Ocean, the Sea of Azov, the Baltic, Black, Capsian, Mediterranean, and North Sea and in estuaries (Fig. 1.5). Støttrup et al. (1986) cultivated *A. tonsa* in the laboratory at the Danish Institute for Fisheries and Marine Research for over 70 generations. In laboratory experiments, adults and nauplii of the calanoid copepod *A. tonsa* feed on phytoplankton. Holste et al. (2006) reared *A. tonsa* in the laboratory using a wide ranges of temperatures and salinities, to explore egg production and hatching success. Marcus & Wilcox (2007) have successfully cultured this species in a large-scale, and in meso-scale system.

In recent decades, with the development of industry, marine pollution is getting more and more serious, making hugely influence on plankton populations. Due to its role in the marine trophic web, *A. tonsa* was proposed for toxicity tests and marine pollution studies in 1977 (Lee, 1977) and is now widely used as a model organism being one of the recommended species for the evaluation of acute lethal toxicity of marine contaminants (ISO 1999; Gorbi et al., 2012). In order to meet high requirements of suitable organisms for aquaculture and toxic test, the development of cost-effective culture systems need to be made and the collection of eggs from the continuously cultured calanoid copepod *A. tonsa* have recently been exploited (Drillet et al., 2006a, 2006b; Hansen et al., 2016).

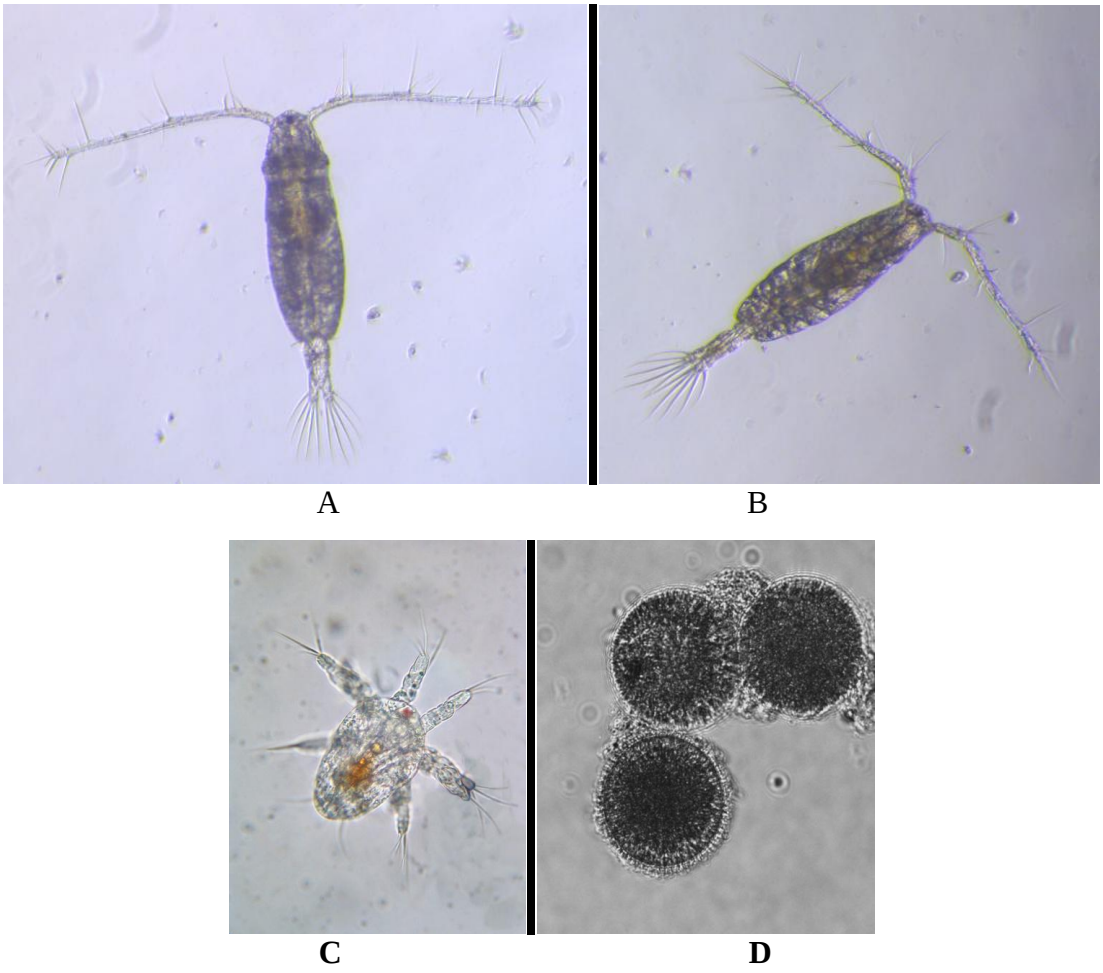


Figure 1.5 *A. tonsa*: (A) female; (B) male (x 40); (C) nauplius (x 400); (D) eggs (x 400).

1.5 References

Buttino I (1994) The effect of low concentrations of phenol and ammonia on egg production rates, fecal pellet production and egg viability of the calanoid copepod *Acartia clausi*. Marine Biology 119(4): 629-634.

Buttino I, Ianora A, Buono S, Vitello V, Sansone G, Miralto A (2009) Are monoalgal diets inferior to plurialgal diets to maximize cultivation of the calanoid copepod *Temora stylifera*?. Marine biology 156(6): 1171-1182.

Blaxter JH, Douglas B, Tyler PA, Mauchline J. The Biology of Calanoid Copepods: The Biology of Calanoid Copepods (Vol. 33). Academic Press, 1998

Camus T, Zeng C (2009) The effects of stocking density on egg production and hatching success, cannibalism rate, sex ratio and population growth of the tropical calanoid copepod *Acartia sinjiensis*. Aquaculture 287: 145-151.

Carotenuto Y, Dattolo E, Lauritano C, Pisano F, Sanges R, Miralto A, Procaccini G, Ianora A (2014) Insights into the transcriptome of the marine copepod *Calanus helgolandicus* feeling on the oxylipin-producing diatom *Skeletonema marinoi*. Harmful Algae 31: 153-162.

Danilo Calliari D, Borg M, Thor P, Gorokhova E, Tiselius P (2008) Instantaneous salinity reductions affect the survival and feeding rates of the co-occurring copepods *Acartia tonsa* Dana and *A. clausi* Giesbrecht differently. Journal of Experimental Marine Biology and Ecology 362: 18-25.

http://champs.cecs.ucf.edu/Library/Journal_Articles/pdfs/17_Calliari_et_al_Instantaneous_salinity_reductions_affect_the_survival.pdf

Drillet G, Frouël S, Sichlau MH, Jepsen PM, Højgaard JK, Joarder AK, Hansen BW (2011b) Status and recommendations on marine copepod cultivation for use as live feed. Aquaculture 315: 155-166.

Drillet G, Iversen MH, Sørensen TF, Ramløv H, Lund T, Hansen BW (2006a) Effect of cold storage upon eggs of a calanoid copepod, *Acartia tonsa* (Dana) and their offspring. Aquaculture 254:714-729.

Drillet G, Jørgensen NOG, Sørensen TF, Ramløv H, Hansen BW (2006b) Biochemical and technical observations supporting the use of copepods as relevant live feed organisms in marine larviculture. Aquac. Res. 37: 756-772.

Drillet G, Lindley LC, Michels A, Wilcox J, Marcus NH (2007) Improving cold storage of subitaneous eggs of the copepod *Acartia tonsa* Dana from the Gulf of Mexico (USA-Florida). Aquacult. Res. 38: 457-466

Drillet G, Rabarimanantsoa T, Frouël S, Lamson JS, Christensen AM, Kim-Tiam S, Hansen BW (2011a) Do microbial preparations improve life history traits of the copepod *Acartia tonsa*? Mar. Biotechnol. 13: 831-836.

Encyclopedia of Life (2013). *Acartia tonsa*. http://eol.org/pages/1020212/details

Gorbi G, Invidia M, Savorelli F, Faraponova O, Giacco E, Cigar M, Buttino I, Leoni T, Prato E, Lacchetti I, Sei S (2012) Standardized methods for acute and semichronic toxicity tests with the copepod *Acartia tonsa*. Environ Toxicol Chem 31(9): 2023-2028.

Lenz PH, Roncalli V, Hassett RP, Wu LS, Cieslak MC, Hartline DK, Christie AE (2014) De Novo Assembly of a Transcriptome for *Calanus finmarchicus* (Crustacea, Copepoda) – The Dominant Zooplankter of the North Atlantic Ocean. PLOS one 9(2): e88589.

Hansen BW, Buttino I, Cunha ME, Drillet G (2016) Embryonic cold storage capability from seven strains of *Acartia* spp. isolated in different geographical areas. Aquaculture 457:131-139.

Hubareva E, Svetlichny L, Kideys A, Isinibilir M (2008) Fate of the Black Sea *Acartia clausi* and *Acartia tonsa* (Copepoda) penetrating into the Marmara Sea through the Bosphorus. Estuarine, Coastal and Shelf Science 76(1): 131-140.

Huys R, Boxshall GA. Copepod evolution. The Ray Society, London, 1991

Holste L, Peck MA (2006) The effects of temperature and salinity on egg production and hatching success of Baltic *Acartia tonsa* (Copepoda: Calanoida): a laboratory investigation. Marine Biology 148(5):1061-1070

Hook SE (2010) Promise and progress in environmental genomics: a status report on the applications of gene expression‐based microarray studies in ecologically relevant fish species. Journal of fish biology 77(9): 1999-2022.

ISO International Organization for Standardization (1999) Water quality - Determination of acute lethal toxicity to marine copepods (Copepoda, Crustacea). Draft International Standard ISO/DIS 14669. ISO, Genève, Switzerland.

Jakobsen H, Halvorsen E, Hansen B, Visser A (2005) Effects of prey motility and concentration on feeding in *Acartia tonsa* and *Temora longicornis*: the importance of feeding modes. Journal of Plankton Research 27(8): 775-785.

Kim B, Rhee JS, Jeong CB, Seo JS, Park GS, Lee YM, Lee JS (2014) Heavy metals induce oxidative stress and trigger oxidative stress-mediated heat shock protein (hsp) modulation in the

intertidal copepod *Tigriopus japonicus*. Comp. Biochem. and Physiol., Part C 166: 65–74.

Kiørboe T, Saiz E, Viitasalo M (1996) Prey switching behaviour in the planktonic copepod *Acartia tonsa*. Marine Ecology Progress Series 143: 65-75. http://www.int-res.com/articles/meps/143/m143p065.pdf.

Lee JS, Rhee JS, Kim RO, Hwang DS, Han J, Choi BS, Park GS, Kim IC, Park HG, Lee YM (2010) The copepod Tigriopus japonicus genomic DNA information(574 Mb) and molecular anatomy. Mar. Environ Res. 69: S21–S23.

Lee W (1977) Some laboratory cultured crustaceans for marine pollution studies. Mar. Pollut. Bull. 8: 258-259.

Marchus NH, Wilcox JA (2007). Guide to the meso-scale production of the copepod *Acartia tonsa*. http://www.flseagrant.org/program_areas/aquaculture/copepod/about.htm

Marcus N, Wilcox J (2007) A Guide to the Meso-scale Production of the Copepod. *Acartia tonsa*.

Marshall SM, Orr AP. The Biology of a Marine Copepod: *Calanus finmarchicus* (Gunnerus). Springer-Verlag Berlin Heidelberg GmbH 1972, pp. 17.

Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bemben LA, ... & Dewell SB (2005) Genome sequencing in micro fabricated high-density picolitre reactors. Nature 437(7057): 376-380.

McKinnon AD, Duggan S, Nichols PD, Rimmer MA, Semmens G, Robino B (2003) The potential of tropical paracalanid copepods as live feeds in aquaculture. Aquaculture 223: 89-106.

Milione M, Zeng C (2008) The effects of temperature and salinity on population growth and egg hatching success of the tropical calanoid copepod, *Acartia sinjiensis*. Aquaculture 275: 116-123.

Miller C, Roman M (2008) Effects of food nitrogen content and concentration on the forms of nitrogen excreted by the calanoid copepod, *Acartia tonsa*. Journal of Experimental Marine Biology and Ecology 359(1): 11-17.

Ning J, Wang M, Li C, Sun S (2013) Transcriptome sequencing and de novo analysis of the copepod Calanus sinicus using 454 GS FLX. PloS one 8(5): e63741.

Nikkinmaa M, Rytkonen KT (2011). Functional genomics in aquatic toxicology-donot forget the function. Aquat. Toxicol. 105: 16–24.

Norsker NH, Støttrup JG (1994) The importance of dietary HUFAs for fecundity and HUFA content in the harpacticoid, *Tisbe holothuriae* Humes. Aquaculture 125: 155-166.

Ohs CL, Chang KL, Grabe SW, DiMaggio MA, Stenn E (2010a) Evaluation of dietary microalgae for culture of the calanoid copepod Pseudodiaptomus pelagicus. Aquaculture 307: 225-232.

Ohs CL, Rhyne, AL, Grabe SW, DiMaggio MA, Stenn E (2010b) Effects of salinity on reproduction and survival of the calanoid copepod *Pseudodiaptomus pelagicus*. Aquaculture 307: 219-224.

Olivotto I, Gaiot G, Holste L, Tulli F, Cardinaletti G, Piccinetti CC, Gioacchini G, Carnivali O (2012) Are *Acartia tonsa* cold-stored eggs a suitable food source for the marine ornamental species *Amphiprion polymnus*? A feeding study. Aquacult. Nutr. 18, 685-696.

Peck MA, Ewest B, Holste L, Kanstinger P, Martin M (2008) Impacts of light regime on egg harvests and 48-h egg hatching success of *Acartia tonsa* (Copepoda: Calanoida) within intensive culture. Aquaculture 275:102-107.

Peck MA, Holste L (2006) Effects of salinity, photoperiod and adult stocking density on egg production and egg hatching success in *Acartia tonsa* (Calanoida: Copepoda): Optimizing

intensive. Aquaculture 255: 341-350

Pérez-Casanova A, Noel Jr RJ, Rivera-Amill V, Husain K, Kumar A (2007). Morphine-mediated deterioration of oxidative stress leads to rapid disease progression in SIV/SHIV-infected macaques. AIDS research and human retroviruses 23(8): 1004-1007.

Poynton HC, Vulpe CD (2009) Ecotoxicogenomics: emerging technologies foremerging contamiantns. J. Am. Water Resour. Assoc 45: 83–96.

Richmond C, Marcus N, Sedlacek C, Miller G, Oppert C (2006) Hypoxia and seasonal temperature: Short-term effects and long-term implications for *Acartia tonsa* dana. Journal of Experimental Marine Biology and Ecology 328(2): 177-196.

Roman M, Reaugh M, Zhang X (2006) Ingestion of the dinoflagellate, *Pfiesteria piscicida*, by the calanoid copepod, *Acartia tonsa*. Harmful Algae 5(4): 435-441.

Saiz E (1994) Observations of the free-swimming behavior of *Acartia tonsa*: Effects of food concentration and turbulent water motion. Limnology and Oceanography 39(7): 1566-1578. http://www.aslo.org/lo/toc/vol_39/issue_7/1566.pdf

Saiz E, Kiørboe T (1995) Predatory and suspension feeding of the copepod *Acartia tonsa* in turbulent environments. Marine Ecology Progress Series 122: 147-158. http://www.int-res.com/articles/meps/122/m122p147.pdf

Sei S, Invidia M, Gorbi G (2006) Near anoxia and sulfide as possible factors influencing the spatial distribution of *Acartia tonsa* and *Acartia clausi:* Comparative evaluation of egg tolerance. Journal of Experimental Marine Biology and Ecology 337(2): 121-130.

Stillman JH, Colbourne JK, Lee CL, Patel NH, Philips MR, Towle DW, Eads BD, Gelembiuk GW, Henry RP, Johnson EA, Pfrender ME, Terwilliger NB, 2008. Recent advances in crustacean genomics. Integr. Comp. Biol. 48: 852-868.

Stoecker D, Eglof D (1987) Predation by *Acartia tonsa* Dana on planktonic ciliates and rotifers. Journal of Experimental Marine Biology and Ecology 110(1): 53-68.

Støttrup JG, Bell JG, Sargent JR (1999) The fate of lipids during development and cold-storage of eggs in the laboratory-reared calanoid copepod, *Acartia tonsa* Dana, and in response to different algal diets. Aquaculture 176: 257-269.

Støttrup JG, Richardson K, Kirkegaard E, Pihl NJ (1986) The cultivation of *Acartia tonsa* Dana for use as a live food source for marine fish larvae. Aquaculture 52(2): 87-96.

Taylor RC, Webb Robertson BJM, Markillie LM, Serres MH, Linggi BE, Aldrich JT, Hill EA, Romine MF, Lipton MS, Wiley HS (2013) Changes in trans-lational efficiency is a dominant regulatory mechanism in the environmental response of bacteria. Integr. Biol. 5: 1393-1406.

Thor P (2003) Elevated respiration rates of the neritic copepod *Acartia tonsa* during recovery from starvation. Journal of Experimental Marine Biology and Ecology 283(1-2): 133-143.

Valenzuela-Muñoz V, Nuñez-Acuña G, Gallardo-Escárate C (2014) Molecular characterization and transcription analysis of P-glycoprotein gene from the salmon louse *Caligus rogercresseyi*. Journal of Aquaculture Research & Development 2014.

Van Straalen NM, Feder ME (2012) Ecological and evolutionary function algenomics-how can it contribute to the risk assessment of chemicals? Environ.Sci. Technol. 46: 3-9.

Walter TC, Boxshall G (2016) World of Copepods database. http://www.marinespecies.org/copepoda on 2016-03-22

Watanab T (1993) Importance of Docosahexaenoic Acid in Marine Larval Fish. Journal of the World Aquaculture Society 24: 152-161.

Wiseman SB, Anderson JC, Liber K, Giesy JP (2013) Endocrine disruption and oxidative stress in larvae of Chironomus dilutus following short-term exposure to fresh or aged oil sands process-affected water. Aquatic toxicology 142: 414-421.

Zhang J, Wu C, Pellegrini D, Romano G, Esposito F, Ianora A, Buttino I (2011) Effects of different monoalgal diets on egg production, hatching success and apoptosis induction in a Mediterranean population of the calanoid copepod *Acartia tonsa* (Dana). Aquaculture 400-401: 65-72.

Zhou C, Vitiello V, Casals E, Puntes VF, Iamunno F, Pellegrini D, Wu CW, Benvenuto G, Buttino I (2016a). Toxicity of nickel in the marine calanoid copepod *Acartia tonsa*: Nickel chloride versus nanoparticles. Aquatic Toxicology 170: 1-12.

Zhou C, Vitiello V, Pellegrini D, Wu CW, Morelli E, Buttino I (2016b). Toxicological effects of CdSe/ZnS quantum dots on marine planktonic organisms. Ecotoxicology and environmental safety 123: 26-31.

2 Aims of the study

The aims of this thesis were focused on three topics:

TOPIC 1 Find a system on how to obtain great amount of eggs (cold storage eggs). To better understand the reproductive physiology of this copepod, *A. tonsa* species was reared in a small-scale laboratory conditions. Cultivation of this species is not easy, requiring in depth knowledge of life traits and specific equipment and continuous and time consuming laboratory activities. Here the thesis explored the possibility to store copepod eggs over the time at low temperatures. This technique should insure sufficient quantities of biological materials which can be used as alternative live feed in aquaculture, or in ecotoxicology studies. I have analyzed the reproductive success, measured as the number of eggs produced per female per day (Er), and their hatching success, measured as the number of hatched nauplii.

Furthermore, the thesis was also focused on possible stresses which can reduce *A. tonsa* productivity. In particular, the aim was to verify if an exposure to environmental stress, like toxicants, should interfere with copepod reproduction and larval viability. This is the **TOPIC 2: Possible stress affecting *A. tonsa* reproduction (toxicants).** I have tested three different toxicants: dissolved Nickel, as $NiCl_2$, Nickel nanoparticles (NiNPs) and lumidot orange quantum dots (QDs). Nickel (Ni), which is an ubiquitous element naturally present in the biosphere and in the water, is considered an essential trace component for living organisms (US EPA, 1986). Different chemical forms of Ni are introduced in the environment by industries (alloys, electroplating, batteries, coins and stainless-steels), oil and coal combustion and by refinery and incineration (Munoz & Costa, 2012; US EPA, 1986). Moreover, particulate forms (dusts, NiNPs) are generated by nanoparticle industries or by industrial processes. As a consequence, an increasing level of environmental contamination occurred in the last decade with a concomitant increasing exposure of living

organisms to this metal (Munoz & Costa, 2012). The highest dissolved Ni concentration reported for European marine waters was 3.75 $\mu g\ L^{-1}$ (Heijerick & Van Sprang, 2008), but values up to 2000 $\mu g\ L^{-1}$ were found in water near the industrial sites (Eisler, 1998). The toxicity of this metal is well documented: it induces nephrotoxicity, hepatotoxicity and teratogenesis in invertebrates and vertebrates (DeForest & Schlekat, 2013; Denkhaus & Salnikow, 2002; Haber et al., 2000; Vijayavel et al., 2009). Nickel sulfate, sulphides and oxides have been classified as human carcinogens and the elemental nickel as a possible carcinogenic metal (IARC International Agency for Research on Cancer, 1990).

Engineered nanomaterials (NM) are widely used in different industry. Recent developments in the field of nanotechnology have led to an increased interest to understand the effect of nanomaterials on the human health and on natural environment (Handy et al., 2008; Corsi et al., 2014). Nanoparticles can reach estuarine and coastal waters, and undergo physicochemical transformation towards aquatic organisms at different levels of the food chain (Matranga & Corsi, 2012). In particular, in marine environment, the high ionic strength of sea water facilitates aggregation of nanoparticles, thereby favouring settlement on the sediment or mixing with the water column, following marine movements (Klaine et al., 2008). The toxicity of nanoparticles needs to be verified to ensure the safe manufacturing and use of nanomaterials.

Nanoparticles (NPs), classified as materials with dimension between 1 anda few hundred nm (Handy et al., 2008; Handy & Shaw, 2007), are emerging as new, potentially toxic compounds, discharged in the aquatic ecosystem derived by pharmaceuticals, personal careproducts, renewable energy components and electronic devices (Fabrega et al., 2011). The majority of researches regarding NP toxicity has been conducted using freshwater filter-feeding organisms as model organisms. These animals could ingest NPs from the watercolumn and seem more sensitive than aquatic vertebrates (Handy et al., 2008; Stolpe & Hassellov, 2007). However, bioassays to test nanoparticle toxicity in seawater have not yet been defined.

Among nanoparticles, QDs are semiconductor nanocrystals, exhibiting unique photophysical properties, very attractive for biomedical applications as well as for manufactured products, including image sensors, emitting materials in LEDs and solar cells (Winnik & Maysinger, 2013). Due to their growing industrial production, increasing amounts of QDs are expected to enter the aquatic ecosystems where their effects on biological communities are poorly understood (Hardman, 2006). There is a growing body of literatures regarding the effects of QDs on aquatic organisms, such as bacteria, microalgae, invertebrates and fishes (Gagnè et al., 2008; Domingos et al., 2011; Zhang et al., 2012; Yang et al., 2012; Ambrosone et al., 2012; Leigh et al., 2012). Although it has been reported that QDs can partially degrade in aqueous environments and release free Cd, it seems

that QDs cause greater and specific toxicity than equivalent concentrations of dissolved Cd (Domingos et al., 2011; Ambrosone et al., 2012; Morelli et al., 2012). So far, few studies have investigated the effects of QDs on marine organisms, such as algae, bivalves, polychaetes and crustaceans (Jackson et al., 2012; Mouneyrac et al., 2014; Rocha et al., 2014).

Finally, this thesis focused on the **TOPIC 3: *A. tonsa* transcriptome and gene expression analyses**, to explore microarrays and qPCR assays and gene expression in adults exposed to the above mentioned contaminants. Data generated by this thesis was useful for both aquaculture and ecotoxicological studies to better understand the biology of these crustaceans and improve their productivity. This study provide insights into the response of copepods to a harmful toxicants at the transcriptome level, in order to support the hypothesis that the expore to chemicals can induce a stress response in copepods with a specific reduction of their reproductive fitness. This is the first study which investigate on the transcriptome and gene expression of *A. tonsa* copepod.

2.1 References

Ambrosone A, Mattera L, Marchesano V, Quarta A, Susha AS, Tino A, Rogach AL, Tortiglione C (2012) Mechanisms underlying toxicity induced by CdTe quantum dots determined in an invertebrate model organism. Biomaterials 33: 1991-2000.

Corsi I, Cherr GN, Lenihan HS, Labille J, Hassellov M, Canesi L, Pondero F, Frenzilli G, Hristozov D, Puntes V, Della Torre C, Pinsino A, Libralato G, Marcomini A, Sabbioni E, Matranga V (2014) Common strategies and technologies for the ecosafety assessment and design of nanomaterials entering the marine environment. ACS Nano 8: 9694-9709.

DeForest DK, Schlekat CE (2013) Species sensitivity distribution evaluation for chronic nickel toxicity to marine organisms. Integrated environmental assessment and management 9(4): 580-589.

Denkhaus E, Salnikow K (2002) Nickel essentiality, toxicity, and carcinogenicity. Critical reviews in oncology/hematology 42(1): 35-56.

Domingos RF, Simon DF, Hauser C, Wilkinson KJ (2011) Bioaccumulation and effects of CdTe/CdS quantum dots on *Chlamydomonas reinhardtii* – Nanoparticles or the free ions? Environ. Sci. Technol. 45: 7664-7669.

Eisler R (1998) Nickel hazards to fish, wildlife, and invertebrates: a synoptic review (No. USGS/BRD/BSR--1998-0001). Geological survey washington dc.

Fabrega J, Luoma SN, Tyler CR, Falloway TS, Lead JR (2011) Silvernanoparticles. behaviour and effects in the aquatic environment. Environ. Int. 37: 517-531.

Gagnè F, Auclair J, Turcotte P, Fournier M, Gagnon C, Sauvè S, Blaise C (2008) Ecotoxicity of CdTe quantum dots to freshwater mussels: impact on immune system, oxidative stress and genotoxicity. Aquatic Toxicology 86: 333-340.

Haber LT, Diamond GL, Zhao Q, Erdreich L, Dourson ML (2000) Hazard identification and dose response of ingested nickel-soluble salts. Regulatory Toxicology and Pharmacology 31(2): 231-241.

Handy RD, Shaw BJ (2007) Toxic effects of nanoparticles and nanomaterials:implications for public health, risk assessment and the public perception ofnanotechnology. Health Risk Soc 9: 125-144.

Handy RD, Owen R, Valsami-Jones E (2008) The ecotoxicology of nanoparticles and nanomaterials: current status, knowledge gaps, challenges and future needs. Ecotoxicology 17: 315-325.

Hardman R (2006) A toxicologic review of quantum dots: toxicity depends on physicochemical and environmental factors. Environmental Health Perspectives 114: 165-172.

Heijerick DH, Van Sprang PA (2008). Determination of reasonable worst case (RWC) ambient PEC concentrations for nickel in the marine environment. Final report. Prepared by EURAS for the Nickel Producers Environmental Research Association.

IARC International Agency for Research on Cancer (1990) Monographs on the evaluation of carcinogenic risks to humans. Chromium, Nickel and Welding, 49. IARC, Lyon.

Jackson BP, Bugge D, Ranville JF, Chen CY (2012) Bioavailability, toxicity, and bioaccumulation of quantum dot nanoparticles to the amphipod *Leptocheirus plumulosus*. Environ. Sci. & Technol 46: 5550-5556.

Klaine SJ, Alvarez PJJ, Batley GE, Fernandes TF, Handy RD, Lyon DY, Mahendra S, McLaughlin MJ, Lead JR (2008) Nanomaterials in the environment: behavior, fate, bioavailability, and effects, Environmental Toxicology & Chemistry 27: 1825-1851.

Leigh K, Bouldin J, Buchanan R (2012) Effects of exposure to semiconductor nanoparticles on aquatic organisms. Journal of Toxicology, Article ID 397657, pp. 9.

Muñoz A, Costa M (2012) Elucidating the mechanisms of nickel compound uptake: a review of particulate and nano-nickel endocytosis and toxicity. Toxicology and applied pharmacology 260(1): 1-16.

Morelli E, Cioni P, Posarelli M, Gabellieri E (2012) Chemical stability of CdSe quantum dots in seawater and their effects on a marine microalga. Aquatic Toxicology 122–123: 153-162.

Mouynerac C, Buffet PE, Poirier L, Zalouk-Vergnoux A, Guibbolini M, Risso-de Faverney C, Gilliland D, Berhanu D, Dybowska A, Chatel A, Perrein-Ettajni H, Pan JF, Thomas-Guyon H, Reip P, Valsami-Jones E (2014) Fate and effects of metal-based nanoparticles in two marine invertebrates, the bivalve mollusc *Scrobicularia plana* and the annelid polychaete *Hediste diversicolor*. Environ. Sci. Pollut. Res. 21: 7899-7912.

Rocha TL, Gomes T, Cardoso C, Letendre J, Pinhero JP, Serrao Sousa V, Ribau Teixeira M, Bebianno MJ (2014) Immunocytotoxicity, cytogenotoxicity and genotoxicity of cadmium based quantum dots in the marine mussel *Mytilus galloprovincialis*. Mar. Env. Res. 101: 29-37.

Stolpe B, Hassellov M (2007) Changes in size distribution of fresh waternanoscale colloidal matter and associated elements on mixing in seater.Geochim. Cosmochim. Acta 71: 3292-3301.

US Environmental Protection Ageny (1986) Test methods for the evaluation of solid wastes, SW-846. US EPA, Cincinnati.

Vijayavel K, Gopalakrishnan S, Thiagarajan R, Thilagam H (2009). Immunotoxic effects of nickel in the mud crab Scylla serrata. Fish & shellfish immunology 26(1): 133-139.

Winnik FM, Maysinger D (2013) Quantum dot cytotoxycity and ways to reduce it. Accounts of Chemical Research 46: 672-680.

Yang Y, Wang J, Zhu H, Colvin VL, Alvarez PJ (2012) Relative susceptibility and transcriptional response of nitrogen cycling bacteria to quantum dots. Environmental Science & Technology 46:

3433-3441.

Zhang W, Lin K, Miao Y, Dong Q, Huang C, Wang H, Guo M, Cui X (2012). Toxicity assessment of zebrafish following exposure to CdTe QDs. J. Haz. Mat. 213-214: 413-420.

3 TOPIC 1: Find a system on how to obtain great amount of eggs (cold storage eggs)

3.1 Materials and methods

3.1.1 Phytoplankton cultures

Two cryptophytes *Rhodomonas baltica* (Zimmermann) (FE 202) and *Rhinomonas reticulate* (FE 208 corresponding to CCAP 995/2) and the prymnesiophyte *Isochrysis galbana* (FE 207 corresponding to CCMP 1323) were used as feed for *A. tonsa* (Dana) adult copepods. These algae were cultured in a temperature-controlled room at 20 ℃, using 500 mL flasks filled with seawater filtered on 0.22 µm membrane filters (Millipore, Milan, Italy) (FSW), at the salinity of 30‰. Seawater was collected in an uncontaminated area and after filtration, was stored in the dark at 4 ℃ for further use. Filtered seawater was previously treated for 24h with HClO (0.04% v:v) and then with sodium thiosulphate 12.5% (v:v) aerated for further 24h to remove chloride residues(Lavens & Sorgeloos, 1996). All algae, were cultured at ISPRA laboratory in Leghorn, in F/2 medium without silicate (Guillard, 1975). The algae were cultured on a 14 h light: 10 h dark cycle. Stock cultures were maintained in exponential growth by inoculating cells weekly into a fresh sterilized medium.

3.1.2 Copepod culture

The copepod *A. tonsa*, originally obtained from cultures coming from the Northern Adriatic Sea (Comacchio Valley, courtesy of Gorbi G. and Sei S.), were reared through multiple generations over the last 8 years, at the ISPRA laboratory and were cultured continuously in the three PhD years (2013-2016), and also at the Zhejiang Ocean University (People's Republic of China) for gene expression and transcripton analyses.

Copepods were reared in 20 L tanks containing 0.22 µm mesh net FSW (30 mg/L salinity, pH 8.2 ± 0.1) and fed with algal cultures supplied to copepods during their exponential growth phases, at the final concentrations corresponding to 500 µg C L^{-1}: *I. galbana* (prymnesiophyceae), 3.8 × 10^4 cells mL^{-1} (~65 μm^3 volume); *R. reticulata* (cryptophyceae), 0.90 × 10^4 cells mL^{-1} (~321 μm^3 volume); *R. baltica* (Cryptophyceae), 0.4 × 10^4 cells mL^{-1} (~942 μm^3 volume). Carbon contents were converted from cell volumes according to the formula reported by Strathmann (1967). The algae cultures were made as described in Zhang et al. (2013). Adults used for the experiment were collected from the cultures using a 300 µm mesh.

Density was estimated from three replicate subsamples of 40 mL taken after a gentle homogenization of the copepod distribution in the 20 L tank using a paddle, and checked every month. Copepod cultures were maintained in a climate-controlled chamber at 20 ± 1 ℃ and at 14:

10 h light: dark photoperiod. Salinity of culture system needed to be tested everyday and water in the tanks was partially renewed every month.

The eggs (diameter of 70-80 μm) were harvested daily by siphoning the bottom of the tank, through a 55 μm mesh net filter, and were subsequently rinsed into crystallizing dish to separate the impurities (fecal pellets and algae). In this process, most of the bigger particles like feces, empty eggs, nauplii, ciliates and aggregations of algae were removed with a pipette. Collected eggs could be used for making new culture system or for later use as live prey for fish larvae.

Everyday, approximately 100 fresh eggs were harvested and cleaned, placed in a 60 ml crystallizing dish filled with 50 ml FSW (30 ‰) and sealed with glass cover, to evaluate the egg viability. The samples were kept at 20 ℃ under 14 h light: 10 h dark and egg hatching success was evaluated after 48 hours. The hatched nauplii and unhatched eggs were fixed with 5 ml 100% ethyl alcohol and counted. under a Leica stereomicroscope (model: MDG41, Italy) using the formula:

$$\%\ hs = [he / (he + nhe)] * 100$$

where *hs*, *he* and *nhe* represent the egg hatching success, the number of hatched and non-hatched eggs, respectively.

3.1.3 Copepod collection and cold storage eggs

Adult copepods were collected from the main culture by filtration through 300 μm mesh net filter, and checked and selected under the Leica stereomicroscope to select healthy and mature adults. They were incubated at a density of 1 adult/ml in 800 mL beakers containing a mixture of exponentially growing algae *R. reticulata* and *R. baltica*, at concentration > 500 μg Carbon L^{-1}. Algae were manually added once a day to the becker. After 18-24h, eggs at the bottom of the beaker were collected, through a water filtration with a 55 μm mesh net filter, gently rinsed with FSW and sorted using the Leica stereomicroscope. From 5 to 7 groups of about 250 eggs were stored in 50 mL Corning flasks with FSW at 3 ± 1 ℃ for later use. The adults were transferred to the beakers with a new medium, in order to collect eggs for the following 3 days.

As a control group, the percentage of egg hatching success was evaluated in 30-50 early-spawned eggs before their storage eggs were incubated in FSW crystallizers in a climate-controlled chamber at 20 ± 1 ℃ at 14:10 h light: dark and the percentage of egg hatching success was calculated as in the formula reported above: ***% hs = [he / (he + nhe)] * 100.***

The percentage of eggs lost (*% le*) during the cold storage period was evaluated on a monthly basis for up to 150 days and then after 240 days. Stored eggs were moved from the fridge to 20 ℃ room temperature and the total content of 50 mL was transferred to the crystallizers. After settling, the number of non-hatched eggs (*nhe*) at the bottom of crystallizers was evaluated under the inverted microscope (Olympus, Milan, Italy; x4 and x10 objectives) and the percentage of lost eggs

was calculated as follows:

$$\%\ le = 100 - (nhe * 100 / tse)$$

Where *nhe* and *tse* represent the number of non-hatched eggs and the total number of stored eggs, respectively.

The number of empty (hatched or dead) eggs was recorded at the end of cold storage (t0) and after a further 48h at 20 °C (t48) and the percentage of egg hatching success (*% hst*48) was evaluated as follows:

$$\%\ hst48 = [(eet48 - eet0) / nhet0] * 100$$

Where *eet*48 represents the number of empty eggs after 48h at 20 °C and *eet*0 and *nhet*0 represent the numbers of empty and non-hatched eggs respectively, recorded at the end of cold storage period (t0).

3.1.4 Sensitivity of cold-stored eggs to toxicants

To evaluate the suitability of cold-stored eggs in ecotoxicological tests, eggs stored for 30, 60, 90 and 120 days were moved from the fridge to 20 °C room temperature and incubated as reported in Gorbi et al. (2012) for the acute test. The sensitivity of cold storage eggs to the toxicants was evaluated with the acute test using $NiCl_2$ as a referent toxicant. Groups of about 50 eggs were randomly transferred into 10 mL crystallizers containing FSW (control eggs) or toxicant solutions, using a Leica stereomicroscope (AE2000, Motic, x4 and x10 objectives). Afterwards, 1 to 2 eggs were transferred in 24-well plates containing 2.5 mL of FSW or toxicant solutions. Plates were then placed in a climate-controlled chamber (14:10 light:dark photoperiod) at 20 ± 1 °C for 48h. Hatched eggs and naupliar viability were calculated by counting empty membranes, non-hatched eggs and immobilized/dead nauplii (endpoint) on the bottom of the wells. Three replicates for each test and treatment were performed, for a total of 48 eggs.

Nauplii were considered immobilized when they were unable to actively move vertically and/or horizontally during a 15 sec of observation period, after stimulation with a pipette tip.

For all the eggs the effect concentration inducing 50% naupliar mortality and/or immobilization (EC_{50}) was calculated using nickel chloride as a referent toxicant. Nickel chloride hexahydrate solution ($NiCl_2$) in HCl was obtained from Carlo Erba Reagents (Milan, Italy); stock solution of 1000 mg L^{-1} was diluted in bidistilled water to obtain a nominal concentration of 10 mg L^{-1}, corresponding to 10.0 ± 0.09 mg L^{-1} calculated with ICP OES 720 (Agilent Technologies, Santa Clara, CA, USA). The pH value was adjusted up to 8.0 ±0.1 using 0.1 M NaOH solution. Corresponding dilutions in FSW were performed to obtain final concentrations of 0.05, 0.10, 0.20 and 0.40 mg L^{-1}.

Analysis of variance and Student *t*-test's, used to analyse significant differences between

experimental groups, were performed using Past Program- Version 2.17. Effect concentration value (EC_{50}) was estimated using Probit Analysis - Version 1.5. Data in the graphs and tables were presented as means ± standard deviation (s.d.).

3.2 Results and discussion

3.2.1 Copepod culture

There are many challenges in building intensive cultivating systems of copepods, as the egg production is the main bottle neck in intensive cultivation of copepod. Therefore, we need to evaluate all the variables involved in the culture system, in order to maximize egg production. Commonly, *A. tonsa* has been found to produce between 11 and 50 eggs female^{-1} day^{-1} (Støttrup, 2003), in our system, egg production was the highest recorded, with a maximum of > 30 eggs female^{-1} day^{-1}, increasing 4-5 fold after the first 10 days, and the mean egg production was 24.21 ± 8.57 eggs female^{-1} day^{-1} during the entire spawning period for our Mediterranean strain. Egg production per female per day recorded for our Mediterranean *A. tonsa* population, did not reach the production rates recorded by Holste and Peck (2006) for the Baltic population (almost 50 eggs female^{-1} day^{-1}). However, this production was recorded supplying 5 times the concentration used in our experiments. Our culture system better balanced the feed and egg output. The fecal pellet production was also very high with a mean of 138 fecal pellets couple^{-1} day^{-1}.

The copepod densities in the tanks were 4.0~4.3 ± 1.5 ind/ ml, respectively, and the egg production was stabilized at 17 thousand eggs L^{-1} day^{-1} in the tanks. The maximum *A. tonsa* densities of Norway strain was up to 10.0 ± 1.5 ind/ml, but the egg production of *A. tonsa* were found to tend to increase largely with decreasing copepod density in another kind of culture system (Salveson, 2013). Our results showed that the *% hs* slowly declined during the culture period from greater than 87% to 60% after 15 days. Our results indicate that the *% hs* declined after the half month, perhaps suggesting that the diet could not keep the cultivation of *A. tonsa* in a continually highest reproductive level, for periods longer. On average, egg hatching success calculated over the whole period was 76%, still higher than that from the culture system of Salveson (2013). Therefore, our culture system is regarded as a balanced system, which can keep a high level of copepod density, suitable algae concentraions and abundant egg production. So choosing the right culture conditions will benefit the ambition of mass cultivation of calanoids for aquaculture and bioassay purposes.

3.2.2 Viability of cold storage eggs

In copepods, different types of eggs have been described: subitaneous diapauses and oligopause (delayed hatching eggs -DHE) (Grice & Marcus 1981; Chen & Marcus 1997). Subitaneous eggs hatch within hours to a few days after being spawned and, in some species, these eggs can remain in

a quiescent stage if environmental conditions are unsuitable. Quiescent eggs resume development as soon as environmental conditions are optimal once more. Diapause eggs stop their development for long period of time and this mechanism is under maternal control; they will not resume development before a specific refractory period even if conditions are optimal. The DHE have a very short refractory phase, under maternal control, part way between the subitaneous and diapauses eggs and require more time to hatch with respect to the subitaneous.

A. tonsa was found to produce both types of eggs, diapause or quiescent, which survive in poor environmental conditions (Zillioux & Gonzales, 1972; Castro-Longoria, 2001) such as at low temperatures (Toledo et al., 2005; Holmstrup et al., 2006; Drillet et al., 2008a). Delayed hatching eggs were observed in *A. tonsa* coming from the Baltic Sea (Katajisto, 2006; Drillet et al., 2008b, 2011a). Such eggs would take more than 72 h up to 1 month and exhibit a significantly slower metabolism when compared to subitaneous eggs (Hansen & Drillet, 2013). No evidence of DHE was found for the Floridian population of *A. tonsa* (Scheef & Marcus, 2010; Drillet et al., 2011a). Hansen & Drillet (2013) and Drillet et al. (2011a) found some eggs of *A. tonsa* Baltic strains required more than 48h to hatch, depending on the duration of storage.

Our results show that the percentage of eggs lost (due to mortality or hatching) occurring during the cold storage period (t0) increased with the duration of storage (Fig. 3.1, Table 3.1); during the first 30 days almost 40% (40.8 ± 11.5%) of eggs hatched and the percentage increased to above 50% for eggs stored from 60 to 120 days (54.3 ± 25.9%, 60.1 ± 32.5%, respectively), but these percentages are not statistically different. However, a significant higher percentage of hatched eggs (> 80%, $p < 0.05$) were recorded after 150 and 240 days of cold storage, compared to eggs stored for 30 days.

		Control	***Days 30***	***60***	***90***	***120***	***150***	***240***
t0	Replicates		7	15	26	9	3	6
	% Egg lost (mean ± s.d.)		40.9±11.5^{a}	54.3±25.9^{a,b}	56.4±19.6^{a,b,c}	60.1±32.5^{a,b,c,d}	82.9±1.0^{b,d}	82.5±11.5^{d}
	p values (t-test)*			0.2088	0.0551	0.1586	0.0003	4.42E-005
t48	Replicates	18	6	10	12	5	6	5
	% Egg hatching (mean ± s.d.)	87.5±4.2^{1}	82.12± 9.8^{1,2}	76.3±5.2^{2}	59.1±10.1^{3}	50.8±11.8^{3,4}	55.0±23.8^{3,4}	43.7±22.2^{3,4}
	p values (t-test)**		0.0698	1.73E-06	2.30E-11	2.00E-10	8.26E-06	4.31-E08

Table 3.1 *A. tonsa* percentage of egg hatching success after cold storage. Upper part (t0) percentage of eggs lost (hatched or died) during the cold storage period. Lower part (t48) percentage of egg hatching after 48h at 20 ℃ at the end of storage period. Values with the same apical letters or numbers were not significantly different ($p \geq 0.05$). Control = non-stored eggs; d=days. * p values

(Student t-test's) calculated comparing each treatment with eggs stored 30 days. ** p values (Student t-test's) calculated comparing each treatment with non-stored eggs (Control).

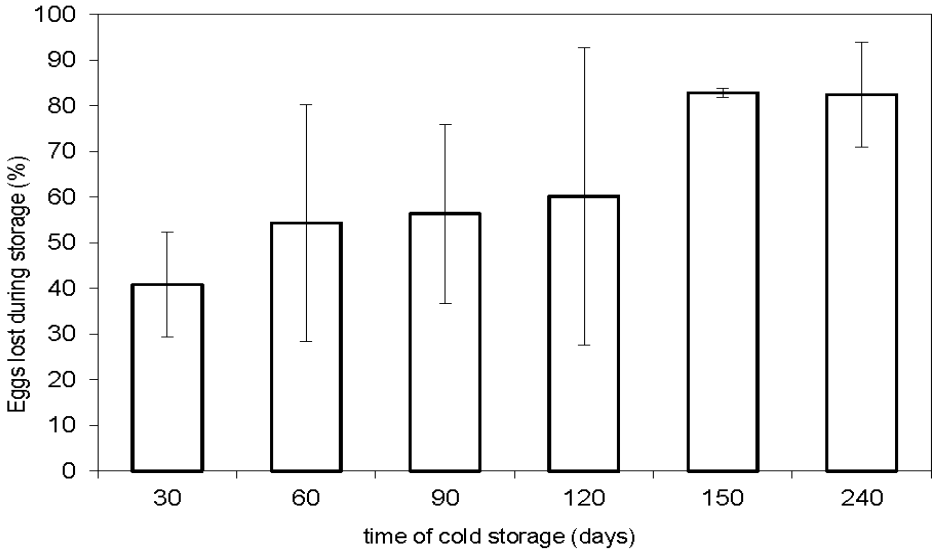


Figure 3.1 *A. tonsa* percentage of eggs lost recorded at the end of the cold storage period (t0) (mean ± s.d.).

The percentage of egg hatched recorded after 48h at 20 ℃ (t48) is reported in Fig. 3.2 and in Table 3.1. Before storage, newly spawned eggs showed a high percentage of hatching success (87.5 ± 4.24%), similar to the eggs stored for 30 days (82.12 ± 9.80%, $p = 0.0688$), whilst a significant decrease of egg hatching success occurred after 60 days of cold storage (76.33 ± 5.24%, $p < 0.001$). A further increase of cold storage time for 90, 120, 150 and 240 days, induced a statistically significant reduction of egg hatching success, compared to the control and to the other eggs stored for less than 90 days (59.08 ± 10.12%, 50.76 ± 11.77%, 54.99 ± 23.80% and 43.69 ± 22.19%, respectively) (Table 3.1).

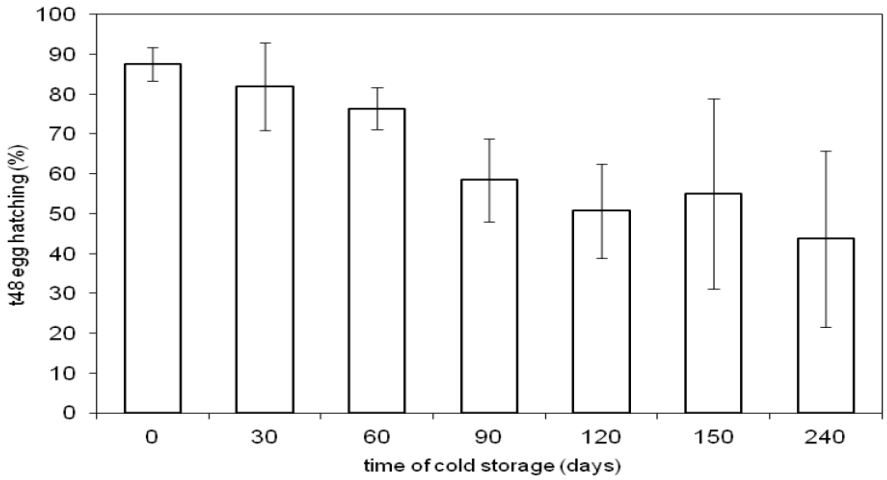


Figure 3.2 *A. tonsa* percentage of egg hatching success after 48h at 20 ℃ at the end of different periods of cold storage (t48) (mean ± s.d.).

In our study the mean percentage of hatching success recorded in non-stored eggs after 48 hours always exceeds 80% (see Control in Table 3.1), suggesting that the majority of eggs from *A. tonsa* Mediterranean strain was subitaneous. However, eggs stored from 90 to 240 days required more time to hatch (data not showed); therefore we cannot exclude that part of these eggs which need more time to hatch could be DHE. Furthermore, authors have found that cold storage does not stop the metabolism of subitaneuous *A. tonsa* eggs (Drillet et al., 2006a, 2006b; Nielsen et al., 2006). Therefore, although cold storage can delay egg development, it cannot be extended indefinitely. Also our results showed that eggs are able to hatch during the cold storage period, with a consequent reduction of materials available for future experiments. Almost 50% of eggs hatched or died during the cold storage period from 2 to 4 months, over this period more than 80% of eggs were lost at the end of storage. Therefore, the loss of eggs during the period of cold storage must be taken into account in planning future use.

Peck & Holste (2006) reported that the viability of subitaneous eggs of *A. tonsa* Denmark strain, stored at 4 ℃, decreased linearly by 4% for every 20 days of storage. Drillet et al. (2006a) found that 70% of *A. tonsa* eggs of Denmark strains, stored at 2–3 ℃, remained viable for up to 11 months, their viability dropping significantly after 12 months with no viable eggs found after 20 months. In our study the percentage of lost eggs was evaluated at the end of each cold storage period (t0) and the percentage of hatching success after 48h was calculated excluding those which hatched or died during the storage period. The viability of stored eggs from our Mediterranean strain decreased by about 8% every 20 days of storage, up to 120 days. The highest viability observed by Drillet et al. (2006a) should be due to a different storage protocol used; in fact, the eggs were incubated in anoxic conditions which probably increase cold storage tolerance of eggs by slowing their metabolism.

Recent studies revealed that there are important differences between populations of *A. tonsa* coming from different geographical areas. Genetically distinct clades have been reported within the nominal species *A. tonsa* (Caudill & Bucklin, 2004) with ecologically relevant differences (Hill, 2004; Chen & Hares, 2008; Drillet et al., 2008a). Cultures originating from subtropical American regions (Alabama and Florida strains) and from boreal Europe (Denmark and Germany strains) showed significant variability of storage tolerance (Drillet et al., 2008a). Moreover, significant differences in terms of mortality, egg production, hatching success, and biochemical composition of eggs and adults between strains were also reported (Drillet et al., 2008b). Hansen et al. (2016) compared cold storage capacity of eggs produced by five different *A. tonsa* strains isolated either in the Mediterranean and Baltic Sea (Europe) or in subtropical seas (Florida-USA) and found a longer storage capacity (> 100 days) for the European strains compared to the subtropical ones. Our

Mediterranean strain also showed storage capability similar to that recorded for the German strain with about 45% of eggs hatched after 6 months of cold-storage (Olivotto et al., 2012).

The viability of eggs would decrease after storage and might lead to a problem for using as live prey. These storage conditions should be investigated in order to further improve egg storage and thereby promoting the copepod use in aquaculture. The present study indicates that our strain of *A. tonsa* subitaneous eggs can be stored at low temperatures for up to 240 days, making them a good material as first feed of fish larvae.

3.2.3 Suitability of cold stored eggs for aquaculture and bioassays

Fukusho (1980) cultured copepods as live prey of various flatfish larvae, and recorded an excellent survival and high growth rates. Larvae of turbot showed a preference for copepod nauplii of *Brachionus plicatilis* as the first feeding, then turned to adult copepods as feeding after 14 days. As a consequence, the survival rate of the turbot larvae was much higher than other feeding, and the fry grew faster, at day 26, reaching 12 mg dry weight (17 mm TL) (Nellen et al., 1981). Therefore copepods can be used as an effective first feed in aquaculture. Developing methods to store copepod eggs is necessary to increase the availability of copepods as a live feed for aquaculture industry. Drillet et al. (2006a) found that the total quantity of fatty acids in eggs tended to decrease with the period of cold storage. These changes in biochemical composition during cold storage obviously decreased the egg viability. Based on this condition, cold stored eggs need to get further test, to verify the quality of cold stored eggs for aquaculture.

To verify the sensitivity of cold stored eggs, toxicity tests with reference chemicals have been performed. In acute exposure experiments eggs, stored for up to 120 days, and the control group were exposed for 48h to increasing concentrations of $NiCl_2$ and the EC_{50} was calculated considering naupliar immobilization or mortality as an endpoint (Table 3.2). EC_{50} calculated for eggs stored for 30, 60, 90 and 120 days range from 0.130 to 0.221 mg L^{-1} and are similar to the EC_{50} calculated for non-stored eggs (0.152 mg L^{-1}). A percentage of egg hatching success ed for non-stored eggs eggs stored for 30 and 60 days, while a decrease to 54 and 68% was observed in eggs stored for 90 and 120 days, respectively. These values are in the range of those found by Gorbi et al. (2012) in inter-calibrating experiments involving different laboratories (0.12 - 0.36 mg L^{-1}). Our results suggest that nauplii hatched from stored eggs have similar sensitivity to the pollutant, compared to the control group (nauplii hatched from non-stored eggs).

	Control	*Days 30*	*60*	*90*	*120*
EC_{50} (95% C.I.)	0.152 (0.079-0.240)	0.181 (0.150-0.216)	0.130 -	0.190 (0.132-0.254)	0.221 (0.167-0.305)

Table 3.2 *A. tonsa* acute test. Effect concentrations (mg L^{-1}) of nickel chloride inducing 50% naupliar immobilization/death (EC_{50}) after 48h of exposure. *95% C.I.* 95% confidence intervals.

In our study, naupliar survival was not affected by 120 days of cold-storage and the acute test, performed with stored eggs, showed EC_{50} for the referent toxicant in the range of that found for non-stored eggs. However, all the parameters required for the acute test by standardized protocols (Gorbi et al., 2012) are satisfied only for *A. tonsa* eggs stored for 30 days at low temperatures and therefore, we suggest to use these eggs for future use. Eggs stored longer than 30 days, which did not satisfied the required parameters, can be used to start new laboratory cultures, to improve culture density and genetic variability or for aquaculture purposes (Marcus & Murray, 2001).

3.3 Conclusions

Copepod has often been suggested that using copepods as live feeds might be a durable strategy for raising small mouthed fish species in aquaculture, which experience high larval mortalities (Drillet et al., 2006a). Copepod eggs in a cold storage situation enter arrested development that lasts viable for months. This is considered promising for future mass production of copepod for aquaculture (Drillet et al., 2006a). The present study indicates that eggs from *A. tonsa* Mediterranean strain can be stored at low temperatures for months. This strain does not produce diapauses eggs and has a limited number of delayed hatched eggs, making it a good candidate for aquaculture and ecotoxicology applications. Choosing the right culture conditions will benefit the ambition of mass cultivation of calanoids for aquaculture and bioassay. our results demonstrate that the copepod culture system can keep a high level of copepod density, suitable algae concentraions and abundant egg production, which can be effectively used for mass collection of eggs for cold storage. We concluded that the studied eggs from *A. tonsa* could all be stored for different time periods and hence are relevant for being used as egg banks and further studies for developing future live feed items. The stored eggs can be sent to other researchers or the hatched nauplii to establish new copepod culture systerm. The standardised protocol for the acute test needs batches of eggs with a high percentage of hatching and the possibility of storing and distributing *A. tonsa* eggs to other laboratories open new horizons for a more widespread use of this strain.

3.4 References

Castro-Longoria E (2001) Comparative observations on the external morphology of subitaneous and diapause eggs of *Acartia* species from Southampton water. Crustaceana 74: 225-236.

Caudill CC, Bucklin A (2004) Molecular phylogeography and evolutionary history of the estuarine copepod, *Acartia tonsa*, on the Northwest Atlantic coast. Hydrobiologia 511: 91-102.

Chen F, Marcus NH (1997) Subitaneous, diapause, and delayed-hatching eggs of planktonic copepods from the northern Gulf of Mexico: morphology and hatching success. Mar Biol 127: 587-597.

Chen G, Hare MP (2008) Cryptic ecological diversification of a planktonic estuarine copepod, *Acartia tonsa*. Mol Ecol 17(6): 1451-1468.

Drillet G, Iversen MH, Sørensen TF, Ramløv H, Lund T, Hansen BW (2006a) Effect of cold storage upon eggs of a calanoid copepod, *Acartia tonsa* (Dana) and their offspring. Aquaculture 254: 714-729.

Drillet G, Jørgensen NOG, Sørensen TF, Ramløv H, Hansen BW (2006b) Biochemical and technical observations supporting the use of copepods as relevant live feed organisms in marine larviculture. Aquac Res 37: 756-772.

Drillet G, Goetze E, Jepsen PM, 364 Højgaard JK, Hansen BW (2008a) Strain specific vital rates in four *Acartia tonsa* cultures, I: Strain origin, genetic differentiation and egg survivorship. Aquaculture 280: 109-116.

Drillet G, Jepsen PM, Højgaard JK, Jørgensen NOG, Hansen BW (2008b) Strain specific vital rates in four *Acartia tonsa* cultures II: life history traits and biochemical contents of eggs and adults. Aquaculture 279: 47-54.

Drillet G, Hansen BW, Kiørboe T, (2011a). Resting egg production induced by food limitation in the calanoid copepod *Acartia tonsa*. Limnol Oceanogr 56: 2064-2070.

Fukusho K (1980) Mass production of a copepod, *Tigriopus japonicus* in combination culture with a rotifer *Brachionus plicatilis*, fed omega-yeast as a food source. Bulletin of the Japanese Society of Scientific Fisheries 46: 625-629.

Gorbi G, Invidia M, Savorelli F, Faraponova O, Giacco E, Cigar M, Buttino I, Leoni T, Prato E, Lacchetti I, Sei S (2012) Standardized methods for acute and semichronic toxicity tests with the copepod *Acartia tonsa*. Environ Toxicol Chem 31(9): 2023-2028.

Grice GD, Marcus NH (1981) Dormant eggs of marine copepods. Oceanogr Mar Biol Annu Rev 19: 125-140.

Guillard RRL. Cultures of phytoplankton for feeding marine invertebrates. In: Culture of marine invertebrates animals. Smith W.L., Chanley M.H. Ed. New York, Plenum Press 1975, pp. 29-60.

Hansen BW, Drillet G (2013) Comparative oxygen consumption rates of subitaneous and delayed hatching eggs of the calanoid copepod *Acartia tonsa* (Dana). J Exp Mar Biol Ecol 442: 66-69.

Hansen BW, Buttino I, Cunha ME, Drillet G (2016) Embryonic cold storage capability from seven strains of *Acartia* spp. isolated in different geographical areas. Aquaculture 457: 131-139.

Hill RS (2004) Genetic diversity and structure of calanoid copepods: molecular evolutionary patterns in coastal estuaries (*Acartia tonsa*) and the open ocean (*Calanus* spp.). PhD Dissertation, University of New Hampshire, UK.

Holmstrup M, Overgaard J, Sørensen TF, Drillet G, Hansen BW, Ramløv H, Engell-Sørensen K (2006) Influence of storage conditions on viability of quiescent copepod eggs (*Acartia tonsa*

Dana): effects of temperature, salinity and anoxia. Aquac Res 37: 625-631.

Holste L, Peck MA (2006) The effects of temperature and salinity on egg production and hatching success of Baltic *Acartia tonsa* (Copepoda: Calanoida): a laboratory investigation. Marine Biology 148: 1061-1070.

Katajisto T (2006) Benthic resting eggs in the life cycles of calanoid copepods in the northern Baltic Sea. PhD Thesis, University of Helsinki, Finland.

Lavens, Patrick, Patrick Sorgeloos. Manual on the production and use of live food for aquaculture. No. 361. Food and Agriculture Organization (FAO)., 1996.

Marcus NH, Murray M (2001) Copepod diapause eggs: a potential source of nauplii for aquaculture. Aquaculture 201:107-115

Nellen W, Quantz G, Witt U, Kuhlmann D, Koske PH (1981) Marine fish rearing on the base of an artificial food chain. Eur Maric Soc Spec Publ, 6, 133-147.

Nielsen P, Mortensen J, Vismann B, Hansen BW (2006) Physiological tolerance of marine calanoid copepod eggs to sulphide. Mar Ecol-Prog Ser 435 328: 171-182.

Olivotto I, Gaiot G, Holste L, Tulli F, Cardinaletti G, Piccinetti CC, Gioacchini G, Carnivali O (2012) Are *Acartia tonsa* cold-stored eggs a suitable food source for the marine ornamental species *Amphiprion polymnus*? A feeding study. Aquacult Nutr 18: 685-696.

Peck MA, Holste L (2006) Effects of salinity, photoperiod and adult stocking density on egg production and egg hatching success in *Acartia tonsa* (Calanoida: Copepoda): Optimizing intensive. Aquaculture 255: 341-350.

Salveson E (2013) Effects of copepod density and water exchange on the egg production of *Acartia tonsa* Dana (Copepoda: Calanoida) feeding on *Rhodomonas baltica*. Norwegian University of Science and Technology.

Scheef LP, Marcus NH (2010) Occurrence and significance of copepod resting egg accumulation in seagrass sediments. Mar. Ecol. Prog. Ser. 407: 125-134.

Støttrup JG (2003) Production and nutritional value of copepods. In: Live feeds in marine aquaculture (ed. by Støttrup JG, McEvoy L). Oxford: Blackwell Science Ltd. 168-194.

Strathmann RR (1967). Estimating the organic carbon content of phytoplankton from cell volume of plasma volume. Limnol. Oceanogr. 12: 411-418.

Toledo JD, Golez M, Ohno A (2005) Studies on the use of copepods in the semi-intensive seed production of Grouper *Epinephelus coioides*. In: Lee C S, O'Bryen PJ, Marcus NH (Eds.), Copepods in Aquaculture. Blackwell publishing, Iowa.

Zhang J, Wu C, Pellegrini D, Romano G, Esposito F, Ianora A, Buttino I (2013) Effects of different monoalgal diets on egg production, hatching success and apoptosis induction in a Mediterranean population of the calanoid copepod *Acartia tonsa* (Dana). Aquaculture 400-401: 65-72.

Zillioux EJ, Gonzalez JG (1972) Egg dormancy in a neritic calanoid copepod and its implications to overwintering in boreal waters. – In: Battaglia, B. (ed.), Fifth European Marine Biology Symposium 217-230.

4 TOPIC 2: Possible stress affecting *A. tonsa* reproduction (toxicants)

4.1 Materials and methods

To verify if the exposure to toxicants affect the productivity of *A. tonsa* copepods, in terms of egg production per female, egg hatching success and naupliar viability, I tested different chemical

compounds which can find in marine ecosystem. Toxic substances tested were nickel chloride hexahydrate, nickel nanopowder (NiNP) and Lumidot orange quantum dots -QDs).

Nickel is an ubiquitous element naturally present in the biosphere and in the water (USEPA, 1986). Different chemical forms of Ni are introduced in the environment by industry process (Munoz & Costa, 2012), moreover, particulate forms are generated by nanoparticle industries, and in the last decade with a concomitant increasing exposure of living organisms to this metal (Munoz & Costa, 2012). The toxicity of Ni is well documented: it induces nephrotoxicity, hepatotoxicity and teratogenesis in invertebrates and vertebrates (DeForest & Schlekat, 2013; Denkhaus & Salnikow, 2002; Haber et al., 2000; Vijayavel et al., 2009). It is very important to evaluate the effect of NiNPs to environment.

Due to QDs growing industrial production, increasing amounts of QDs are expected to enter the aquatic ecosystems where their effects on biological communities are poorly understood (Hardman, 2006). Although QDs can partially degrade in aqueous environments and release free Cd, and make greater and specific toxicity than equivalent concentrations of dissolved Cd (Domingos et al., 2011; Ambrosone et al., 2012; Morelli et al.,2012). So far, few studies have investigated the effects of QDs on marine organisms (Jackson et al., 2012; Zhang et al., 2013; Mouneyrac et al., 2014; Rocha et al., 2014).

4.1.1 Nickel chloride

$NiCl_2*H_2O$ was obtained from Carlo Erba Reagents (Milan, Italy); stock solution of 1000 mgL^{-1} was diluted in distilled water to obtain a nominal concentration of 10 mgL^{-1}, corresponding to 10.0 ± 0.09 mg L^{-1} calculated with ICP OES 720 (Agilent Technologies, Santa Clara, CA, USA). Dilutions in FSW were made to obtain final concentrations of 0.10, 0.25, 0.40 and 0.65 mg L^{-1} for the acute test and 0.025, 0.065 and 0.10 mg L^{-1} for the semichronic test. Stock solutions were prepared immediately before use and dilutions were made in FSW.

4.1.2 Nanoparticles

NiNP was obtained from Aldrich (Milan, Italy) (Ni<100 nm diameter, purity >99%). Nickel chloride hexahydrate ($NiCl_2$ $6H_2O$) was purchased by Carlo Erba Reagents (Milan, Italy).

Due to the chemical characteristics of the nanomaterials and their dissolution properties in seawater, two different protocols were used for the acute and semichronic tests: i) in the so-called "sonicated experiment" (SON) the stock solution of 1000 mg L^{-1} NiNPs was dissolved in bidistilled water (BdW) and sonicated using a sonicator bath (Bandelin sonoplus HD2070) at room temperature for 30 min with two consecutive pulses at 100 Watt. In the "non-sonicated experiment" (NON-SON) the stock solution of 1000 mg L^{-1} . NiNP was vigorously shaken 5 min by hand before the dilutions in seawater. Final concentrations of 5, 10, 50, and 1, 5, 10 mg L^{-1} NiNPs were assayed

for the acute and semichronic tests, respectively. In the chronic test copepods were exposed to 8.5 and 17 mg L^{-1} NiNPs, preparing 850 mg L^{-1} solution in BdW, vigorously shaken 5 min by hand as for the NON-SON.

The commercial NiNPs were resuspended in ultrapure water (Millipore, 18.2 MΩ cm resistance, ion free) (BdW) to obtain a concentration of 10 mg L^{-1} and sonicated 10 minutes for a proper dispersion with a 40 kHz Branson 2510 (Dambury, CT) sonication bath at 100 Watt. The water was degassed before the ultrasound treatment by running the ultrasound for 5 min, as recommended by the manufacturer of the sonication bath. Transmission Electron Microscopy (TEM) images were acquired with a JEOL 1010 Electron Microscope operating at an accelerating voltage of 80 kV.

Non-sonicated seawater solutions (NON-SON) of 10 mg L^{-1} NiNPs, collected after 48h from the semichronic test incubation (see below) were centrifuged twice (Universal Refrigerated Centrifuge Hermle Z36HK) at 40,000 g for 30 min and resuspended in BdW for TEM analyses. Samples were prepared soon after pellet resuspension by drop casting on carbon coated copper TEM grids and dried at room temperature.

Dynamic Light Scattering (DLS) of NiNPs was performed to determine the diameter of colloidal particles. Measurements were made with a Malvern Zeta Sizer Nano ZS Instrument operating with a light source wavelength of 532 nm and a fixed scattering angle of 173 °. A volume of 0.8 mL of the colloidal seawater non-sonicated solutions, at the concentrations of 1, 5, 10 and 50 mg L^{-1} , were collected soon after the preparation (time = 0) and after 48h. This last solution was filtered through a 55 µm mesh net to remove fecal pellets produced by copepods. Samples were centrifuged twice at 40,000 x g for 30 min and the pellet fractions containing NPs were resuspended in BdW. Each analysis was conducted in triplicate.

Zeta Potential, the electrical potential of colloidal particles, was measured with a Malvern Zeta Sizer Nano ZS Instrument. A volume of 0.8 mL of NiNP non-sonicated seawater solutions at the concentrations of 5, 10 and 50 mg L^{-1} was collected soon after the preparation (t=0), after 24 and 48 h. Moreover, Z potential of the medium alone (sea water and microalgae) and of BdW containing NiNPs at the concentration of 50 mg L^{-1} were also measured soon after their preparation (t=0). Each analysis was conducted in triplicate.

Induced Coupled Plasma–Mass Spectroscopy (ICP-MS) was performed to analyze the amount of ions released after 48h in seawater. NP concentrations of 1, 5 and 10 mg L^{-1}, collected from the non-sonicated and sonicated experiments were filtered through a 55 µm mesh net to remove fecal pellets produced by copepods. Total Ni ions released were analyzed using an ICP-MS Agilent instrument (7500cx) with a detection limit of 0.02386 ppb. Aliquots of the NiNP samples were dissolved in concentrated aqua regia, heated to ensure complete dissolution of all the metals and

diluted for ICP-MS analyses. Gallium was used as the internal standard and the integration time/point and time/mass were 0.1 sec and 0.3 sec, respectively with a 3x repetition. Each analysis was conducted on two different samples.

Lumidots orange quantumdots (QDs) emitting at 590nm, constituted by a CdSe core with a ZnS shell and stabilized by hexadecylamine (HDA), were purchased by Sigma-Aldrich (Milan, Italy). QDs were encapsulated with the amphiphilic polymer poly (styrene-co-maleic anhydride) terminated with cumene (PSMA) and ethanolamine (EA) and transferred in deionized water following the procedure reported in (Morelli et al., 2012).

The same protocol was performed with HDA, PSMA and EA solutions without QDs, in order to obtain a blank medium (named HDA/PSMA), useful to evaluate if these organic constituents of QDs affected algae and copepods. Nanoparticle concentration was measured by Jasco V-550UV/vis spectrophotometer (Lecco, Italy) using the molar absorption coefficient (ε - 1.6×10^5 M^{-1} cm^{-1}) provided by Sigma-Aldrich. Total Cd was measured by Atomic Absorption Spectrometry equipped with a graphite furnace (AAS, Perkin Elmer, Ueberlingen, Germany), after acidification with HNO_3 (0.3% v/v) (Table 4.1 and 4.2).

Emission (λ_{max})	Absorption (λ_{max})	Core size (nm)	Hydrodynamic diameter (nm)	ε_M (M^{-1} cm^{-1})	[QDs] (μM)	[Cd] (μM)	[Zn] (μM)
591 nm	569 nm	3.5	17 ± 3	$1.6x10^5$	1.23	590	112

Table 4.1 Chemical and optical properties of the water-soluble stock supsension of CdSe/ZnS QDs.

	QDs in deionized water (Cd, μM)	QDs in seawater (Cd, μM)
Total Cd	1.16± 0.18	1.24± 0.08
Cd>1200 nm	0.012± 0.01	0.79± 0.16
Cd<1200 nm	1.06± 0.22	0.11± 0.09
Cd<3 kDa	0.07± 0.07	0.08± 0.04

Table 4.2 Supplementary Information. CdSe/ZnS QD water-soluble stock suspensions were properly diluted with deionized water or seawater, as to obtain a final QD concentration of 2.5 nM, and processed after 1h.Concentration of Cd, as a tracer of QDs, in fractions obtained after filtration onto a 1.2 μm membrane filter, followed by centrifugation with a centrifugal filter device with a 3 kDa cutoff (Amicon Ultra-0.5 mL, Millipore, USA). Total Cd was measured by AAS.

4.1.3 Acute test incubation

Two days before the acute tests, adult females and males of *A. tonsa* were selected from the main tank and isolated in 1 L FSW at a density of 40 couples, fed with an algal mixture of 1500 μg C L^{-1}. Copepods were maintained at the same temperature and light conditions of the main culture (see above). After 24 h the medium was renewed, removing the eggs at the bottom of the beaker,

and after further 24h, adults and eggs were collected and incubated for the acute test.

Almost 200 *A. tonsa* eggs were randomly transferred in 10 ml crystallizers containing FSW (control eggs) or in toxicant solutions, using an inverted microscope (Olympus, Milan, Italy) equipped with 4x and 10x objectives. Afterward, 1 to 3 eggs were incubated in a 24-well plates containing 2.5 ml of FSW with and without toxicants; four replicates per concentration were performed. Plates, covered with their lids, were then placed in a climate-controlled chamber (14:10 light: dark photoperiod) at 20 ± 1 ℃. After 48h hatched eggs and naupliar viability were calculated by counting empty membranes and immobilized/dead nauplii on the bottom of the wells. Nauplii were considered immobilized when they were unable to actively move vertically and/or horizontally during a 15 sec of observation period, after stimulation with a pipette tip. At least three replicates were performed for each treatment. Naupliar viability, was the end-point and the effective concentration affecting 50% of hatched nauplii (EC_{50}) was calculated (Gorbi et al., 2012; ISO International Organization for Standardization, 1999). The EC_{50} values for copepod in the acute test were estimated using Probit Analysis -Version 1.5. For all eggs, the EC_{50} was calculated using nickel chloride as a referent toxicant, compared with NiNPs.

One-way and two way ANOVA analysis of variance and *t*-test's Student were used to analyze significant differences among experimental groups. Daily data in the graphs are means ± standard error (s.e.). Tests were considered valid when the copepods in FSW (control) showed a percentage of egg hatching success after 48h after 48hudent were used to analyze significantwas er 48h (Gorbi et al., 2012).

Three different protocols were used for preparing test solutions in the acute test:

i) In the SON, the stock solution of 1000 mg L^{-1} NiNPs was dissolved in BdW and sonicated using a sonicator bath (Bandelin sonoplus HD2070) at room temperature for 30 min with two consecutive pulses at 100 W. In the NON-SON, the stock solution of 1000 mg L^{-1} NiNP was vigorously shaken 5 min by hand before the dilutions in seawater. Final concentrations of 5, 10 and 50 mg L^{-1} NiNPs were used for the acute test.

ii) Nickel chloride hexahydrate was obtained from Carlo Erba Reagents (Milan, Italy); $NiCl_2*H_2O$ stock solution of 1000 mg L^{-1} was diluted in BdW to obtain a nominal concentration of 10 mg L^{-1}, corresponding to a measured value of 10 ± 0.09 mg L^{-1} analyzed with ICP OES 720 (Agilent Technologies, Santa Clara, CA, USA). Dilutions in FSW were made to obtain final concentrations of 0.10, 0.25, 0.40and 0.65 mg L^{-1} for the acute test.

iii) QD and total Cd concentrations in the stock suspension used in this study were 1.23 µM and 590 + 40 µM (n-4), respectively. The size of the water-soluble QD suspension was17 + 3 nm, as stated by fluorescence correlation spectroscopy measurements (Morelli et al., 2013). Stable

suspensions of water-soluble QDs were stored in the dark at 4 °C for a maximum of 3 months and used for exposure experiments. Dilutions in FSW were made to obtain final nominal concentrations of 0.15, 0.25, 0.50, 1.0, 1.5nM for the acute test.

Stock solutions were prepared immediately before use and dilutions were made in FSW.

4.1.4 Semichronic test incubation

Almost 120 *A. tonsa* eggs, collected after acclimation described above, were randomly transferred in 10 ml crystallizers (10 eggs in each crystallizers) containing FSW (control eggs) or toxicant solutions and a suitable aliquot of the microalgae *I. galbana* at a final cell density of 5.72 x 10^4 cells mL^{-1} (Gorbi et al., 2012). After 48h, 5 days and 7 days hatched eggs and swimming nauplii were transferred in new crystallizers containing fresh solutions and algae. Crystallizers, incubated at 20±1 °C and on a 14h: 10h light: dark photoperiod, were kept in slight agitation (Ika Labortechnik KS125), to favor particle suspension. After 7 days the number of viable nauplii was counted and the EC_{50} was calculated (Gorbi et al., 2012). The percentage of naupliar mortality was calculated as the ratio of those immobilized or dead versus the number of hatched eggs. Immobilized nauplii were unable to actively move vertically and/or horizontally in a period of 15 sec of observation, also after stimulation with pipette tips.

The EC_{50} values were estimated using Probit Analysis -Version 1.5. One-way and two way ANOVA analysis of variance and *t*-test's Student were used to analyze significant differences among experimental groups. Daily data in the graphs are means ± standard error (s.e.). Tests were considered valid when the copepods in FSW (control) showed a percentage of egg hatching success ≥ 80% and the EC_{50} for naupliar immobilization in $NiCl_2$ was naupliar i < 30% in semichronic test (Gorbi et al., 2012).

Two different protocols were used for preparing test solutions in the semichronic tests:

i) In the SON, the stock solution of 1000 mg L^{-1} NiNPs was dissolved in BdW and sonicated using a sonicator bath (Bandelin sonoplus HD2070) at room temperature for 30 min with two consecutive pulses at 100 W. In the NON-SON, the stock solution of 1000 mg L^{-1} NiNP was vigorously shaken 5 min by hand before the dilutions in seawater. Final concentrations of 1, 5, and 10 mg L^{-1} NiNPs were used for the semichronic tests, respectively.

ii) $NiCl_2*H_2O$ stock solution of 1000 mg L^{-1} was diluted in BdW to obtain a nominal concentration of 10 mg L^{-1}, corresponding to a measured value of 10 ± 0.09 mg L^{-1} analyzed with ICP OES 720 (Agilent Technologies, Santa Clara, CA, USA). Dilutions in FSW were made to obtain final concentrations of 0.025, 0.04, 0.065 and 0.10 mg L^{-1} for the semichronic test.

Stock solutions were prepared immediately before use and dilutions were made in FSW.

4.1.5 Chronic test incubation

This bioassay was performed to verify if *A. tonsa* reproduction was affected by QDs and Ni compounds during 4 days of exposure. Almost 60 adult mature males and females, obtained according to the above described procedures after acclimation, were sorted under a stereomicroscope and transferred pair-wise into 60 mL crystallizing dishes containing 50 mL FSW, the algae *R. baltica* at a cell density of 3.6×10^4 cells mL^{-1} with and without toxicant solutions. All the experimental groups were kept at 20±1 ℃ on a 14h: 10h light: dark photoperiod and kept in slight agitation as described above. Each 24h couples were gently transferred to new crystallizers containing fresh medium and algae; the eggs at the bottom of the crystallizer were counted under the inverted microscope (x 10 magnification) and left undisturbed to hatch for further 48h. Egg hatching success was analyzed by counting the number of empty egg membranes, hatched nauplii and unhatched eggs. A total of 20 and 22 couples were incubated for each concentration of toxicants and the control. One-way and two way ANOVA analysis of variance and *t*-test's Student were used to analyze significant differences among experimental groups. Daily data in the graphs are means ± standard error (s.e.). Tests were considered valid when the copepods in FSW (control) showed a percentage of egg hatching success ≥ 80%.

Three different protocols were used for preparing test solutions in the chronic tests:

i) The stock solution of 850 mg L^{-1} NiNPs was dissolved in BdW, at room temperature, which was vigorously shaken 5 min by hand before the dilutions in seawater. Final concentrations of 8.5 and 17 mg L^{-1} NiNPs were used for the chronic tests, respectively.

ii) $NiCl_2*H_2O$ stock solution of 1000 mg L^{-1} was diluted in BdW to obtain a nominal concentration of 10 mg L^{-1}, corresponding to a measured value of 10 ± 0.09 mg L^{-1} analyzed with ICP OES 720 (Agilent Technologies, Santa Clara, CA, USA). Dilutions in FSW were made to obtain final concentrations of 0.025, 0.065 and 0.10 mg L^{-1} for the chronic test.

iii) QD and total Cd concentrations in the stock suspension used in this study were 1.23 µM and 590 + 40 µM (n-4), respectively. Stable suspensions of water-soluble QDs were stored in the dark at 4 ℃ for a maximum of 3 months and used for exposure experiments. Dilutions in FSW were made to obtain final nominal concentrations of 0.5, 1.5 and 2.5 nM for the chronic test.

Stock solutions were prepared immediately before use and dilutions were made in FSW.

Transmission electron microscopy (TEM)

Adult *A. tonsa* females incubated 4 days in 17 mg L^{-1} NiNPs were fixed at 4 ℃ in 2.5% glutaraldehyde and 1% paraformaldehyde buffered to pH 7.2 with 0.2 M sodium cacodylate (Sigma–Aldrich, Milan, Italy) and 20% sea water (Arnaud et al., 1978). After 24 h copepods were rinsed with sodium cacodylate and sea water buffer, post-fixed with 1% osmium tetroxide at 4 ℃

for 1 h, rinsed five times with 0.1 M sodium cacodylate buffer, dehydrated in a graded ethanol series, further substituted by propylene oxide and embedded in Epon 812 (TAAB, TAAB Laboratories Equipment Ltd., Berkshire, UK) at room temperature for 1 day and polymerized at 60 ℃ for 2 days. Resin blocks were sectioned with a Ultracut UCT ultramicrotome (Leica, Vienna, Austria). Semithin sections (1 µm) were stained with toluidine blue and observed with a Leica DMI6000B microscope. Ultrathin sections were contrasted with 4% aqueous uranyl acetate for 30 min, rinsed once with a mix of methanol and bidistilled water (1:1), twice with BdW, placed on nickel grids and observed with a Zeiss LEO 912AB EFTEM (Zeiss, Oberkochen, Germany). Ultrathin sections (50–60 nm) were analyzed by EFTEM equipped with in column energy loss Omega spectrometer, first order aberration corrected, with energy dispersion of 0.75 µm/eV on the spectrometer dispersive plane. The Ni-L3 energy loss spectrum was acquired in parallel mode, at 100× spectrum magnification, with bottom mount 2K × 2K TRS Peltier cooled CCD camera and analyzed with Olympus iTem image analyzer software.

4.1.6 Gene expression of *A. tonsa* exposed to toxicants

Almost 100 adults were exposed 4 days in 1L FSW with $NiCL_2$ at the final concentration of 0.065 mg/L, NiNP at the final concentration of 8.5 and 17 mg/L, and QDs at the final concentration of 0.5 nM under the same experimental conditions reported above, respectively.

Exposure experiments were designed to follow the temporal pattern of different genes (heat shock protein (hsp70) and ferritin) expression in *A. tonsa*. Almost 200 adults were exposed 4 days in 1L FSW with toxicants at the different concentrations under the same experimental conditions reported above. At 0h, 24h, 48h, 72h, 96h, 10 to 20 adults were daily collected and transferred in 2 mL tubes with 0.5 mL of TRIZOL® Reagent (InVitrogen, San Diego, CA, US), frozen in liquid nitrogen and stored at -80 ℃.

Frozen animals were thawed and homogenized with Tissue Lyser (Qiagen, Austin, TX, US) using 3 mm sterile tungsten carbide beads at 20.1 Hz for 3 min. A further break of the copepod body was obtained by passing 5-6 time through a 0.1 mm syringe-needle. After centrifuging at 12,000 x g for 10 min at 4 ℃, samples were processed by following the manufacturer instructions to isolate RNA. And purified further with a TURBO DNA free Kit (Ambion, Austin, TX, USA). The quantity and quality of total RNA was analyzed using a 1% agarose gel and an Agilent 2100 bioanalyzer. The amount of RNA used for the reverse transcription was about 5 µg. This amount of RNA was used to convert into cDNA with the Statistical analysis.

PrimeScriptTM II 1st strand cDNA Synthesis Kit (Takara, Japan). Real Time amplification was performed in a 7500 Fast Real-Time PCR System (ABI, USA) using TAKARA SYBR® Premix Ex

TaqTM II (Tli RNaseH Plus) (Takara, Japan). Each quantitative RT-PCR reaction contained DNA from 50 to 100 ng.

Primers **(**Nilsson et al., 2013)**:**

The β-actin gene of *A. tonsa* was used as a reference house keeping gene to normalize the level of gene expression. The primers for the β-actin gene were:

Forward: 5‘-CTTCTGCATACGGTCAGCAA-3’;

Reverse: 5‘-ACCCGTACGCCAACACTG-3’.

Hsp70:

The RT-PCR conditions were as follows: 94 °C/10 min; 40 cycles of 94 °C/20 s, 60 °C/45 s. The RT-PCR primers for the hsp70 gene were:

Forward: 5‘-TTCAATGATTCACAGAGACAAGC-3’;

Reverse: 5‘-TCCTTGTGATGTTAAGACCAGCTAT-3’.

Ferritin:

The RT-PCR conditions were as follows: 94 ° C/10 min; 40 cycles of 94 ° C/20 s, 60 ° C/45 s. The RT-PCR primers for the Ferritin gene were:

Forward: 5‘-CATYAACAAGCARATCAA-3’;

Reverse: 5‘- AGYACAAYYCTTCCWCCWCG-3’.

Fold change for the gene expression relative to controls was determined by the $2^{-\Delta\Delta CT}$ method of Livak & Chmittgen (2001). Comparison between groups was made by one-way ANOVA and independent sample t-tests, followed by a Tukey test for identification of the statistically distinct groups. Significant differences were accepted for $p \leq 0.05$. The statistical analyses were performed using SPSS 18 software. Experiments were performed in triplicates.

4.2 Results and discussion

The calanoid copepod *A. tonsa* is considered a model organism to test toxicant matrices and specific protocols for determination of the toxic effects of chemical substances on eggs and early life developmental stages have been standardized (ISO International Organization for Standardization, 1999, 2013). This species is also the main food for fish larvae and other invertebrates therefore, any variation in copepod biomass can affect fish recruitment at sea (Beaugraund et al., 2003).To the best of our knowledge, this is the first study which explores the toxicity of NiNPs and QDs on the calanoid copepod *A. tonsa*.

These results have been published in the following journals:

C. Zhou et al. / Aquatic Toxicology 170 (2016a) 1–12;

C. Zhou et al. / Ecotoxicology and Environmental Safety 123 (2016b) 26–31.

4.2.1 Nickel chloride toxicity

The percentage of egg hatching success and naupliar mortality recorded in the acute test for $NiCl_2$ is shown in Fig. 4.1 Egg hatching success was significantly reduced at the highest concentration compared to the control, with only 68% of hatched embryos ($p < 0.0001$) (Fig. 4.1a). On the contrary, a significant increase in naupliar mortality occurred already at the lowest $NiCl_2$ concentration tested (>30% at 0.1 mg L^{-1}), and more than 94% nauplii died at 0.4 and 0.65 mg L^{-1} (Fig. 4.1b) ($p<0.001$) The effective concentration inducing 50% of naupliar mortality (EC_{50}) was 0.164 mg L^{-1} $NiCl_2$ (0.149-0.249 mg L^{-1}, 95% confidential interval CI, Table 4.3).

NiNP nominal concentration (mg L^{-1})	Sonicated			Non sonicated		
	Total Ni (mg L^{-1})	Ni released from NPs (mg L^{-1})	% Nireleased	Total Ni (mg L^{-1})	Ni released from NPs (mg L^{-1})	% Ni released
1 mg L^{-1}	0.030	0.010	33.3	0.059	0.015	25.4
5 mg L^{-1}	0.311	0.075	24.1	0.187	0.055	29.4
10 mg L^{-1}	0.153	0.105	68.6	0.265	0.139	52.4

Table 4.3 ICP-MS quantitative analysis of nickel in water samples collected after 48 h. From Zhou et al. (2016a).

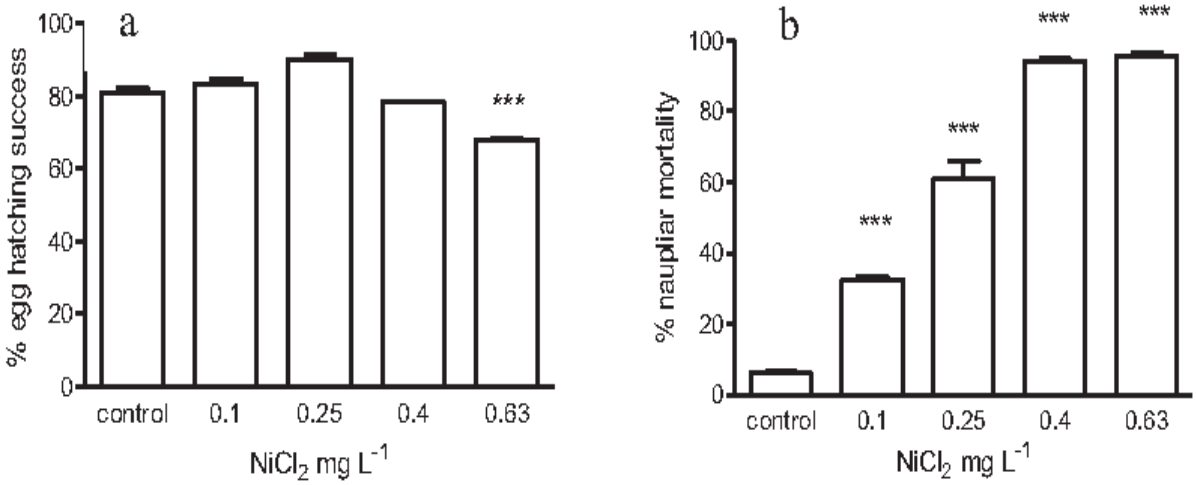


Figure 4.1 Acute test with *A. tonsa*. Percentage of egg hatching (a) and naupliar mortality (b) after exposure of eggs for 48 h to 0.1, 0.25, 0.4 and 0.63 mg L^{-1} $NiCl_2$ and the control in sea water (mean ± s.e.). Naupliar mortality was calculated as percentage [100 × (total number of eggs-hatched embryos)/total number of eggs)] (***significant differences between the exposed eggs and the control $p < 0.001$). From Zhou et al. (2016a).

In the semichronic test the percentage of naupliar mortality increased in a dose dependent manner; at the lowest concentration tested (0.025 mg L^{-1}) 38% of nauplii died and at 0.04 and 0.065 mg L^{-1} the percentage of dead nauplii increased to 54 and 77%, respectively (Fig. 4.2). At the highest concentration (0.1 mg L^{-1}) more than 90% of nauplii died and the EC_{50} was 0.039 mg L^{-1} (0.032-0.045, 95% CI).

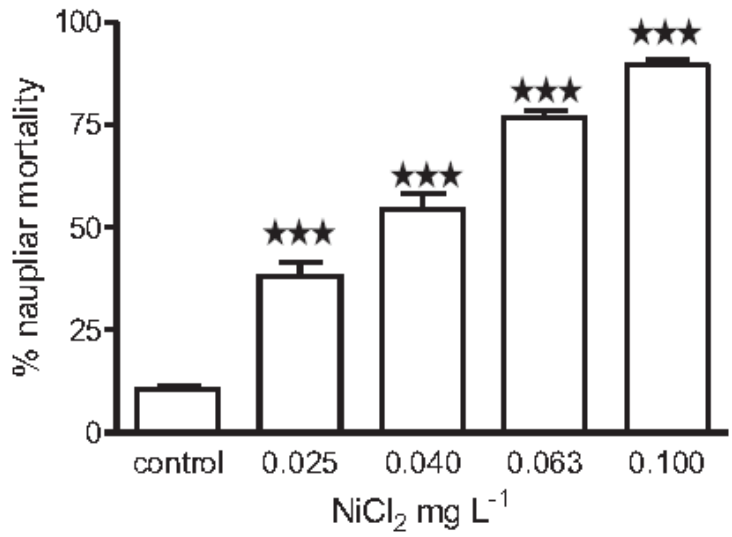


Figure 4.2 Semichronic test with *A. tonsa*. Naupliar mortality (in%) after exposure of eggs and nauplii 7 days to 0.025, 0.04, 0.063 and 0.1 mg L^{-1} NiCl2 and the control in sea water (mean ± s.e.). Naupliar mortality was calculated as percentage [100 × (total number of eembryos-hatched embryos)/total number of embryos)] (***significant differences between the exposed embryos and the control $p < 0.001$) From Zhou et al. (2016a).

Daily egg production per female exposed 4 days to $NiCl_2$ is reported in Fig. 4.3a. At the lowest concentration a significant increase in egg production was recorded the first day respect to the control (32.7 eggs f^{-1}, $p<0.05$, versus 18.3 eggs f^{-1} in the control). On average, the total fecundity calculated over the 4 days was similar to the control (21.17; 19.76 and 22.14 eggs f^{-1} day^{-1} for 0.025, 0.065 and 0.10 mg L^{-1} $NiCl_2$, respectively). On the contrary, daily egg hatching success was significantly reduced the first day (68.7%) at the highest concentration tested of 0.10 mg L^{-1} $NiCl_2$, with respect to the control (90%, $p<0.05$), and remains lower the following days (Fig. 4.3b). Considering the mean value calculated over the total 4-day incubation, egg hatching success recorded at 0.025 and 0.1 mg L^{-1} $NiCl_2$ (73.2% and 68.7%, respectively) was significantly different from the control (88%) ($p< 0.05$).

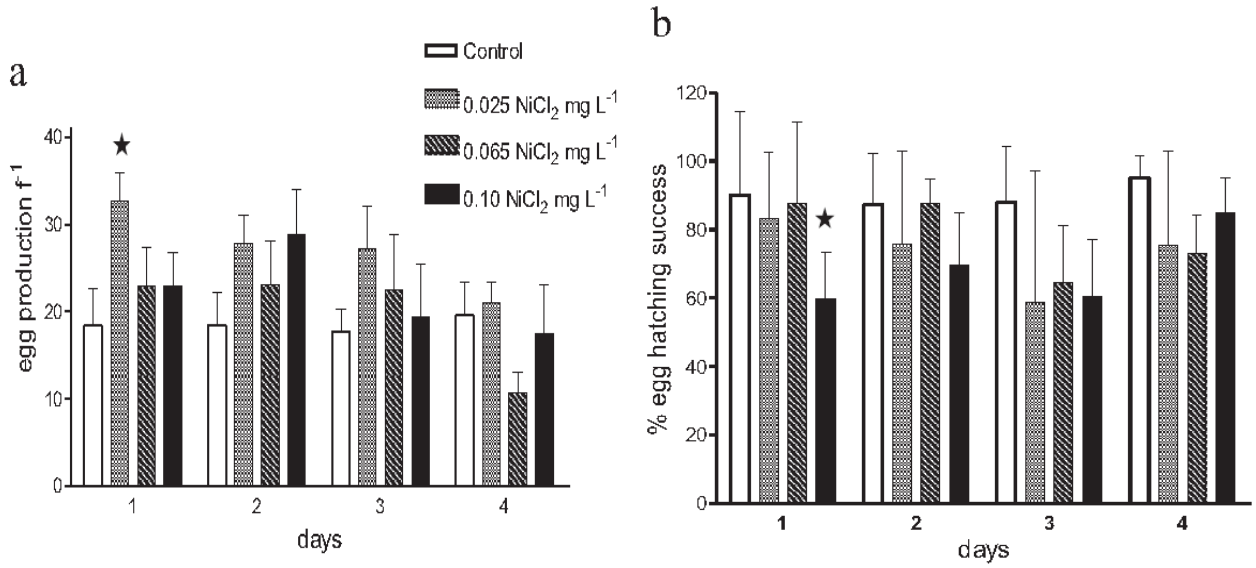


Figure 4.3 Chronic test with *A. tonsa*. Egg production per female per day (a) and the percentage of eggs hatching (b) after exposure of adults for 4 days to 0.025, 0.065and 0.10 mg L^{-1} $NiCl_2$ and the control in sea water (mean ± s.e.). The percentage of eggs hatching is calculated with respect to the total number of embryos (* significant differences between the exposed eggs and the control $p < 0.05$). From Zhou et al. (2016a).

4.2.2. Nanoparticle toxicity

In the last decade, toxicological studies on nanoparticles in the aquatic environment have been approached with different protocols, making a comparison of the results difficult (Corsi et al., 2014; Handy et al., 2008). Commonly, NPs are dispersed into aqueous suspensions using sonication, but this method adds further misinterpretations of the results (see Petersen et al., 2014 for a review). In fact, the dispersion protocol is difficult to be replicated with consequent incorrect conclusions about the toxicological effects. Therefore, we first focused our attention to verify if sonication of NiNP solutions induced different chemical behavior of NPs in seawater or different toxicity on *A. tonsa* eggs.

The behavior of Nickel nanoparticles was analyzed with different techniques and at different protocols of dissolution in seawater. The diameter of NiNPs was analyzed with DLS in the sonicated (SON) and non-sonicated (NON-SON) seawater solutions. In both treatments the diameter of NiNPs was similar with values of 146.9 ± 51.4, 151.4 ± 25.8 and 128.8 ± 22.4 nm recorded for NPs in the SON solutions and values of 138.6 ± 12.2, 176.1 ± 50.4 and 156.6 ± 35.2 in NON-SON solutions at the concentrations of 1, 5 and 10 mg L^{-1} NiNP, respectively. Furthermore, NON-SON solutions, collected soon after the preparation (time=0) and after 48h were also analyzed with DLS (Fig. 4.4). NiNP size did not change over time at all the concentrations tested, except for the highest concentration of 50 mg L^{-1}, in which the diameter increased up to 843 nm after 48h (Fig 4.5). Nanoparticles dissolved in seawater at a concentration of 10 mg L^{-1} and collected after 48h from the acute test incubation in the NON-SON experiment, were observed with TEM (Fig. 4.4a).

Colloidal agglomerates were evident and NPs appeared included in a mucus. A change of morphology is evident with respect to the same NP concentration dissolved in ultrapure water (Fig. 4.4b). The intensity of darkness of NPs after 48h in seawater decreased, suggesting that an oxidation of the metallic core occurred.

Quantitative analysis of Ni released from the NiNPs solutions and measured by ICP-MS at the different concentrations is reported in Table 4.4. Dissolved Ni increased with NPs concentration and was similar for SON and NON-SON solutions. Differences between nominal concentrations of NiNPs (i.e. 1, 5, 10 mg L^{-1}) and ICP-MS values found in the samples were due to the pre-filtration of the samples. In our experimental conditions results indicated that the sonication did not change significantly the dimension of NiNPs, neither the concentration of dissolved Ni ions.

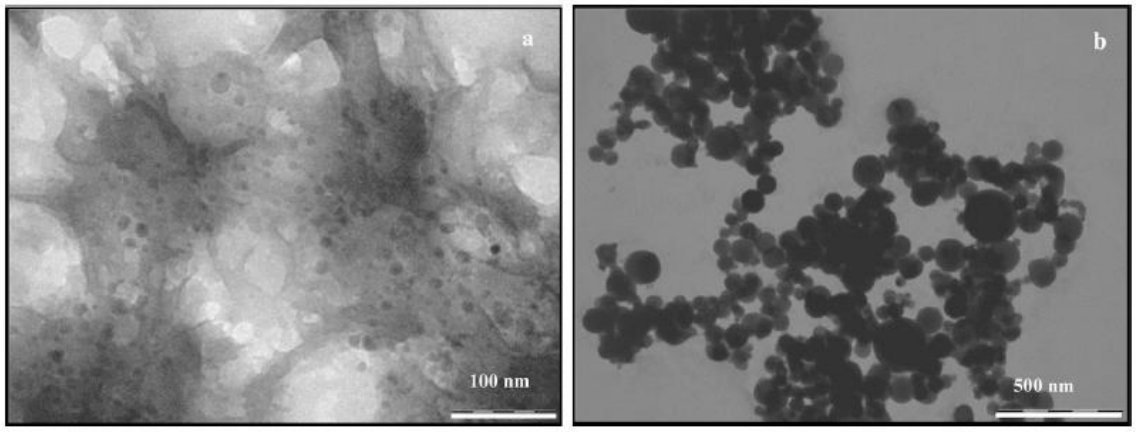


Figure 4.4 Nickel nanoparticle solution at the concentration of 10 mg L^{-1} observed with transmission electron microscopy. (a) NiNPs dissolved in seawater and collected after48 h in seawater from the semichronic non-sonicated test solution. (b) The same nickel nanoparticle concentration dissolved in double distilled water and sonicated for30 min (control). From Zhou et al. (2016a).

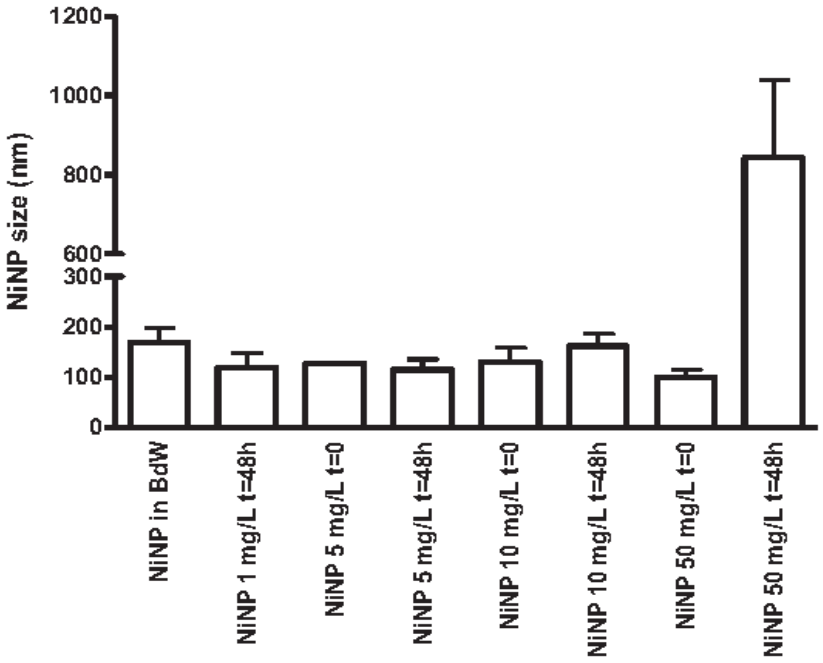


Figure 4.5 Nickel nanoparticle diameter (nm) of non-sonicated solutions collected from acute and semichronic tests soon after the preparation (t = 0) and after 48 h, calculated by Dynamic Laser Scattering. From Zhou et al. (2016a).

Test	Duration (days)	End-point	Toxicant	Concentration tested (mg L^{-1})	EC50 mg L^{-1}(CI 95% $p < 0.05$)	Ni released from NPs (mg L^{-1})	Extrapolated EC_{50} (mg L−1)
Acute	2	Naupliar viability	NiNPs	5		0.075	
				10	S 22.145 (16.86/28.854)	0.10	0.275
				50	NS 20.2 (13.17/30.11)	0.75	
			$NiCl_2$	0.10			
				0.25	0.164 (0.149/0.249)		
				0.40			
				0.63			
Semichronic	7	Naupliar viability		1.0		0.010	
			NiNPs	5.0	S 7.45 (4.25/10.36)	0.075	0.021
				10	NS 6.97 (3.71/8.57)	0.105	
				0.025			
			NiCl2	0.040	0.039 (0.032/0.045)		
				0.063			
				0.100			
Chronic	4	Female fecundity (EP) Egg hatching success (EH)	NiNPs	8.5	No effects on EP Effects on EH		
				17.0	No effect on EP Effect on EH		
			NiCl2	0.025	No effect on EP and Effect on EH		
				0.063	No effect on EP and EH		
				0.1	No effect on EP and Effect on EH		

Table 4.4 Summary of the bioassays, duration of the experiments, end-points, toxicants, concentrations tested, and EC_{50} values. Nanoparticle solutions were prepared, before the acute and semichronic tests. EP = egg production $female^{-1}$ day^{-1}; EH = percentage of egg hatching success. Extrapolated EC_{50} is referred to nickel released by nanoparticles. From Zhou et al. (2016a).

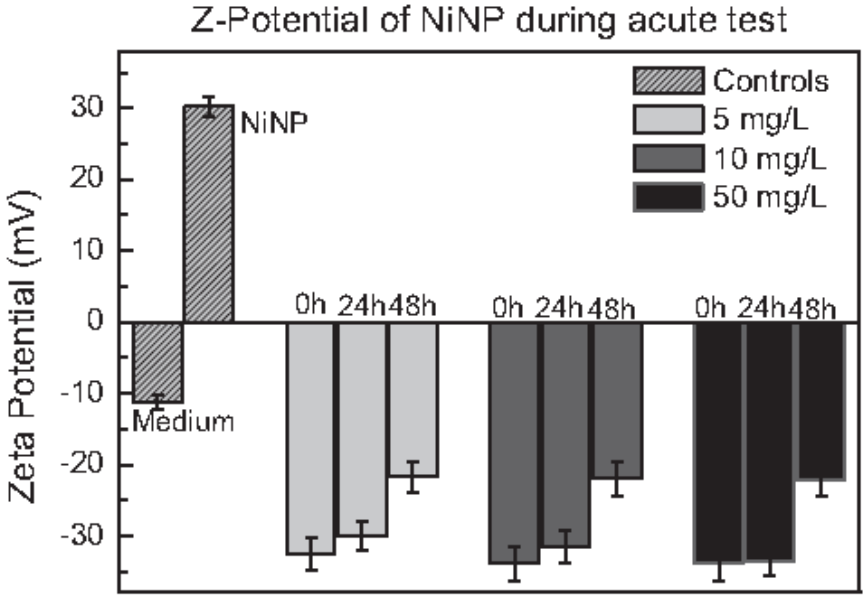


Figure 4.6 Zeta potential measurements of nickel nanoparticle samples from acutetests collected soon after the preparation (t = 0), after 24 h and 48 h. Positive zeta-potential corresponds to nanoparticles dissolved in distilled water (NiNP). Medium corresponds to zeta-potential of the biological media (seawater and microalgae). From Zhou et al. (2016a).

As expected, behavior and morphology of NiNPs are different if they are dissolved in seawater or in fresh water; our results indicate that colloidal agglomerates appeared after 48 h of incubation in seawater at the concentration of 10 mg L^{-1} NiNPs. Z-Potential (surface charge) measurements are commonly used to determine the stability of a colloidal suspension of NPs. Fig. 4.6 shows that NiNPs have a positive Z-Potential when they are dissolved in BdW and a negative Z-Potential after incubation in seawater. The highest negative value was recorded during the first 24h of incubation in seawater (~-30 mV) and decreased to ~-20 mV after 48h, at all the concentrations tested, suggesting that the stability of the solutions occurred later in the time (> 48h) and was independent by NiNP concentrations. It is well known that a medium with high ion levels leads to the aggregation of nanoparticles and to an inversion of their Z-potential, with respect to the same NPs dissolved in de-ionized water (Nel et al., 2006; Sillampää et al., 2011). We showed that, when incubated in seawater, NiNP Z-potential reverses its sign from positive to negative values; furthermore, NiNPs in seawater have a series of alterations that modify the way they are encountered by species (see Corsi et al., 2014 for a review). The formation of NP–organic matter complexes is driven mainly by electrostatic interaction between the positively charged NPs and negatively charged organic matter in the water (Casals et al., 2011; Hughes & Kim, 2007). The high concentration of ions in sea water induces aggregation and corrosion; NP surface rapidly oxidizes during the corrosion process, as observed in our NP solution (10 mg L^{-1}) that lost darkness intensity over time. Moreover, NPs interact with organic matter, with a consequent aggregation. Therefore,

as aggregation and corrosion occur at different times (aggregation being normally much faster than dissolution), toxicity can change over time. Large aggregates may be toxic after a short time of exposure, while the leached ions should be responsible for toxicity at the longer time of incubation.

In order to assess if the toxicity of NiNPs was dependent by a preliminary treatment of the incubation medium, two different protocols were used for the acute and semichronic tests with *A. tonsa* eggs. In the SON protocol, stock solution was sonicated before egg incubation while, in NON-SON protocol, stock solution was simply re-suspended in seawater and shaked by hand.

In our study we tested NP toxicity on *A. tonsa* copepods using different protocols and time of incubation, also various end-points were considered in order to verify the sensitivity of this species. We exposed copepod eggs following the classical acute and semichronic ecotoxicological tests utilized for dissolved chemicals, as described by Gorbi et al. (2012). Moreover, to verify the impact of Ni exposure on copepod fecundity, we conducted a chronic test exposure, incubating adults and using fecundity and egg hatching success as end-points, as also suggested by Bielmyer et al. (2006) and Hook & Fisher (2001).

Testing nanoparticle toxicity is complex due to their behavior in the environment matrices, to the interaction with biological materials and to the uncertainty of laboratory procedures (Petersen et al., 2014). Moreover, the presence of natural organic matter in seawater (e.g., algae and their exudates) modifies NP behavior with yet unknown toxicological effects on marine ecosystems (Navarro et al., 2008; Stolpe & Hassellov, 2007). It has been shown that polymeric substances in the water, such as those produced by microalgae, can reduce NP toxicity and their bioavailability to filter-feeding organisms (Gong et al., 2011; Quigg et al., 2013). Since the incubation medium of our semichronic and chronic experiments included algae, we used a slight agitation to reduce the settlement of algae and NPs on the bottom of culture vessels to avoid there duction of nanoparticle availability.

In the acute test, egg hatching success was never reduced by NiNPs in experimental protocols (Fig.4.7a), with the percentage varying from 87% in the controls to 78% in eggs exposed to 50 mg L^{-1} NiNPs. On the contrary, naupliar mortality increased significantly in a dose-dependent manner, in experiments (Fig. 4.7b). Comparing the two different protocols NON-SON solution induced significantly higher naupliar mortality only at 5 mg L^{-1} (24 ± 3.7%) with respect to the sonicated one (1.6 ± 0.5%) ($p<0.001$) while the opposite occurred at the highest NiNP concentration when more than 88% of mortality was recorded in the SON experiment ($p<0.001$) versus 51% in NON-SON. Overall, mean EC_{50} values were similar for NON-SON (20.2 mg L^{-1}, 13.17-30.11, 95% confidential interval CI) and SON experiments (22.145 mg L^{-1}, 16.893-28.854, 95% CI).

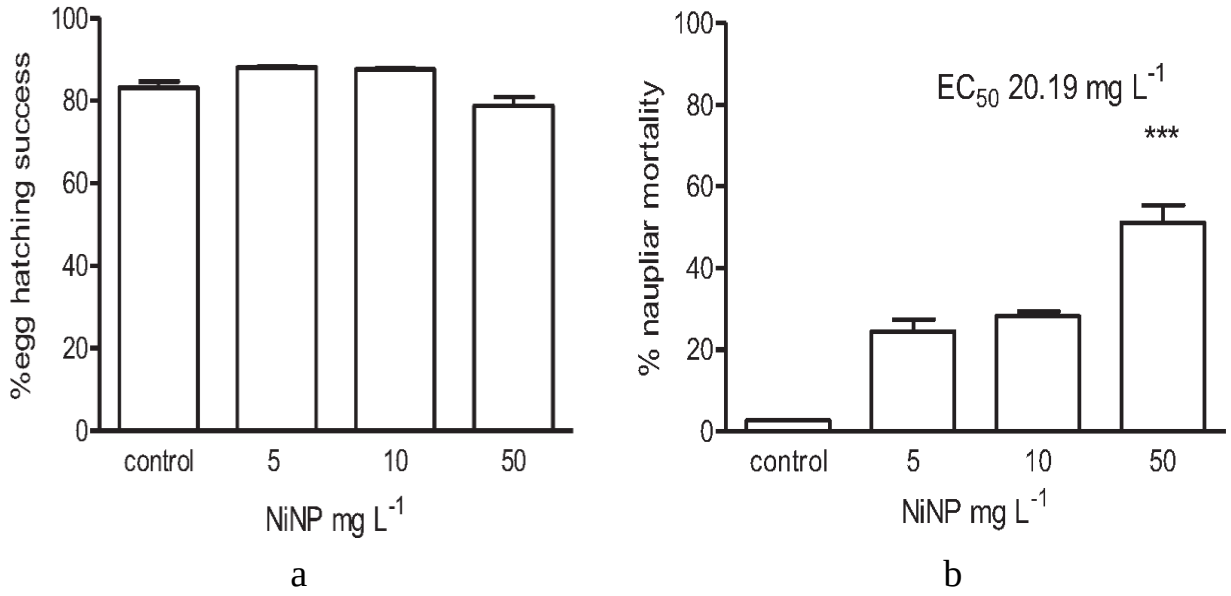


Figure 4.7 Acute test with *A. tonsa*. Percentage of eggs hatching (a) and naupliar mortality (b) after exposure of embryos 48 h to 5, 10, and 50 mg L^{-1} nickel nanoparticles solutions (mean ± s.e.). Naupliar mortality was calculated as percentage [100 × (total number of eembryos-hatched embryos)/total number of embryos)] (*** significant differences between the exposed embryos and the control $p < 0.001$). From Zhou et al. (2016a).

The percentage of naupliar mortality recorded in the semichronic test is shown in Fig. 4.8. As for the acute test, no differences occurred between the two different protocols and the EC_{50} was 7.45 mg L^{-1} (4.258-10.36, 95% CI) and 6.97 mg L^{-1} (3.71-8.575, 95% CI) for NON-SON and SON experiments, respectively. Comparing the effects with the control groups, exposure from 1 to 5 mg L^{-1} NiNPs did not affect significantly naupliar mortality in both SON and NON-SON groups, whereas at 10 mg L^{-1} a significant increase was recorded for both experiments ($p<0.0001$), where only 10 and 20% of nauplii survived, respectively.

The effects observed on *A. tonsa* eggs incubated in the SON and NON-SON NiNP solutions were comparable; in fact, the EC_{50} found in the acute and semichronic tests were similar for both protocols. For this reason we used the non-sonicated protocol for further chronic exposure of *A. tonsa* adults. Also Manusadzianas et al. (2012) showed that the toxicity of CuO nano suspensions on the macrophitic algae, shrimps and rotifers did not change between the non-sonicated and sonicated suspensions.

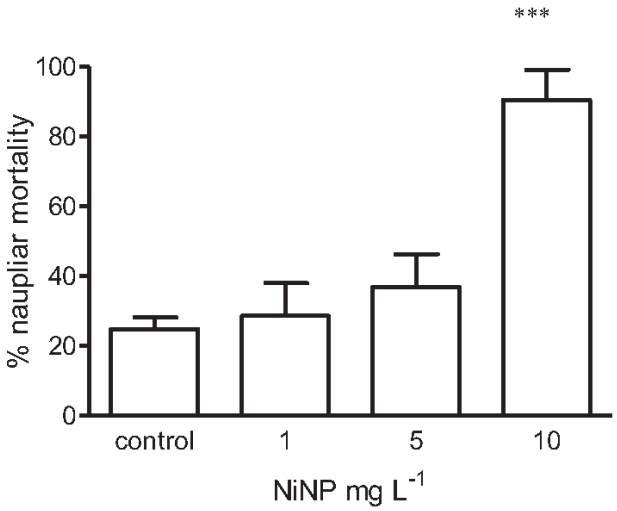


Figure 4.8 Semichronic test with *A. tonsa*. Naupliar mortality after exposure of embryos and nauplii for 7 days to 1, 5 and 10 mg L^{-1} nickel nanoparticles and the control in sea water (mean ± s.e.). Naupliar mortality was calculated as percentage [100 × (total number of eembryos-hatched embryos)/total number of embryos)] (*** significant differences between the exposed embryos and the control $p < 0.001$). From Zhou et al. (2016a).

NP aggregation (with themselves and with algae) increases sedimentation rates with two different possible scenarios afterwards: i) a reduction of the available food, which will sink rapidly on the bottom (Campos et al., 2013), and ii) an accumulation of NPs on the algal surface can increase their toxicity (Jarvis et al., 2013). In the first hypothesis adult survival should be a more sensitive end-point than copepod reproduction due to the depletion of the suspended algae. However, because we used a slight agitation of the medium, we can exclude this possibility. In the second hypothesis the effect should be much more visible on the progeny even at the lowest NiNP concentration tested, as a maternal effect due to the ingestion of NiNPs by the females (Jarvis et al., 2013). Our results seem to exclude this effect. In fact, the reduction of EH success occurred only at the highest NiNP concentrations and this effect is probably due to the Ni released into the water. Alternatively, the ingestion of NiNPs could interfere with the feeding activity of females, with a consequent reduction of the ingested food. As a consequence, the production of good quality embryo and yolk component could be reduced, with a reduction of egg viability. It is interesting to note hat the percentage of naupliar mortality observed in semichronic test (82.3 ± 8.2%) at the nominal concentration of 10 mg L^{-1} NiNPs, corresponding to almost 0.10 mg L^{-1} of total Ni in the water, was similar to that recorded in the same test at the same concentration of Ni salts (89.6 ± 7.6%). This is in agreement with Gong et al. (2011) who suggested that toxicity of NiO, which inhibits the growth of the marine microalgae *Chlorella vulgaris*, was mainly due to Ni ions dissolved into the water. The EC_{50} calculated considering the concentration of nickel dissolved by NP solutions at 1, 5, 10 and 50 mg L^{-1} are very similar to those calculated for $NiCl_2$ in both the

acute and semichronic tests, suggesting that the toxicity of NPs is mainly due to the metal dissolved in the water.

To study the effects of NiNPs on copepod fecundity, two different concentrations were tested: 8.5 and 17 mg L^{-1}. These concentrations were chosen as they are below the EC_{50} of acute test (20-22 mg L^{-1}) and in the range of EC_{50} of semichronic test (6.97-7.45 mg L^{-1}) (Fig. 4.9). At both concentrations no significant differences were recorded for daily egg production rate, with respect to the control group. On the contrary, egg hatching success was a more sensitive end point (Fig. 4.9b) and, in fact, the percentage of egg viability was significantly reduced at both the concentrations tested. At the highest concentration the percentage of hatching success declined sharply from 70 to 9%, after 4 days. On verage, only 42% of the eggs hatched and these nauplii did not survive, as most of them were found immobilized at the bottom of the crystallizers. Adult mortality observed over 4 days of incubation in both $NiCl_2$ and NiNPs was negligible and similar to the control, with a percent-age below to 5% at the highest concentrations tested (0.10 mg L^{-1} for $NiCl_2$ and 17 mg L^{-1} for NiNPs).

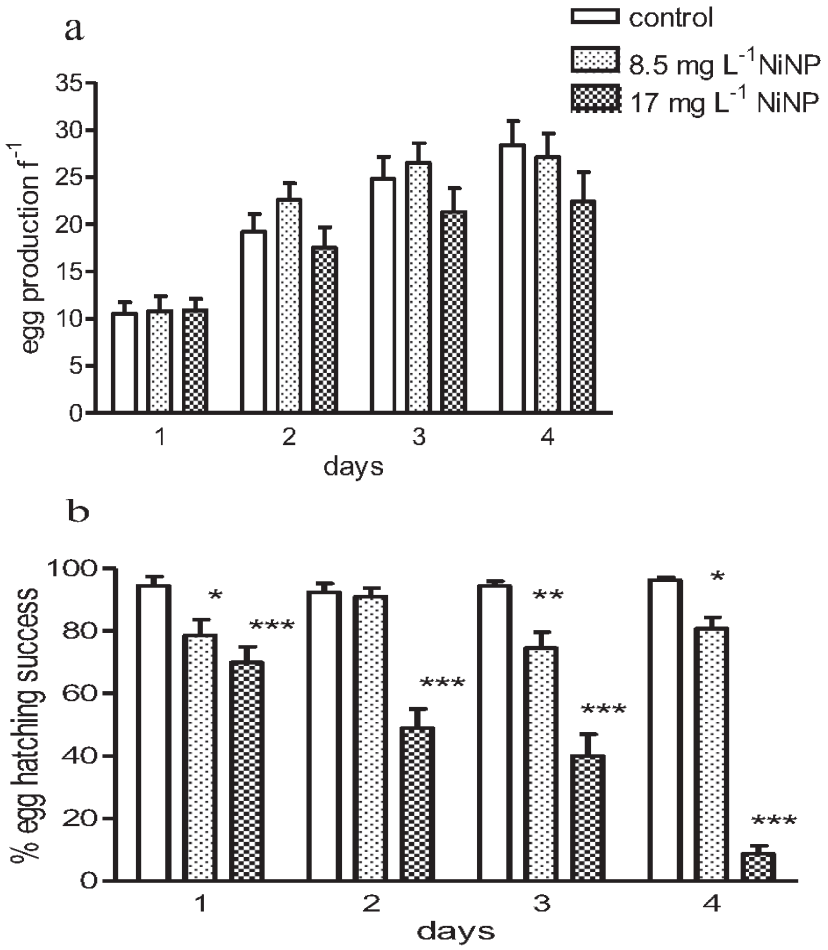


Figure 4.9 Chronic test with *A. tonsa*. Egg production per female per day (a) and the percentage of eggs hatching (b) after exposure of adults for 4 days to 8.5 and 17 mg L^{-1} nickel nanoparticles and

the control in sea water (mean ± s.e.).The percentage of eggs hatching is calculated with respect to the total number of eggs (significant differences between exposed and control * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). From Zhou et al. (2016a).

The toxicity of Ni salts has been investigated by Bielmyer et al. (2006) on *A. tonsa*; the authors reported that adult survival was not affected by Ni supplied with the food via phytoplanktonic cells, in agreement with our results. Whereas a reduction of fecundity (naupli female^{-1}) was observed in a concentration-dependent manner, after 7 days exposure, with the no observable effect (NO EC) of 3.82 μg Ni L^{-1} and the EC20 of 2.43 μg L^{-1}. Our results indicated that fecundity over 4 days of exposure, in terms of number of eggs per female, was not affected at concentration up to 100 ug L^{-1} (Table 4.4), confirming that a previously enriched algae with Ni can increase the sensitivity of *A. tonsa* to this metal. Mohammed et al. (2010) tested $NiCl_2$ toxicity on different calanoid and harpacticoid copepods showing that *Acartia pacifica* adults were the most sensitive species, with the effective concentration inducing 50% adult mortality at 2.36 mg L^{-1} after 48 h in the acute test. Our data showed that *A. tonsa* nauplii were much more sensitive than the adult co-generic species, with an EC_{50} 14-folds lower (164 μg L^{-1}). The same authors (Mohammed et al., 2010) reported that fecundity and percentage of egg hatching success of *A. pacifica* decreased at increasing $NiCl_2$ concentrations, with a significant reduction at 0.1 and 0.01 mg L^{-1} after 10 days exposure. Our results did not show any statistical reduction in *A. tonsa* egg production after a shorter time of incubation (4 days) at 0.1 mg L^{-1} $NiCl_2$. Longer incubation time needs to be tested to compare if the sensitivities of two co-generic species are different.

Considering copepod fecundity, our results indicated that both forms of Ni did not affect egg production rate (EP) at the tested concentrations, excluding the effect due to a poor-quality food uptake. Therefore, egg hatching success remains the most sensitive endpoint. Overall, our results indicate that copepods are more sensitive to Ni salts than to NiNPs with EC_{50} being 120 and 170 fold higher for nanoparticles than for $NiCl_2$, in the acute and semi-chronic experiments, respectively (Table 4.4). Similar EC_{50} values were found for $NiCl_2$ in our previous study (Buttino et al., 2011), in which an apoptotic-like mechanism was found in those survived nauplii exposed at the concentration of 0.025 mg L^{-1}. The EC_{50} calculated for $NiCl_2$ is in the range of dissolved Ni found in polluted water (Eisler, 1998) and the risk of planktonic biomass reduction could be realistic in polluted marine environments. Currently, a safety threshold for NiNPs has not been established.

A summary of the results recorded in the acute, semi-chronic and chronic tests for both $NiCl_2$ and NiNPs exposure, is reported in Table 4.4. Finally, to verify if *A. tonsa* adults ingested NPs, semi thin longitudinal sections of mature females incubated 4 days with 17 mg L^{-1} NiNPs were analyzed (Fig. 4.10a) and internal digestive channel was observed in ultrathin section with the

EFTEM (Fig. 4.10 b, c). Nanoparticles are well visible in the inner part of the gut (upper square in Fig. 4.10) and in the fecal pellet inside the intestinal lumen (bottom square in Fig. 4.10a). Analysis with the EFTEM and the Energy Loss spectra confirmed that those nanoparticles were composed by Nickel (Fig 4.10 b, c).

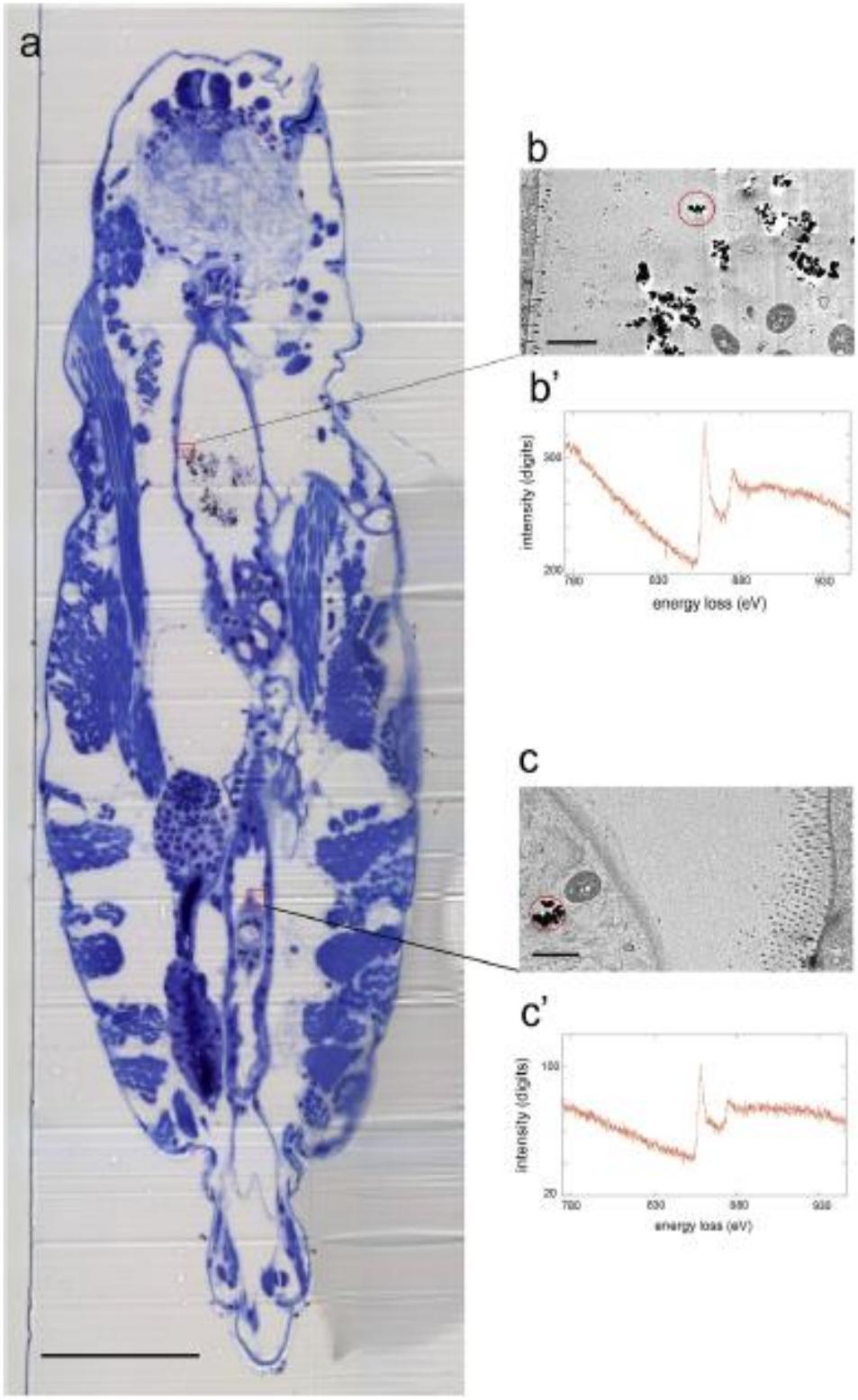


Figure 4.10 *A. tonsa* mature female fed 17 mg L^{-1} nickel nanoparticles for 4 days. (a) Semi thin

longitudinal section of *A. tonsa*, squares indicate the portion of gut (upper)and fecal pellet (lower) analyzed in ultrathin sections with the TEM (scale bar = 100 um). (b) TEM image of the ultrathin section corresponding to the gut and lumen reported in the upper square in (a). (b') Energy Loss spectra confirmed that are composed by nickel. (c) TEM image of the ultrathin section of the fecal pellet inside the intestinal channel in lower square in (a). (c') Energy Loss spectra confirmed that the particles are composed by nickel. Scale bar = 1 um in (b) and (c). From Zhou et al. (2016a).

To better understand the fate of NiNPs during copepod incubation, we analyzed if they were ingested by the adults using electron microscopical analysis. We demonstrated for the first time that NiNPs were ingested and excreted by *A. tonsa*. Copepods lack hepatopancreas as a detoxifying organ, and the digestive tract consists of a one-layered epithelium with cell types which assume different digestive functions (Arnaud et al., 1978; Nott et al., 1985).Vesicles containing heavy metals in the digestive epithelium of copepod have been found (Barka, 2007) and, more generally, metal storage pathways involve cytosolic metalloproteins and/or lysosomal systems in invertebrates (Amiard et al., 2006; Rainbow, 2006). Barka et al. (2001) showed that Ni detoxification involved slight induction of metallothioneins in the copepod *Tigriopus brevicornis* and the major metal-storage tissues are found in the digestive epithelium (vacuolar B-cells). The same authors found granules in the digestive epithelia of the copepod *T. brevicornis*, with high degree of variations in size and morphology, depending on the type of metal to which the organisms were exposed (Barka, 2007). Nickel granules were barely found in copepods exposed to $NiSO_4$, indicating that this metal did not accumulate in the copepods in the soluble form (Costa et al., 1994). Our results are in agreement with these findings, in fact, we did not find lysosomal-like granules inside the epithelium of the gut.

Fig. 4.11 showed the percentage of egg hatching success and naupliar survival of *A. tonsa* after eggs exposure to increasing QD concentrations. The percentage of egg hatching success was not affected by QDs and more than 85% of eggs hatched in all conditions of exposure (Fig. 4.11A). On the contrary, naupliar survival was strongly reduced, starting from 0.25 nM (79% ± 1.41 s.d.), in a dose-dependent manner (Fig. 4.11B). At 1.5 nM QD concentration only 4.5% ± 1.1 of hatched nauplii survived after 48h of exposure. The effective concentration inducing 50% naupliar mortality (EC_{50}) was 0.70 nM (95% CI, 0.47-0.9, n=43).

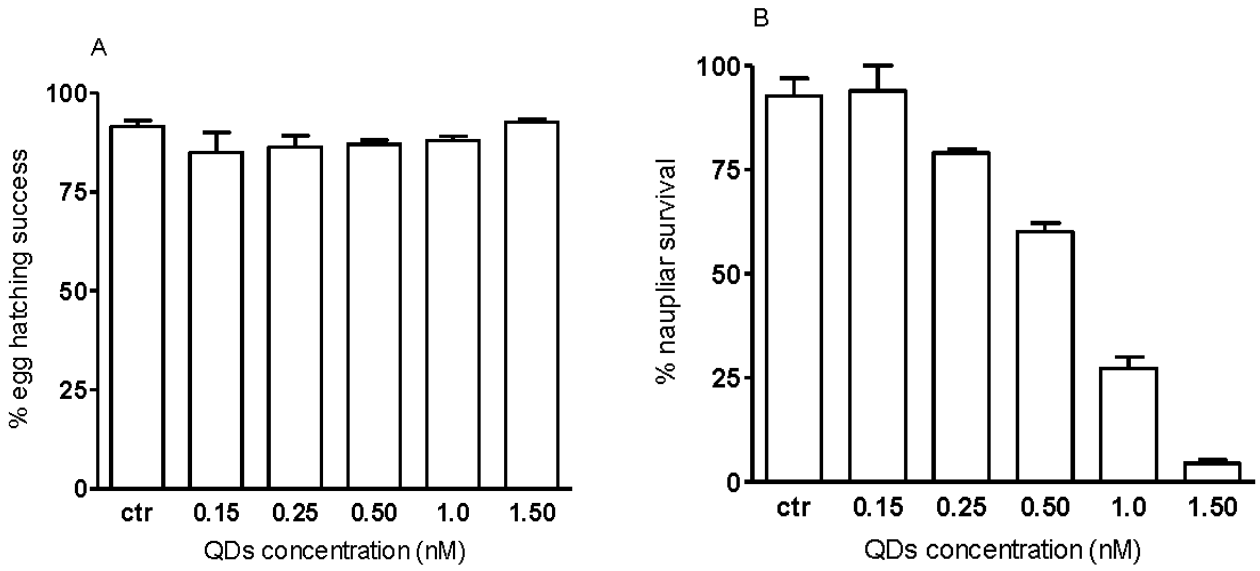


Figure 4.11 *A. tonsa* embryos exposed 48h to different concentrations of QDs. A) Percentage of egg hatching success in exposed and control groups. B) Percentage of naupliar survival calculated on the total number of hatched embryos (mean values ± standard deviation). From Zhou et al. (2016b).

In the chronic test, adult copepods incubated at increasing QDs concentrations showed a reduction of their fecundity after 3 days of exposure at 2.5 nM (Fig. 4.12A). After 4 days, less than 15 eggs female^{-1} were recorded, and this value was significantly lower than that of the control group (mean = 27.3 eggs female^{-1}, p=0.05, F= 6.08, n: 29). Hatching success was not statistically reduced respect to the control at all the exposure conditions (Fig. 4.12B), even though at the highest QDs concentrations the lowest percentage was recorded and less than 80% of eggs hatched after 4-day exposure.

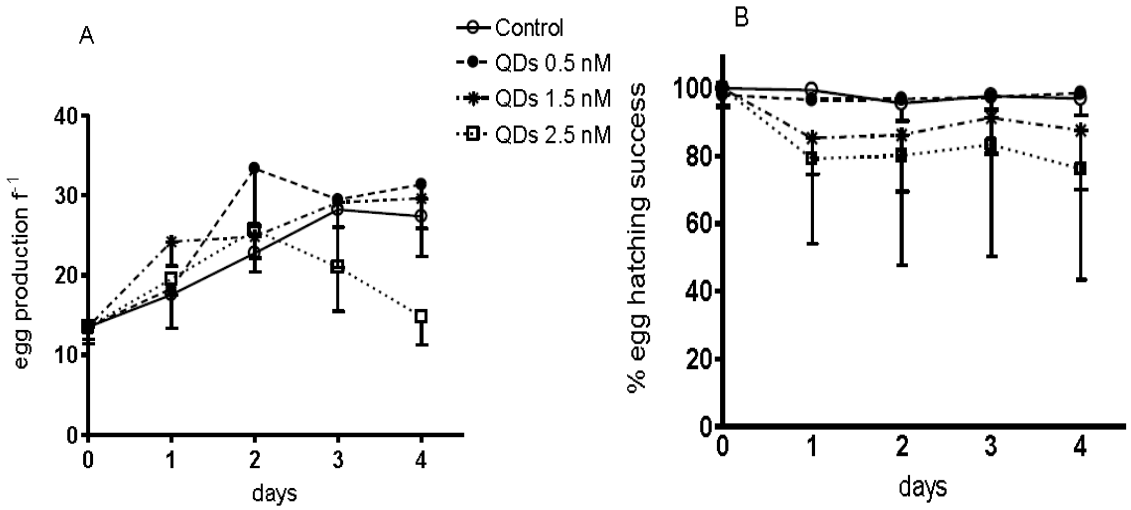


Figure 4.12 *A. tonsa* daily fecundity (A) and percentage of egg hatching success (B) after exposure of adults to 0.5, 1.5, 2.5 nM QDs and control (mean values ± standard deviation). From Zhou et al. (2016b).

To our knowledge, the mechanism of toxicity of QDs in copepods has not yet been investigated. Moreover the amount of QD released into the environment is not known. Uptake of QDs by aquatic organisms is dependent on the feeding physiology and, in particular, filter feeding organisms could be exposed to nanoparticles, which can be transferred to higher trophic levels. In our acute test experiments the observed reduction of *A. tonsa* naupliar survival was due to the presence of QDs in the water, as nauplii were not able to feed at this developmental stage, being their oral apparatus not yet fully formed. Our results confirm the hypothesis that organisms, such as benthic algae or first naupliar stages which mostly live on the bottom, are more sensitive to nanoparticle toxicity.

Taking into account our results of the chronic tests, no evident toxicity of QDs was observed in planktonic *A. tonsa* copepods, suggesting that adults are less sensitive to these nanoparticles. The observed reduction of copepod fecundity, after 4-day exposure at the highest QD concentration, could be due both to a direct uptake of nanoparticles and/or to an indirect ingestion mediated by the algal food. In the first case, nanoparticles could limit the ingestion of food leading to a low egg production (Lee et al., 2013; Teixeira et al., 2010), whereas in the second case, nanoparticles, adhering onto the alga surface, can increase toxicity for filter feeding copepods. Recently, Jackson et al. (2012) investigated the effects of QDs on the amphipod *Leptocheirus plumulosus*, showing a higher toxicity when nanoparticles were ingested with the algae.

Chronic tests performed with *A. tonsa* adults showed weak inhibition on copepod fecundity only at the highest QD concentration tested, but not on egg hatching success. Therefore, QD do not reduce copepod offspring through maternal effect, and on early hatched copepods unable to swim along the water column, probably due to QD accumulation and settling on the bottom.

4.2.3 Gene expression of *A. tonsa* exposed to toxicants

Nickel has been found to induce oxidative damage in the benthic copepod *Tigriopus japonicus* (Wang & Wang, 2010) and a modulation of gene expression in the copepod *Pseudodiaptomus ammamdalei* (Jiang et al., 2013; Lee et al., 2008). Similar damages were also found in copepod exposed to $NiCl_2$ and QDs (Hassan & Mohammed, 2014; Bouldin et al., 2008). For a more precise toxicity assessment of NiNPs, $NiCl_2$ and QDs at the molecular level, we investigated the variation of hsp70 gene and ferritin expression levels by RT-PCR, which were involved in regulation of immunization. The heat shock protein family is constitutively expressed in copepods, i.e. playing a role in the recovery phase between quiescent and subitaneous *A. tonsa* eggs (Aruda et al., 2011; Nilsson et al., 2013), or can be induced by environmental stress, including increased temperature (Voznesensky et al., 2004; Rhee et al., 2009) and heavy metal exposure (Kim et al., 2014). Ferritin was known to play an important iron metabolism (Harrison & Arosio, 1996) and to protect proteins

against oxidative damage in the cellular regulation (Reif, 1992). When exposed to toxicants, the expression of ferritin had a up-regulated, to protect proteins (Tarrant et al., 2008).

Our results indicated that hsp70 and ferritin were up-regulated in adults exposed to NiNPs (8.5 and 17 mg/L), the median $NiCl_2$ concentration (0.065 mg/L) and the lowest QD concentration (0.5 nM), with a significant increase recorded after 3 days (Fig. 4.13). Under this condition, neither egg production nor egg hatching success were affected in $NiCl_2$ and QDs group (Fig. 4.3 & Fig. 4.12), suggesting that at low concentrations of toxicants, a defensive molecular mechanism was activated, before any evident biological effects occurred. The similar egg production results were also found in NiNPs group, no effected by toxicants (Fig. 4.9), but the result of egg hatching success were effected significantly, and declined as the increase of NiNPs concentrations. This results indicated that the concentrations of NiNPs could not disturb the production mechanism of eggs, but activate the defensive molecular mechanism of egg incubation.

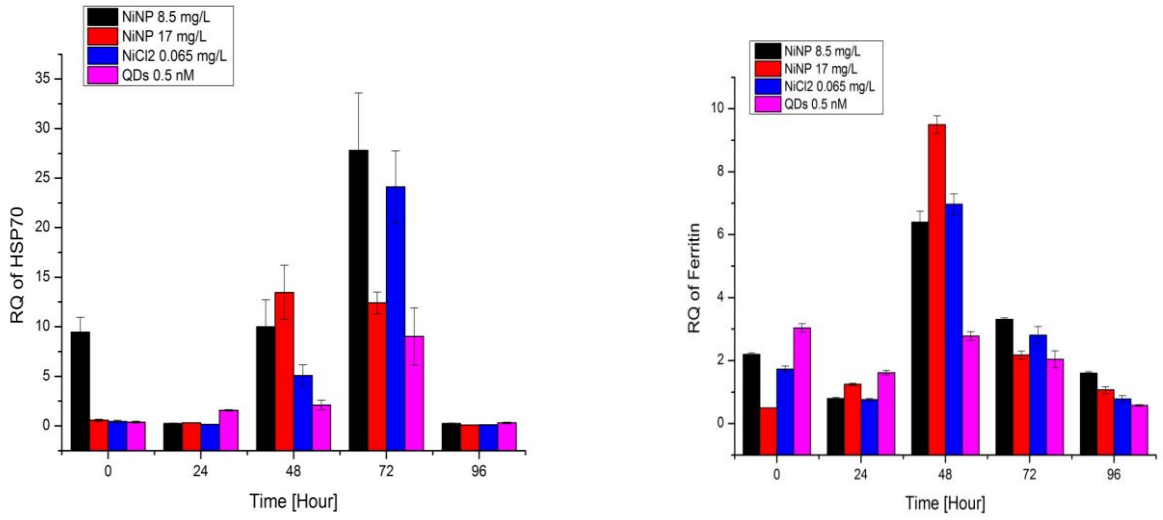


Figure 4.13 Expression levels of hsp70 and ferritin exposed to different toxicants by quantitative RT-PCR analysis. The expression is relative to β-actin, used as reference house keeping gene. Values represent the mean value ± standard error of 3 replicates.

To date, modulation of heat shock proteins was reported in the calanoid species *Calanus finmarchicus* after copepod exposure to pollutants (Hansen et al., 2008). This research of ferritin was also few in copepod field (Nilsson et al., 2013). Gene expression analysis in copepods is still in its infancy, probably due to the difficulties in rearing the organisms necessary for the screening (Ning et al., 2013; Buttino et al., 2012; Lauritano et al., 2012; Carotenuto et al., 2014), although it is considered a powerful tool in ecotoxicology studies (Snell et al., 2003; Lauritano et al., 2015).

Moreover, upregulation of the stress-responsive genes hsp70 and ferritin, observed in QDs, $NiCl_2$ and NiNPs exposed adults of *A. tonsa* in the absence of any evident signal of toxicity, highlights the usefulness of genotoxic biomarkers. Molecular analysis could be an effective early

warning system to evaluate nanoparticle toxic effects in plankton, which can be used as sentinel organisms for the assessment of ecotoxicity in the marine environment.

4.3 Conclusions

Plankton biomass, evaluated in terms of fecundity, should be taken into consideration as its reduction might affect the whole marine productivity. Bioassays carried out with species belonging to different trophic levels are important to gather information about the possible consequences of environmental pollution on biodiversity. For NPs pollution, we have only just started documenting the toxicological effects on marine organisms and their potential impact on marine ecosystems. Furthermore, we do not know the level of contamination in seawater and sediments, neither the amount of NPs discharged.

Considering copepod fecundity, our results indicated that both forms of Ni did not affect egg production rate at the tested concentrations, excluding the effect due to a poor-quality food uptake. Therefore, egg hatching success remains the most sensitive endpoint. Overall, our results indicate that copepods are more sensitive to Ni salts than to NiNPs in the acute and semichronic experiments, respectively.

Chronic tests performed with *A. tonsa* adults showed weak inhibition on copepod fecundity only at the highest QD concentration tested, but not on egg hatching success. Therefore, QD do not reduce copepod offspring through maternal effect, and on early hatched copepods unable to swim along the water column, probably due to QD accumulation and settling on the bottom. The reproductive performance of *A. tonsa* under these stress conditons, which interfered with copepod reproduction and larval viability, provided basic data used for evaluation of the their potential impact on marine ecosystem.

4.4 References

Ambrosone A, Mattera L, Marchesano V, Quarta A, Susha AS, Tino A, Rogach AL, Tortiglione C (2012) Mechanisms underlying toxicity induced by CdTe quantum dots determined in an invertebrate model organism. Biomaterials 33: 1991-2000.

Amiard JC, Amiard-Triquet C, Barka S, Pellerin J, Rainbow PS (2006) Metallothioneins in aquatic invertebrates: their role in metal detoxificationand their use as biomarkers. Aquat. Toxicol 76: 160-202.

Arnaud J, Brunet M, Mazza J (1978) Studies on the midgut of *Centropages typicus* (Copepod, Calanoid). Cell Tissue Res 187: 333-353.

Aruda AM, Baumgartner MF, Reitzel AM, Tarrant AM (2011) Heat shock protein expression during stress and diapause in the marine copepod *Calanus finmarchicus*. J. Insect Physiol 57: 665-675.

Barka S (2007) Insoluble detoxification of trace metals in a marine copepod *Tigriopus brevicornis* (Müller) exposed to copper, zinc, nickel, cadmium, silver and mercury. Ecotoxicology 16: 491-502.

Barka S, Pavillon JF, Amiard JC (2001) Influence of different essential and non-essential metals on MTLP levels in the copepod *Tigriopus brevicornis*. Comp. Biochem. Physiol. C 128: 479-493.

Beaugrand G, Brander KM, Lindley JA, Souissi S, Reid PC (2003) Plankton effect on cod recruitment in the North Sea. Nature 426: 661-664.

Bielmyer GK, Grosell M, Brix KV (2006) Toxicity of silver, zinc, copper, and nickel to the copepod *Acartia tonsa* exposed via a phytoplankton diet. Environ. Sci. Technol 40: 2063-2068.

Bouldin JL, Ingle TM, Sengupta A, Alexander R, Hannigan RE, Buchanan RA (2008) Aqueous toxicity and food chain transfer of quantum dot in freshwater algae and *Ceriodaphnia dubia*. Environmental Toxicology and Chemistry 27(9): 1958-1963

Buttino I, Ianora A, Buono S, Vitiello V, Malzone MG, Rico C, Langellotti A, Sansone G., Gennari L., Miralto A., 2012. Experimental cultivation of the Mediterranean calanoid copepod *Temora stylifera* and *Centropages typicus* in a pilot re-circulating system. Aquaculture Research 43: 247-259.

Buttino I, Pellegrini D, Romano G, Hwang JS, Liu TM, Sartori D, Sun CK, Macchia S, Ianora A (2011) Study of apoptosis induction using fluorescent andhigher harmonic generation microscopy techniques in *Acartia tonsa* naupliiexposed to chronic concentrations of nickel. Hydrobiologia 27 (S2): 97-104.

Campos B, Rivetti C, Rosenkranz P, Navas JM, Barata C (2013) Effects of nanoparticles of TiO2 on food depletion and life-history responses of *Daphnia magna*. Aquat. Toxicol 130–131, 174-183.

Carotenuto Y, Dattolo E, Lauritano C, Pisano F, Sanges R, Miralto A, Procaccini G, Ianora A (2014) Insights into the transcriptome of the marine copepod *Calanus helgolandicus* feeling on the oxylipin-producing diatom *Skeletonema marinoi*. Harmful Algae 31: 153-162.

Casals E, Pfaller T, Duschl A, Oostingh GJ, Puntes VF (2011) Hardening of the nanoparticle–protein corona in metal (Au, Ag) and oxide (Fe3O4, CoO, andCeO2) nanoparticles. Small 7: 3479-3486.

Costa M, Zhuang Z, Huang X, Cosentino S, Klein CB, Salnikow K (1994) Molecular mechanisms of nickel carcinogenesis. Sci. Total Environ 148: 191–199 (special issue).

Corsi I, Cherr GN, Lenihan HS, Labille J, Hassellov M, Canesi L, Pondero F, Frenzilli G, Hristozov D, Puntes V, Della Torre C, Pinsino A, Libralato G, Marcomini A, Sabbioni E, Matranga V (2014) Common strategies and technologies for the ecosafety assessment and design of nanomaterials entering the marine environment. ACS Nano 8: 9694-9709.

DeForest DK, Schlekat CE (2013) Species sensitivity distribution evaluation for chronic nickel toxicity to marine organisms. Integrated environmental assessment and management 9(4): 580-589.

Denkhaus E, Salnikow K (2002) Nickel essentiality, toxicity, and carcinogenicity. Critical reviews in oncology/hematology 42(1): 35-56.

Domingos RF, Simon DF, Hauser C, Wilkinson KJ (2011) Bioaccumulation and effects of CdTe/CdS quantum dots on *Chlamydomonas reinhardtii* – Nanoparticles or the free ions? Environ. Sci. Technol 45: 7664-7669.

Eisler R (1998) Nickel hazards to fish, wildlife, and invertebrates: a synoptic review. U.S. Geological Survey, Biological Resources Division, Biological Science Report USGS/BRD/BSR 1998-0001, Contaminant Hazard Reviews Report No. 34.

Gong N, Shao K, Feng W, Lin Z, Liang C, Sun Y (2011) Biotoxicity of nickeloxide nanoparticles

and bio-remediation by microalgae Chlorella vulgaris. Chemosphere 83: 510-516.

Gorbi G, Invidia M, Savorelli F, Faraponova O, Giacco E, Cigar M, Buttino I, Leoni T, Prato E, Lacchetti I, Sei S (2012) Standardized methods for acute and semi-chronic toxicity tests with the copepod *Acartia tonsa*. Environ. Toxicol. & Chem. 31: 2023-2028.

Haber LT, Diamond GL, Zhao Q, Erdreich L, Dourson ML (2000) Hazard identification and dose response of ingested nickel-soluble salts. Regulatory Toxicology and Pharmacology 31(2): 231-241.

Handy RD, Owen R, Valsami-Jones E (2008) The ecotoxicology of nanoparticles and nanomaterials: current status, knowledge gaps, challenges and future needs. Ecotoxicology 17: 315-325.

Hansen BH, Altin D, Vang SH, Nordthg T, Olsen AJ (2008) Effects of naphthalene on gene transcription in *Calanus finmarchicus* (Crustacea: Copepoda). Aquatic toxicology 86: 157-165.

Hardman R (2006) A toxicologic review of quantum dots: toxicity depends on physicochemical and environmental factors. Environmental Health Perspectives 114: 165-172.

Harrison PM, Arosio P (1996) The ferritins: molecular properties, iron storage function and cellular regulation. Biochimica et Biophysica Acta 1275: 161-203.

Hassan H, Mohammed (2014) Biochemical Response of the Cyclopoida copepod *Apocyclops borneoensis* exposed to Nickel. Jordan Journal of Biological Sciences 7: 41-47.

Hook SE, Fisher NS (2001) Reproductive toxicity of metals in calanoid copepods. Mar. Biol. 138: 1131-1140.

Hughes JB, Kim JH (2007) Monitoring nanoparticles in the environment. Environ. Sci. Technol. 41: 179-184.

ISO International Organization for Standardization, 1999. Waterquality Determination of acute lethal toxicity to marine copepods (Copepoda, Crustacea). In: Draft International Standard ISO/DIS 14669. ISO, Geneve, Switzerland.

ISO International Organization for Standardzation, 2013. Water quality—calanoid copepod development test with *acartia tonsa*. In: Draft International Standard Iso/dis 16778. ISO, Geneve, Switzerland.

Jackson BP, Bugge D, Ranville JF, Chen CY (2012) Bioavailability, toxicity, and bioaccumulation of quantum dot nanoparticles to the amphipod *Leptocheirus plumulosus*. Environ. Sci. & Technol 46: 5550-5556.

Jarvis TA, Miller RJ, Lenihan HS, Bielmyer GK (2013) Toxicity of ZnO nanoparticles to the copepod *Acartia tonsa*, exposed through a phytoplankton diet. Environ. Toxicol. 32: 1264-1269.

Jiang JL, Wang GZ, Mao MG, Wang KJ, Li SJ, Zeng CS (2013) Differential gene expression profile of the calanoid copepod, *Pseudodiaptomus annandalei*, in response to nickel exposure. Comp. Biochem. Physiol. C 157: 203-211.

Kim B, Rhee JS, Jeong CB, Seo JS, Park GS, Lee YM, Lee JS (2014) Heavy metals induce oxidative stress and trigger oxidative stress-mediated heat shock protein (hsp) modulation in the intertidal copepod *Tigriopus japonicus*. Comp. Biochem. and Physiol., Part C 166: 65-74.

Lauritano C, Procaccini G, Ianora A (2012) Gene expression patterns and stress response in marine copepods. Mar. Environ. Res. 76: 22-31.

Lauritano C, Carotenuto Y, Vitiello V, Buttino I, Romano G, Hwang JS, Ianora A (2015) Effects of the oxylipin-producing diatom *Skeletonema marinoi* on gene expression levels of the calanoid

copepod *Calanus sinicus*. Marine Genomics. http://dx.doi.org/10.1016/j.margen.2015.01.007

Lee KW, Raisuddin S, Rhee JS, Hwang DS, Yu IT, Lee YM, Park HG, Lee JS (2008) Expression of glutathione S-transferase (GST) genes in the marinecopepod *Tigriopus japonicus* exposed to trace metals. Aquat. Toxicol. 89:158-166.

Lee KW, Shim WJ, Kwon OY, Kang JH (2013) Size-dependent effects of micropolystyrene particles in the marine copepod *Tigriopus japonicus*. Environ. Sci. Technol. doi:10.1021/es401932b.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25: 402-408.

Manusadzianas L, Caillet C, Fachetti L, Gylyt B, GrigutyteR, Jurkoniene S, Karitonas, R, Sadauskas K, Thomas F, Vitkus R, Férard JF (2012) Toxicity ofcopper oxide nanoparticle suspensions to aquatic biota. Environ. Toxicol. Chem. 31: 108-114.

Mohammed EH, Wang GZ, Jiang JL (2010) The effects of nickel on the reproductive ability of three different marine copepods. Ecotoxicology 19: 911-916.

Morelli E, Cioni P, Posarelli M, Gabellieri E (2012) Chemical stability of CdSe quantum dots in seawater and their effects on a marine microalga. Aquatic Toxicology 122-123: 153-162.

Morelli E, Salvadori E, Bizzarri R, Cioni P, Gabellieri E (2013) Interaction of CdSe/ZnS quantum dots with the marine diatom *Phaeodactylum tricornutum* and the green alga *Dunaliella tertiolecta*: a biophysical approach. Biophys. Chem. 182: 4-10.

Mouynerac C, Buffet PE, Poirier L, Zalouk-Vergnoux A, Guibbolini M, Risso-de Faverney C, Gilliland D, Berhanu D, Dybowska A, Chatel A, Perrein-Ettajni H, Pan JF, Thomas-Guyon H, Reip P, Valsami-Jones E (2014) Fate and effects of metal-based nanoparticles in two marine invertebrates, the bivalve mollusc *Scrobicularia plana* and the annelid polychaete *Hediste diversicolor*. Environ. Sci. Pollut. Res. 21: 7899-7912.

Muñoz A, Costa M (2012) Elucidating the mechanisms of nickel compound uptake: a review of particulate and nano-nickel endocytosis and toxicity. Toxicology and applied pharmacology 260(1): 1-16.

Navarro E, Baun A, Behra R, Hartmann NB, Filser J, Miao AJ, Quigg A, Santsch, PH, Sigg L (2008) Environmental behavior and ecotoxicolgoy of engineered nanoparticles to algae, plants, and fungi. Ecotoxicology 17:372-386.

Nel A, Xia T, Madler L, Li N (2006) Toxic potential of materials at the nano level. Science 311: 622-627.

Nilsson B, Jepsen PM, Rewits K, Hansen BW (2013) Expression of *hsp70* and *ferritin* in embryos of the copepod *Acartia tonsa* (Dana) during transition between subitaneous and quiescent state. doi 10.10937plankt/fbt099

Nott JA, Corner EDS, Mavin LJ, O'Hara SCM (1985) Cyclical contributions ofthe digestive epithelium to faecal pellet formation by the copepod *Calanus helgolandicus*. Mar. Biol. 89: 271-279.

Petersen EJ, Henry TB, Zhao J, MacCuspie RI, Kirschling TL, Dobrovolskaia MA, Hackley V, Xing B, White JC (2014) Identification and avoidance ofpotential artifacts and misinterpretations in nanomaterial ecotoxicity measurements. Environ. Sci. Technol. 48: 4226-4246.

Quigg A, Chin WC, Chen CS, Zhang S, Jiang Y, Miao AJ, Schwehr KA, XuC, Santschi PH (2013) Direct and indirect toxic effects of engineered nanoparticles on algae: role of natural organic matter. ACS Sustain. Chem. Eng. 1: 686–702. http://dx.doi.org/10.1021/sc400103x

Rainbow PS (2006) Trace metal bioaccumulation: model, metabolic availability and toxicity. Environ. Int. 30: 67-78.

Reif DW (1992) Ferritin as a source of iron for oxidative damage. Free Radical Biology & Medicine 12: 417-427.

Rhee LS, Raisuddin S, Lee KW, Seo JS, Ki JS, Kim IC, Park HG, Lee JS (2009) Heat shock protein (hsp) gene responses of the intertidal copepod *Tigriopus japonicus*. Comp. Biochem. and Physiol., Part C 149: 104–112.

Rocha TL, Gomes T, Cardoso C, Letendre J, Pinhero JP, Serrao Sousa V, Ribau Teixeira M, Bebianno MJ (2014) Immunocytotoxicity, cytogenotoxicity and genotoxicity of cadmium based quantum dots in the marine mussel *Mytilus galloprovincialis*. Mar. Env. Res. 101: 29-37.

Sillampää M, Paunu TM, Sainio P (2011) Aggregation and deposition ofengineered TiO2nanoparticles in natural fresh and brackish waters. J. Phys. Conf. Ser. 304: 012018.

Snell TW, Brogdon SE, Morgan MB (2003) Gene expression profiling in ecotoxicology. Ecotoxicology 12: 475-483.

Tarrant AM, Baumgartner MF, Verslycke T, Johnson CL (2008) Differential gene expression in diapausing and active Calanus finmarchicus (Copepoda). Marine Ecology Progress Series 355: 193-207.

Stolpe B, Hassellov M (2007) Changes in size distribution of fresh water nanoscale colloidal matter and associated elements on mixing in seater. Geochim. Cosmochim. Acta 71: 3292-3301.

Teixeira PF, Kaminski SM, Avila TR, Cardozo AP, Bersano JGF, Bianchini A (2010) Diet influence on egg production of the copepod *Acartia tonsa* (Dana, 1896). An. Acad. Bras. Cienc. 82: 333-339.

US Environmental Protection Ageny (1986) Test methods for the evaluation of solid wastes, SW-846. US EPA, Cincinnati.

Vijayavel K, Gopalakrishnan S, Thiagarajan R, Thilagam H (2009). Immunotoxic effects of nickel in the mud crab Scylla serrata. Fish & shellfish immunology 26(1): 133-139.

Voznesensky M, Lenz PH, Spanings-Pierrot C, Towle DW (2004). Genomic approaches to detecting thermal stress in Calanus finmarchicus (Copepoda: Calanoida). Journal of Experimental Marine Biology and Ecology 311(1): 37-46.

Wang M, Wang G (2010) Oxidative damage effects in the copepod *Tigriopus japonicus* Mori experimentally exposed to nickel. Ecotoxicology 19: 273-284.

Zhang J, Wu C, Pellegrini D, Romano G, Esposito F, Ianora A, Buttino I (2013) Effects of different monoalgal diets on egg production, hatching success and apoptosis induction in a Mediterranean population of the calanoid copepod *Acartia tonsa* (Dana). Aquaculture 400-401: 65-72.

5 TOPIC 3: Transcriptome and gene expression analyses (how copepods respond to toxic s tress in genomic response)

5.1 Materials and methods

5.1.1 Sample preparation and sequencing

Almost 3000 healthy *A. tonsa* adults were collected from the main culture tank and acclimatized 3 days in 800 ml beaker, at a density of 1 adult per ml, and fed with a mix algal food as described above (paragraph 3.1.2). Afterward, almost 500 adults were transferred to 1L beaker and exposed to

toxicants at the following concentrations: 0.065 mg/l $NiCl_2$, 8.5 mg/L and 17 mg/L NiNPs and 0.5 nM QDs. A control group were performed without toxicant. After 4 days of exposure, 40 to 60 adults were collected and transferred in 2 mL tubes containing 1 mL of TRIZOL® Reagent (InVitrogen, San Diego, CA, US), then the tubes were frozen in liquid nitrogen and stored at -80 ℃. Almost 6-8 tubes were prepared for each treatment. Two replicates were performed for each exposure and for the control.

Frozen animals were thawed and homogenized with Tissue Lyser (Qiagen, Austin, TX, US) using 3 mm sterile tungsten carbide beads at 20.1 Hz for 3 min. A further break of the copepod body was obtained by passing 5-6 time through a 0.1 mm syringe-needle. After centrifuging at 12,000 x g for 10 min at 4 ℃, samples were processed by following the manufacturer instructions to isolate RNA and purified further with a TURBO DNAfree Kit (Ambion, Austin, TX, USA). The quantity of total RNA was analyzed using a 1% agarose gel. Nanodrop, Qubit 2.0, Aglient 2100 were used to test the purity, concentration and integrity of RNA, respectively.

5.1.2 Transcriptome analysis

Sequencing analyses were performed by Biomarker Technologies Co., LTD (China) Company. The analysis process of transcriptome sequencing bioinformatics were shown in Fig 5.1.

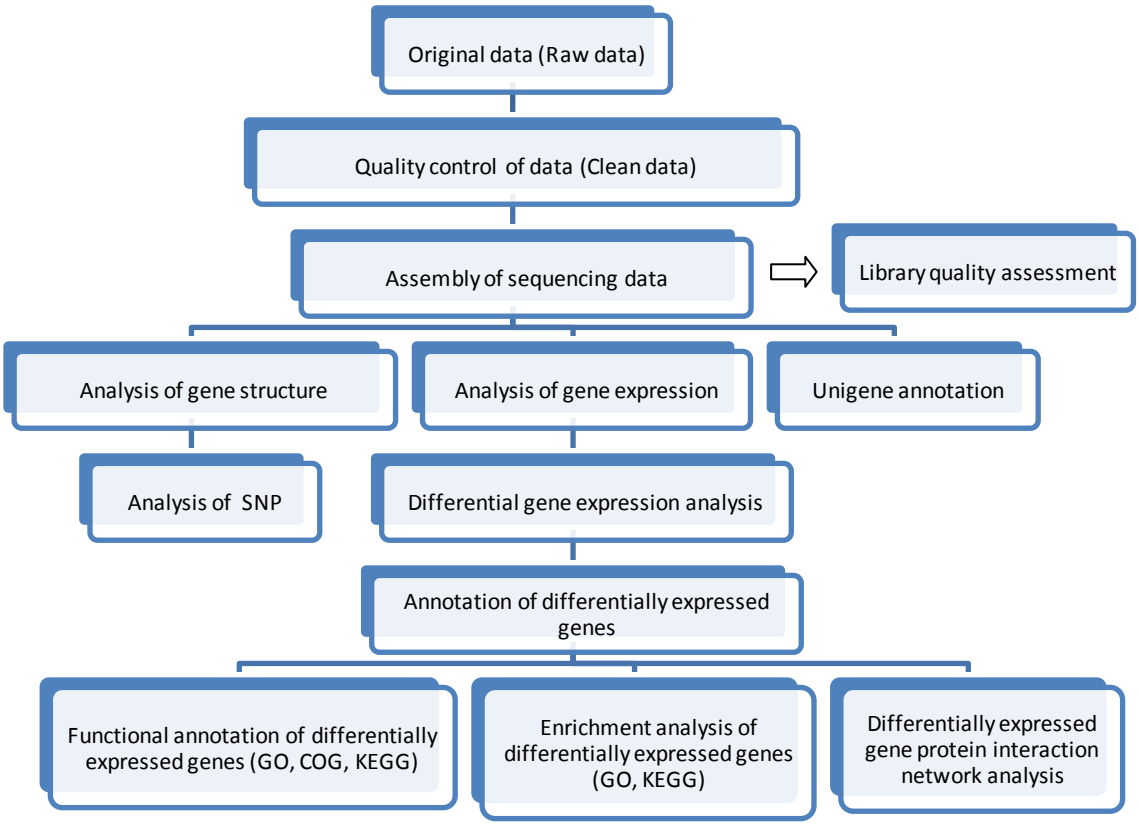


Figure 5.1 Analysis process of transcriptome sequencing bioinformatics

Sequence data analysis and assembly

Five types of cDNA samples, which came from the groups of *A. tonsa* adults exposed to NiNPs, $NiCl_2$, QDs and the control were prepared and sequenced using the Illumina HiSeq2500 platform, which is based on Sequencing By Synthesis technology. By this platform, a lot of reads were obtained, which were called raw data. We got high quality clean reads by filtering raw data. Trinity software, consisting in 4 elements of analysis (short fragments K-mer, contig, components and transcripts) was used to break reads into short fragments and then screened for adapter K-mer and eliminating poor quality sequences with the default parameters. These K-mers were extended to contig and the overlaps were analyzed to make components. Finally, the Trinity software test these components to make transcripts.

The linker sequence and low-quality reads were filtered out from the raw data, to obtain high quality Clean Data. All reads shorter than 40 bp were removed based on an assumption that small reads would fail to assemble or belong to sequencing artifacts. The clean data for sequence assembly were obtained by Trinity assembly program from size-selected reads, where contigs were incorporated into component, then identificated to making the species unigene database for subsequent analyses. Unigenes are groups of components which represents a transcript variant. Therefore, unigenes may represent putative genes.

Sequence annotation

Unigene sequences were mapped against the following databases: National Center for Biotechnology Information (NCBI) non-redundant protein database (Nr), Swiss-Prot, Gene Ontology (GO), Clusters of Orthologous Groups (COG), EuKaryotic Orthologous Groups (KOG) and Kyoto Encyclopedia of Genes and Genomes (KEGG) using BLAST software(e-value $\leqslant 10^{-5}$). Thereafter, the information of amino acid sequence predicted were mapped against Protein families database (Pfam), a large collection of protein families represented by multiple sequence alignments and hidden Markov models (HMMs), using HMMER software (e-value $\leqslant 10^{-10}$), to obtain annotation information of Unigene.

Swiss-Prot is the manually annotated and reviewed section of the UniProt Knowledgebase (UniProtKB). It is a high quality annotated and non-redundant protein sequence database, which brings together experimental results, computed features and scientific conclusions.

Gene ontology (GO) is a major bioinformatics initiative to unify the representation of gene and gene product attributes across all species.

Clusters of Orthologous Groups (COG) is generated by comparing predicted and known proteins in all completely sequenced microbial genomes to infer sets of orthologs.

EuKaryotic Orthologous Groups (KOG) is a eukaryote-specific version of the Clusters of Orthologous Groups (COG) tool for identifying ortholog and paralog proteins.

Kyoto Encyclopedia of Genes and Genomes (KEGG) is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies.

Using the KEGG database, we further studied the complex biological behaviors of genes and determined pathway annotations for sequences.

Differential gene expression analysis

The reads collected from each samples were mapped against Unigene database using Bowtie program (Langmead et al., 2009). According to the comparison results, we used RNA-Seq by Expectation-Maximization (RSEM) to estimate the performed gene expression level (Li et al., 2011). The expression level of a transcript was quantified in fragments per kilobase of transcript per million mapped reads (FPKM) in the transcriptome (Formula 5.1), where FPKM can eliminate the influence of gene sequencing length and the differences of sequencing amount on gene expression, and the results calculated can be directly used for the gene differential expression between different samples.

$$\mathrm{FPKM} = \frac{\mathrm{cDNA\ Fragments}}{\mathrm{Mapped\ Fragments(Millions)} \times \mathrm{Transcript\ Length(kb)}}$$

Formula 5.1 cDNA Fragments: number of fragments mapped to a transcript (number of double-ended Reads); Mapped Fragments (Millions): total number of fragments mapped to transcripts, with 10^6 as one unit; Transcript Length (kb): transcript length, with 10^3 base as one unit.

For biological replicates of experiments, we used DESeq, which is an R package to analyse counting data from high-throughput sequencing assays such as RNA-Seq and test for differential expression, to differentiate groups of samples expression analysis (Anders et al., 2010). R is a free software environment for statistical computing and graphics. Differentially expressed genes were identified in treated groups of *A. tonsa* by a Differentially Expressed Gene sequence (DEGseq) package using the MA-plot-based method with Random Sampling model method (MARS). Genes were considered differentially expressed when changes between gene expressions were more or equal to 2 in the Formula 5.1, and with 1% or less false discovery rate (FDR) considered. Differentially expressed genes were evaluated as up-regulated if the expression levels in a group was significantly higher than in the other group. Down-regulated genes were evaluated as up-regulated if the expression levels in a group was significantly lower than in the other group. A

Fisher's exact test was used with a threshold of 5% as the false discovery rate to determine the differentially regulated genes in the two samples for each GO term.

Real-time PCR (RT-PCR)

The extraction and purification of total RNA followed procedures reported in the paragraph 4.1.7. The quality of total RNA were analyzed as for the transcriptome sequencing reported in the paragraph 5.1.1. From each sample, 5 µg of RNA was purified and reverse-transcribed into cDNA using PrimeScriptTM II 1st strand cDNA Synthesis Kit (Takara, Japan). Real time amplification was performed in a 7500 Fast Real-Time PCR System (ABI, USA) using TAKARA SYBR® Premix Ex TaqTM II (Tli RNaseH Plus) (Takara, Japan). Each quantitative RT-PCR reaction contained DNA from 50 to 100 ng.

The significant up- or down- regulated expressed genes associated with heat shock protein 70 (HSP70) performance were validated by quantitative RT-PCR. β-actin gene was chosen and set as reference, to normalize the target gene quantities, because this gene was used as a referent gene in copepod species by other researchers (Ning et al., 2013; Nilsson et al., 2014).

Primers(Nilsson et al., 2014)**:**

β-actin:

Forward: 5'-CTTCTGCATACGGTCAGCAA-3';

Reverse: 5'-ACCCGTACGCCAACACTG-3'.

HSP70 :

The RT-PCR conditions were as follows: 94 °C/10 min; 40 cycles of 94 °C/20 s, 60 °C/45 s. The RT-PCR primers for the hsp70 gene were:

Forward: 5'-TTCAATGATTCACAGAGACAAGC-3';

Reverse: 5'-TCCTTGTGATGTTAAGACCAGCTAT-3'.

Statistical analysis

Statistical analysis follows the procedure in the paragraph 4.1.6.

Single Nucleotide polymorphism (SNP) discovery

SOAPsnp (Short Oligonucleotide Analysis Package) is an accurate consensus sequence builder based on soap1 and SOAPaligner/soap2's alignment output. It calculates a quality score for each consensus base, which can be used for any latter process to call SNPs. SOAPsnp software was used to identify reliable SNP loci, using an arbitrary criterion of at least 4 reads supporting the consensus or variant and a similarity of 95%. It can be observed polymorphic loci in some of gene sequences, by further analysis, to see if these SNP loci affect the types of gene expression level.

5.2 Results and discussion

Sequencing results and assembly

Illumina sequencing of the five *A. tonsa* samples yielded 17-18 million pair-end reads in 4000 million base read numbers per each group (Table 5.1). From all *A. tonsa* transcriptome data, 17,535,002 reads were generated from the control group, 17,589,899 reads were from the group exposed to NiNPs 8.5 mg/L, 18,949,417 reads were found in the group exposed to NiNPs 17 mg/L, 17,161,508 reads were collected from the group exposed to $NiCl_2$ 0.065 mg/L and 18,032,489 reads were shown in the group exposed to 0.5nM QDs. All groups had high GC content (>46%) and all the clean data were proved to keep a high level (%⩾Q30>87%). In order to obtain a measure of completeness of the assembly from the full set of reads, a series of Trinity assemblies was generated from 17 million to the complete 89 million reads dataset. These results suggest that each assemblies could be obtained from as few as 17 million reads, which is not surprising given that Trinity software is designed to generate good assemblies even when coverage is low as no reference genome (Grabherr et al., 2011).

Samples	Read Number	Base Number	GC Content	%⩾Q30
Control	17,535,002	4,417,479,615	46.64%	87.67%
NiNP 8.5 mg/L	17,589,899	4,431,224,959	47.03%	87.40%
NiNP 17 mg/L	18,949,417	4,774,323,622	46.41%	87.66%
$NiCl_2$ 0.065 mg/L	17,161,508	4,323,324,257	46.57%	87.01%
QDs 0.5 nM	18,032,489	4,542,344,209	47.14%	87.75%

Table 5.1 Assessment statistics of sample sequencing data. Read Number: Number of pair-end Reads in Clean Data; Base Number: Total base number in Clean Data; GC Content: Percentage of content of G base and C base in Clean Data; %≥Q30: Percentage of Clean Data's quality value, which greater or equal to 30 bases occupied.

All reads were assembled into 14,819,154 contigs, 148,845 transcripts and 96,052 unigenes (Table 5.2). The mean number of contigs per transcript was 99.6. The transcript N50 length, which means 50% of the bases were incorporated into transcript r1157 bp, was 1157 bp. We found that 96,052 unigenes have 148,845 transcript, the average number of transcripts per unigene was 1.6.Size distribution of contigs, transcripts and unigenes were shown in Fig. 5.2. Analysis of the frequency distribution of number of reads showed that more than 75% reads mapped to the predicted transcripts. These estimates suggest that the coverage obtained for the *A. tonsa* transcriptome is higher than those obtained in other crustacean transcriptomics studies (Riesgo et al., 2012; Jung et al., 2011; Zheng et al., 2011; Ning et al., 2013).

Mean # Clean reads (pair-end reads)	17,853,663
Unigene	96,052
Transcript	148,845
Transcript N50	1,157
Mean # Transcripts per unigene	1.6
Contigs	14,819,154
Mean # contigs per transcript	99.6

Table 5.2 Summary of the sequencing and assembly of the *A. tonsa* transcriptome

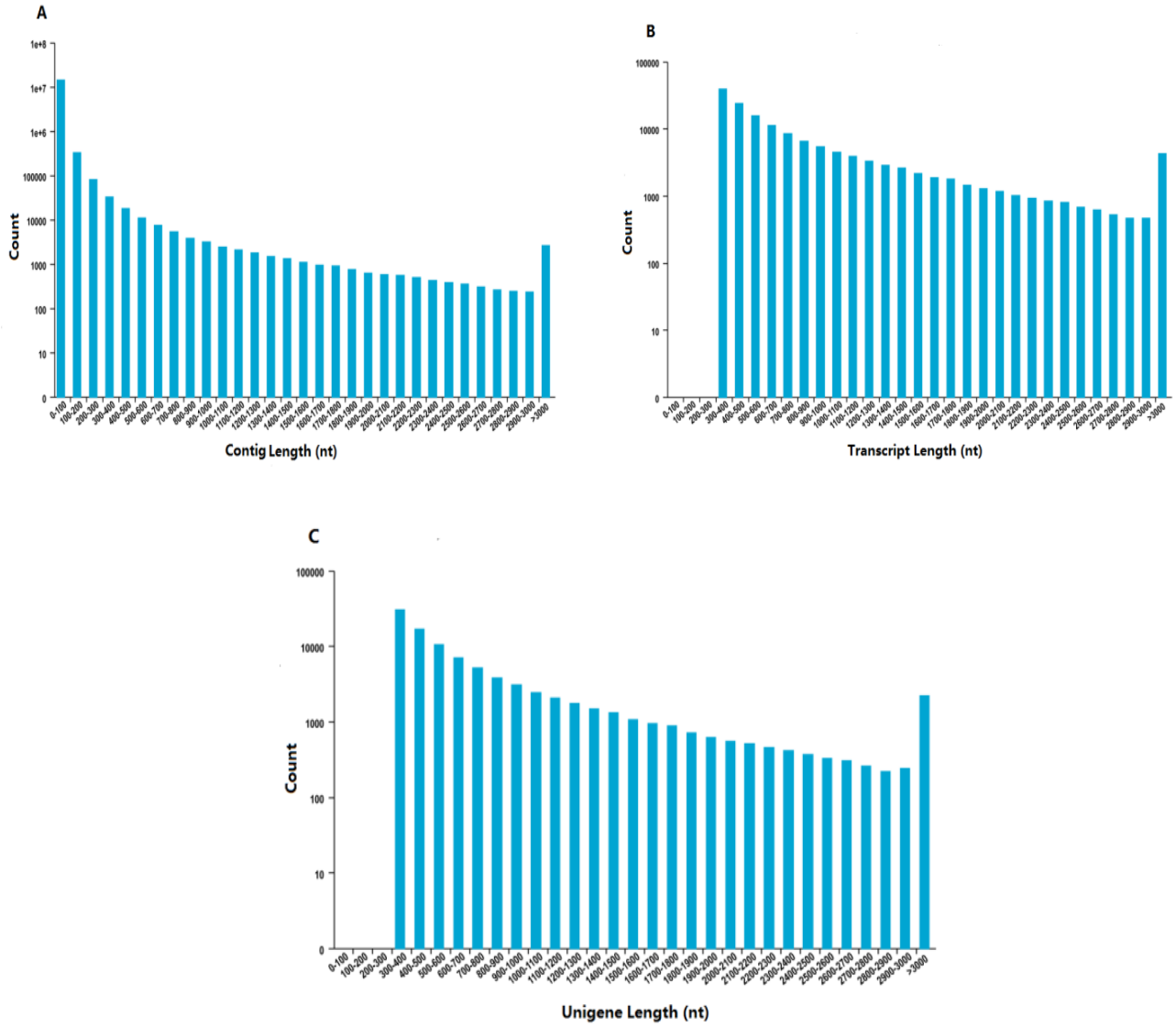


Figure 5.2 Overview of *A. tonsa* transcriptome sequencing and assembly by Illumina HiSeq2500 platform sequencing. (A) Size distribution of contigs. (B) Size distribution of transcripts. (C) Size distribution of unigenes.

Transcriptome annotation: BLAST results

In order to simplify the sequencing data, we just focused on the results of longer length transcripts (≥ 300bp). The total transcript of 148,845 were matched to 96,052 unigenes and 57,102

were matched to proteins with known functions (Table 5.2 & Table 5.3). These obtained data could be used for deeper sequencing, using longer assembled sequences, accordingly to the sequences identified by BLAST against different databases. The annotation informations of unigene in different databases were reported in Table 5.3. For the unidentifiable reads, there were two kinds of possibilities: sequence maybe originate from untranslated regions (UTRs) or should be a non-conserved portions of protein-coding sequences.

Annotated databases	Unigene	≥300 bp	≥1000 bp
COG	18,636	18,636	6,748
GO	14,836	14,836	5,051
KEGG	19,162	19,162	7,048
KOG	35,707	35,707	12,680
Pfam	29,824	29,824	12,682
Swiss-Prot	38,457	38,457	14,008
nr	54,212	54,212	16,822
All	57,102	57,102	17,081

Table 5.3 Annotation statistics of unigene. the results are referred to transcripts, all m 300bp. bp: base-pairs, COG: Clusters of Orthologous Groups, GO: Gene ontology, KEGG: Kyoto Encyclopedia of Genes and Genomes, KOG: EuKaryotic Orthologous Groups, Pfam: Protein families database and Nr: non-redundant protein database.

GO database is a major bioinformatics initiative to unify the representation of gene and gene product attributes across all species. In this study, this database was used to analyze the functional categories of the genes sequenced. In order to assess the representation of cellular components, molecular functions and biological processes among the assembled compounds in the *A. tonsa* transcriptome, the distribution of GO terms were collected and analyzed in Fig. 5.3. We obtained total 49,044 GO terms: 15.6% related to cellular components, 40.5% to molecular functions, and 43.9% related to biological processes.

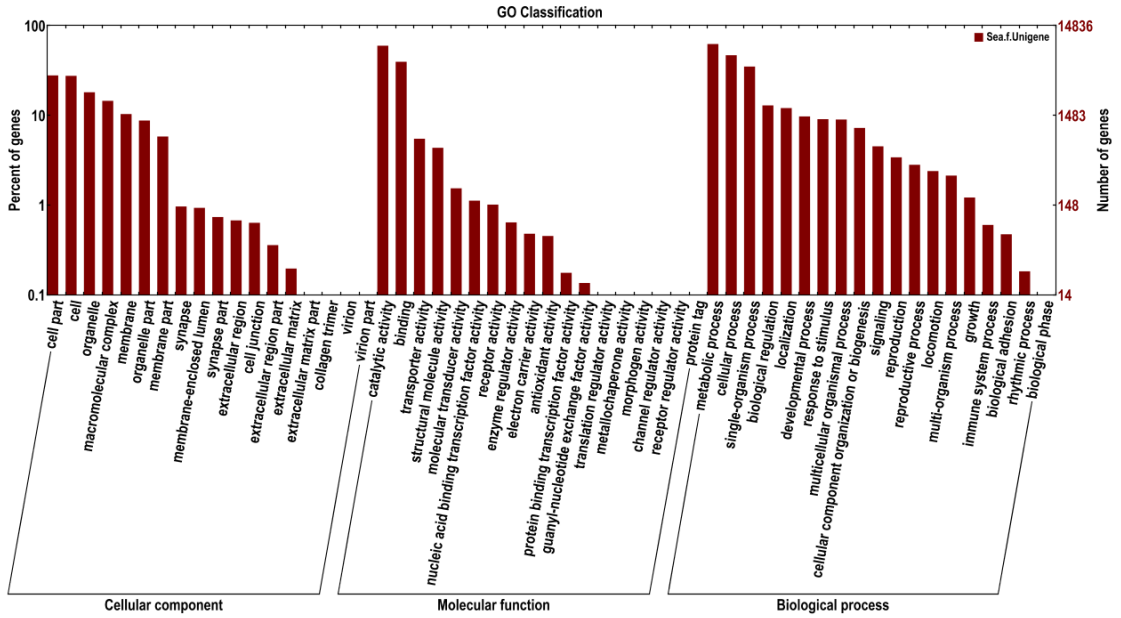


Figure 5.3 GO term distribution of BLAST hits from the *A. tonsa* transcriptome. Selected GO categories are shown within the top-level divisions of Cellular Component, Biological Process, and Molecular Function. The relative percentages of genes fall into GO categories.

The automated BLAST2GO annotation for GO database was not able to process any of the very long components, which was limited to nucleotide sequences shorter than 8,000 bp. Thus, we translated these components ≥8,000 bp) into predicted proteins using an online translation tool (web.expasy.org/translate/?). These translated sequences were manually entered into BLAST protein online and searched against Nr protein database. The unigenes were against Nr protein database for annotation, by blast. The assembled sequences were searched against the Nr and Swiss-Prot protein databases using the blastx algorithm. Searching against the nr database resulted in 54,212 unigenes (54,212 ≥300 bp, 16,822 ≥1000 bp) (Table 5.3). A large of the comps with no blast hits were short., less than 300 bp, probably represent partial transcripts, which no produce the ‘‘no blast hit’’ result. Blastx results using Swiss-Prot database as the reference database, for annotated and reviewed, comprised 38,457 unigenes.

19,162 annotated sequences were significant matched in the KEGG database. Among the matched sequences, 6,396 sequences with enzyme commission (EC) numbers were assigned to metabolic pathways. Meanwhile, 1752, 2001, 7517, 3003 sequences were assigned to environmental information processing, organismal systems, genetic information processing and cellular processes, respectively (Table 5.3 and Fig. 5.3).

The metabolic pathways that were well represented in the *A. tonsa* sequences were energy metabolism, carbohydrate metabolism, lipid metabolism, nucleotide metabolism and amino acid metabolism. Storage lipids in zooplankton play an important role during its reproduction, as shown by studies of spices distributed in various oceanic regions (Irigoien, 2004; Lee et al., 2006). In order

to explore reproductive response of *A. tonsa* under stress conditions in transcriptome level, we focused greater attention on lipid metabolism. Genes were found in several pathways involved with Lipid biosynthesis (Table 5.4).

KEGG pathway	Number of genes	Number of sequences
Metabolism	**4378**	**6396**
Energy metabolism	440	553
Global and overview maps	165	209
Xenobiotics biodegradation and metabolism	120	200
Biosynthesis of other secondary metabolites	8	11
Glycan biosynthesis and metabolism	269	464
Carbohydrate metabolism	919	1324
Lipid metabolism	501	778
Metabolism of terpenoids and polyketides	50	82
Nucleotide metabolism	454	654
Metabolism of other amino acids	296	403
Metabolism of cofactors and vitamins	321	490
Amino acid metabolism	835	1228
Environmental Information Processing	**1170**	**1752**
Signaling molecules and interaction	188	313
Signal transduction	878	1295
Membrane transport	104	144
Organismal Systems	**1342**	**2001**
Endocrine system	383	572
Excretory system	47	94
Immune system	330	461
Circulatory system	96	144
Environmental adaptation	11	21
Sensory system	99	143
Digestive system	174	277
Nervous system	85	114
Development	117	175
Genetic Information Processing	**5594**	**7517**
Replication and repair	315	459
Folding, sorting and degradation	1315	1947
Translation	3208	4056
Translation	756	1055
Cellular Processes	**2078**	**3003**
Transport and catabolism	1249	1942
Cellular community	350	439
Cell growth and death	193	276

Cell motility	286	346
Total	**14562**	**20669**

Table 5.4 KEGG biochemical mappings for *A. tonsa*.

Estimating the number of genes expressed in A. tonsa

Estimating the number of expressed genes is one of the main aims for transcriptome sequencing projects. Through blasting all the sequencing data against different databases, we estimated gene numbers matched by sequences, then assigned gene names to matching assembled sequences, with gene name annotation of the best BLAST match for that sequence. We identified 148,845 transcripts in the *A. tonsa* transcriptome and found 96,052 unigenes expressed in *A. tonsa*. This estimate was much higher than that reported in a previous transcriptome study of another marine copepods (*Tigriopus californicus*: 15,402 unigenes; *Calanus sinicus*: 15,000 unigenes) (Barreto et al., 2011; Ning et al., 2013). By high throughput sequencing technology progress, more unigene information of transcriptome were obtained in *A. tonsa*. Based on the abundant unigenes, we can get increasing number of expressed genes, which are annotation.

From unigenes we successfully collected 57,102 sequences with gene names in the total dataset (57,102 of the sequences ≥300 bp and 17,081 of the sequences ≥1000 bp) (Table 5.3). Among the 57,102 annotated best hits, 10,482 different gene names were assigned. As many, also in our study no fully sequenced genome was produced after sequencing project. In fact, almost 40.6% of genes expressed still did not match well in public sequence databases, failed to be assigned gene names. This is common problem shown in non-reference organism, as no enough annotation genes in online databases (Li & Colin, 2011).

Genes involved with reproduction and immunization

As model species and link between phytoplankton and carnivores, the reproduction of *A. tonsa* is of particular interest to researchers. In this transcriptomic sequencing, a largely diversity of candidate genes involved reproductive process and immunization process genes were identified by BLAST in Swiss-Prot, GO, COG, KOG and KEGG database for annotations (Table 5.3).

Reproductive and immunization processes are important biological processes in the life cycle of copepods. In the sequencing data, *A. tonsa* transcriptome was searched for these two kinds of key related functional genes. Lipids in copepod play an important role during egg production. 13 genes, including 19 sequences, were collected in lipid biosynthesis. Juvenile hormone (JH) in arthropods is key molecules that regulated egg production and egg hatching success (Dubrovsky, 2005). As a key hormone related to reproductive maturation in the regulation of the organism life cycle, JH biosynthesis and response genes were also identified (Riddiford, 2008), and 6 JH sequences were found in *A. tonsa*. Sperm protamines, one of small, arginine-rich, nuclear proteins, can replace

histones in the spermatogenesis and are believed essential for sperm head condensation and DNA stabilization. 5 short sequences of protamines (300-400 bp) were shown in the transcriptome of *A. tonsa* (Table 5.5).

HSP are defined as a family of proteins, which produced by cells in response to exposure to stressful conditions, and firstly known in the heat shock response (Ritossa, 1962), but now found to also be expressed during other stresses, such as cold, UV light and so on (Matz et al., 1995; Cao et al., 1999). Now in the new findings of researcher show that metal toxicity exposure, also including nanoparticles, can regulate response of HSP of organism (Ahameda et al., 2010). As a super family of molecular chaperones, HSP regulate immunization process in organisms, to prevent irreversible protein denaturation during stress, required for stress tolerance (Vos et al., 2008). We identified HSP40, HSP60, HSP70, HSP90, HSP92 and HSP100 in different length range, which response to toxicants (Table 5.5). Ferritin was known to play an important iron metabolism (Harrison & Arosio, 1996) and to protect proteins against oxidative damage in the cellular regulation (Reif, 1992). When exposed to toxicants, the expression of ferritin had a largely up-regulated, to protect proteins (Tarrant et al., 2008). There were 14 sequences with 300-1800 bp found in ferritin gene.

Process	**Num**	**Length (range)**
Reproductive process		
Beta-ketoacyl-CoA synthase	1	2484
Fatty acid hydroxylase domain-containing protein 2-like	3	1632-2085
Fatty acid hydroxylase superfamily	1	2110
Palmitoyl-protein thioesterase 1	1	880
Fatty acyl-CoA desaturase	1	1162
Fatty acid desaturase; Rubrerythrin	3	1471-1521
Putative fatty acyl-CoA desaturase	1	654
Delta(6)-fatty-acid desaturase fat-3	1	615
Omega-6 fatty acid desaturase	1	546
Fatty acid desaturase	1	1470
Delta(12) fatty acid desaturase fat-2	3	584-1289
Elongation of very long chain fatty acids protein	1	1345
Peroxisomal acyl-CoA oxidase	1	1636
Juvenile hormone	6	1152-2505
Sperm protamine	5	303-496
Serine/threonine-protein kinase PLK4	1	482
60S ribosomal protein L5, 6, 7, 10, 10a, 12, 17, 23, 26, 27, 35, 37	14	307-2049

40S ribosomal protein S2, S5, S7, S14, S26, S27a, S30	9	341-545
Actin-3	1	447
Immunization		
Heat shock protein 40	1	1343
Heat shock protein 60	1	644
Heat shock protein 70	20	380-1605
Heat shock protein 90	14	310-2286
Heat shock protein 92	4	376-2471
Heat shock protein 100	1	2286
Ferritin	14	383-1877
CAAX prenyl protease 2	2	486-1188
Catalase	1	1916
Dual oxidase	2	495-5774
Conserved regulator of innate immunity protein 3 (Precursor)	1	1022
Probable GH family 25 lysozyme 3 (Precursor)	1	415
UDP-sugar transporter sqv-7	1	1106
Interleukin enhancer-binding factor 2 homolog	1	1543
PYGGYGW-amide (Precursor)	2	409-664
Profilin	4	494-1284
Cytochrome b5	1	1392
Ras-related protein Ral-a (Precursor)	1	1108
Spore coat protein SP96	1	488
Peroxisomal catalase 1	2	1732-1849
Profilin-A	1	379
Leucine-rich repeat-containing protein let-4 (Precursor)	1	421
Catalase-A	1	1771
ESCRT-I complex subunit tsg101	1	399
Putative UDP-sugar transporter	1	367
Dual specificity mitogen-activated protein kinase kinase mek-2	1	546
Serine/threonine-protein kinase dst4	1	414
SUMO-activating enzyme subunit 2	1	363
Mitogen-activated protein kinase kinase kinase A	1	312
Transcription factor btd	1	482
Protein toll (Precursor)	1	4666
Voltage-dependent calcium channel type A subunit alpha-1	5	310-1136

Peroxisomal catalase	1	415
3-phosphoinositide-dependent protein kinase 1	1	1442
Helicase domino	1	6948
RAC family serine/threonine-protein kinase homolog	1	1545
Vacuolar protein sorting-associated protein 18 homolog	1	605
Sumo-conjugating enzyme ubc9	1	511
Stress-induced-phosphoprotein 1	1	2379

Table 5.5 Selected reproductive and immunization process genes identified in the *A. tonsa* transcriptome.

Differentially expressed genes in different groups

Genes were considered differentially expressed when FPKM changes between expressions were more or equal to 2, and with 1% or less FDR considered. Differentially expressed genes were evaluated as up-regulated if the expression levels in a group was significantly higher than in the other group. Down-regulated genes were evaluated as up-regulated if the expression levels in a group was significantly lower than in the other group. A Fisher's exact test was used with a threshold of 5% as the false discovery rate to determine the differentially regulated genes in the two groups.

96,052 expressed genes were identified in each group. We found 65 genes were differentially expressed between the control and NiNPs 8.5 mg/L groups i.e., 47 sequences were categorized as up-regulated genes, and 18 sequences were categorized as down-regulated genes, with control as reference (Fig. 5.4). Few numbers of differentially expressed genes were found between control and NiNPs 8.5 mg/L groups. 198 genes were differentially expressed between the control and NiNPs 17 mg/L group i.e., 76 sequences were categorized as up-regulated genes, and 122 sequences were categorized as down-regulated genes, with control as reference. More differentially expressed genes were shown between NiNPs 8.5 mg/L and NiNPs 17 mg/L groups, with NiNPs 8.5 mg/L as reference. 263 genes were differentially expressed between these two groups (189 up-regulated genes and 74 down-regulated genes). 986 genes were differentially expressed between the control and $NiCl_2$ 0.065 mg/L groups, with control as reference (115 up-regulated genes and 871 down-regulated genes). 1083 genes were differentially expressed between the control group and 0.5nM QDs group, with control as reference (530 up-regulated genes and 553 sequences down-regulated genes). By evaluating results above, high levels of differentially expressed genes were found in $NiCl_2$ 0.065 mg/L and 0.5nM QDs groups, compared with control.

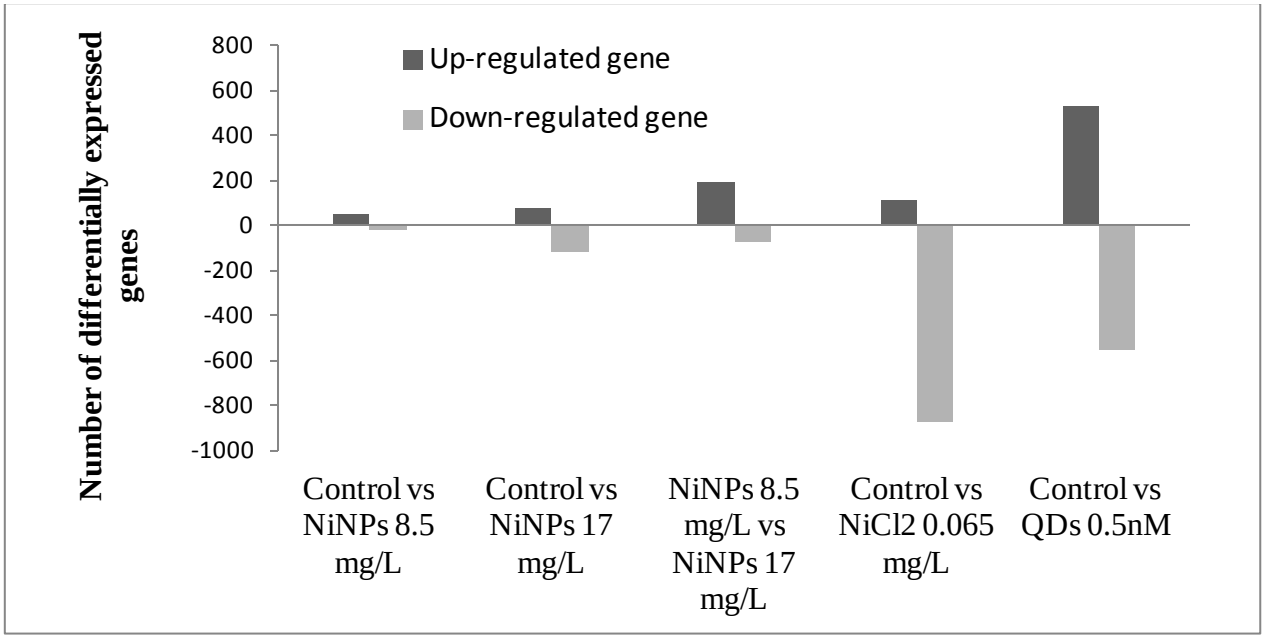


Figure 5.4 Differentially expressed genes between different groups.

We predicted annotations for 47 genes in 65 differentially expressed genes, with 45 matched different proteins, between control and NiNPs 8.5 mg/L groups; 165 genes in 198 differentially expressed genes, with 159 matched different proteins, between control and NiNPs 17 mg/L groups; 863 genes in 986 differentially expressed genes, with 854 matched different proteins, between control and $NiCl_2$ 0.065 mg/L groups; 987 genes in 1083 differentially expressed genes, with 949 matched different proteins, between control and 0.5nM QDs groups; 232 genes in 263 differentially expressed genes, with 221 matched different proteins, between NiNPs 8.5 mg/L and NiNPs 17 mg/L groups. By the GO enrichment of differentially expressed genes, we found some represented GO categories, for instance, catalytic activity, binding, motor activities and kinase activity.

In order to explore the mechanism of reproductive and immunization processes of *A. tonsa* exposed to different contaminants, we searched and identified genes involved with the regulation of these two biology processes. Some regulation genes, like HSP family involved in immunization process, were differentially expressed among different groups (Table 5.6). 15 heat shock protein compounds for immunization response (HSP 40, HSP 60, HSP 70, HSP 90, HSP 92, HSP100) with different expression were identified (Table 5.6). Our results showed that in all groups, HSP70 kept in a high expression level (50-120 FPKM), with moderate expression level in HSP90 and 92, and low expression level in HSP40, HSP60 and HSP100 (Table 5.6). We indicated that no significantly differences were found in HSP70 and HSP90 expression between control and each toxic group after 4 days. The results of egg production in each group also were no effected significantly (see paragraph 4.2). It may mean that these concentrations of toxicants did not activate the production mechanism of eggs.

Some researchers found that lipid are important for organism development, but the regulation mechanism of lipid metabolism is still a fuzzy area and an active area (Vrablik & Watts, 2012). Lipid biosynthesis is one of core regulation reaction for reproductive process of copepod. In *A. tonsa*, lipid store plays an important role in the life history, which refers to reproduction, resistance to stress and so on (Lee et al., 2006; Johnson et al., 2011). As eggs production of copepod need a lot of energy, so, after reproductive process completed, the female shows a lower lipid content and smaller oil sac than the last stage (Fig. 5.5).

Accumulation of lipid droplets and yolk lipovitellin in the developing ovary and oocytes are the reproductive features of copepod (Cuzin-Roudy & Amsler 1991; Ianora & Santella 1991; Laabir et al., 2001). Lee & Walker (1995) found that lipid droplets (wax esters or triacylglycerols) and lipovitellins, associated with yolk spheres or granules within the oocytes, are utilized by copepod eggs for membranes formation, hormones secretion and so on(Lee & Walker, 1995). A part of stored lipids in females firstly convert into phospholipids, then newly formed phospholipids are used to assemble lipovitellin in the developing oocytes. Fig. 5.5 shows this whole process: the large oil sac of female copepod transfer into the eggs with rich lipid, whereby, eggs hatch into nauplii, which are full of lipid droplets and yolk granules with lipovitellin. But now less is known about the lipid specific pathways and their regulation under environmental fluctuations, such as toxicants exposure.

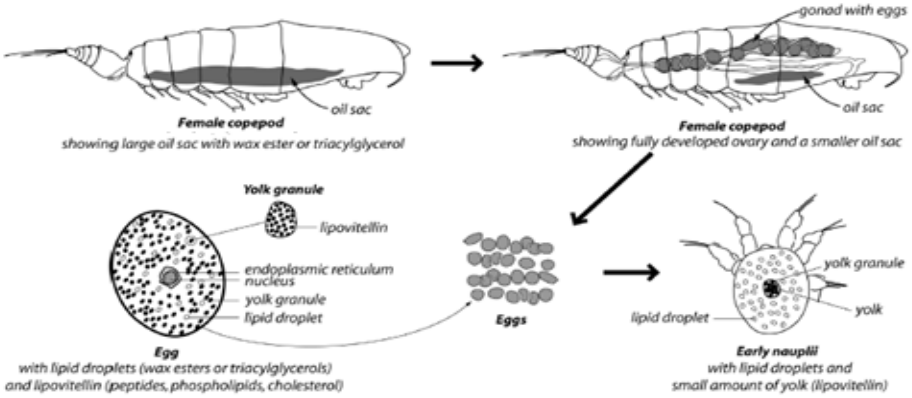


Figure 5.5. Formation of eggs in oil sac containing copepod with lipid droplets (wax esters or triacylglycerols) and lipovitellin associated with yolk granules; note reduction of oil sac size from before egg production to after egg production as lipid in oil sac is utilized to provide lipid for assembly of lipid droplets and lipovitellin. Early nauplii utilize lipid droplets and lipovitellin for energy and to form membranes and organelles (Lee et al., 2006, modified).

For detailed analysis of expression patterns between control and toxic groups, some transcripts of *A. tonsa* were found to be involved in lipid biosynthesis (Table 5.6). Related to lipid metabolism transcripts were tested, there were three metabolism, one fatty acid synthase, one desaturase and one elongase. Among these identifications for lipid biosynthesis were 3 fatty acid synthases, 7 desaturases and 2 elongases (Table 5.6). These lipid biosynthesis protein genes were also involved in the study of *Calanus finmarchicus* (Lenz et al., 2014). These transcripts were differentially expressed in different groups. Egg production of copepod requires the synthesis of wax esters, formed from fatty acids, which generate by the effect of fatty acid synthase (Sargent & Henderson 1986). The most highly expressed fatty acid synthase transcript was identified beta-ketoacyl-coa synthase. The highest expression levels of this transcript was observed in NiNPs 8.5 mg/L, NiNPs 17 mg/L and QDs groups (All >10 FPKM), with moderate levels seen in control and $NiCl_2$ 0.065 mg/L groups (4～5 FPKM). Two others of fatty acid synthase (Fatty acid hydroxylase superfamily and palmitoyl-protein thioesterase 1) were observed highest expression in NiNPs 8.5 mg/L group (1-3 FPKM, Table 5.6).

Fatty acid desaturase, which transcripts involved in desaturase, showed a extreme pattern with highest expression level in QDs group at 28 FPKM.. But the expression pattern seen for the fatty acid desaturase was not representative of the other desaturase. Other four transcripts, putative fatty acyl-coa desaturase, fatty acyl-coa desaturase, delta(6)-fatty-acid desaturase fat-3 and omega-6 fatty acid desaturase showed lowest levels (<0.1 FPKM) in control and toxic groups (NiNPs 8.5 mg/L and NiNPs 17 mg/L), and their peak expressions were observed in the $NiCl_2$ 0.065 mg/L with FPKM values of 2-3 (Table 5.6). Desaturase play an important role in the formation of monounsaturated fatty acids from saturated fatty acids, and it is involved in the biosynthesis of the fatty alcohol component of lipid droplet (Sargent & Henderson, 1986).

In the formation of lipid droplets (wax esters) process, one of latter steps involves chain elongation (Sargent & Henderson 1986). Chain elongation may catalyze the synthesis of very long chain fatty acids, which would likely contribute to an increase in total lipid storage for egg production (Lenz et al., 2014). Higher expression in control and toxic groups ($NiCl_2$ 0.065 mg/L & QDs 0.5 nM) were observed in elongation of very long chain fatty acids protein, which were more than 2 FPKM, but overall expression of groups exposed to NiNPs was lower, the FPKM of NiNPs 17 mg/L was twofold than that of NiNPs 8.5 mg/L. Recent studies on model organisms have focused on expression of proteins involved in lipid metabolism, and have started to reveal the function of elongases (Vrablik & Watts 2012).

These expression results of fatty acid synthase, desaturase and elongase were interesting, and they presented a contrast of lipid biosynthesis among different groups. Their expression patterns are

consistent with lipid storage associated with egg production. These kind of transcripts involved in these processes might be very important and specific, and potentially can be used as good biomarkers for reproductive processes.

Gene	Gene length (bp)	Top Hit species	FPKM				
			Control	NiNPs 8.5	NiNPs 17	$NiCl_2$ 0.065	QDs 0.5nM
heat shock protein family							
Heat shock protein 40	1343	*Paramecium tetraurelia*	**5.8**	**8.3**	**5.3**	0.3	1.9
Heat shock protein 60	644	*Perkinsus marinus*	0.4	＜0.1	0.7	0.7	2.2
Heat shock protein 70	787-1151	*Mytilus coruscus*	1.0	0.4	0.8	1.3	5.1
	480-1062	*Dirofilaria immitis*	1.2	1.7	2.5	2.3	5.6
	380-1605	*Miamiensis avidus*	**123.8**	**114.4**	**55.4**	**58**	**61.8**
Total			**126**	**116.5**	**58.7**	**61.6**	**72.5**
Heat shock protein 90	310-1340	*Tetrahymena thermophila*	1.7	5.8	4.5	0.2	0.3
	1600	*Palpitomonas bilix*	0.5	0.3	0.8	0.5	1.6
	954	*Paramecium tetraurelia*	3.2	0.9	0.1	2.5	0
	488-969	*Toxoplasma gondii*	1.5	＜0.1	0.7	1.2	7.4
	374-823	*Paramecium tetraurelia*	7.7	3.6	＜0.1	6.9	＜0.1
	2286	*Eimeria tenella*	**12.8**	**13.6**	**12.6**	1.0	4.0
Total			**27.4**	**24.2**	**18.7**	**12.3**	**13.3**
Heat shock protein 92	376-2471	*Paramecium caudatum*	**10.2**	**24.8**	**20**	0.6	2.1
Heat shock protein 100	2286	*Trypanosoma brucei brucei*	2.1	1.3	4.0	2..4	8.0
Lipid biosynthesis							
Fatty Acid Synthase							
Beta-ketoacyl-CoA synthase	2484	*Strigomonas culicis*	**5.8**	**13.3**	**12.4**	**4.9**	**11.3**
Fatty acid hydroxylase domain-containing protein 2-	1632-2085	*Saccoglossus kowalevskii*	**1.4**	0.7	**2.7**	**1.3**	**11.2**

Peroxisomal acyl-CoA oxidase	1636	*Tetrahymena thermophila*	0.6	1.3	1.4	＜0.1	0.1
Fatty acid hydroxylase superfamily	2110	*Stegodyphus mimosarum*	0.7	0.4	**1.8**	0.7	**4.7**
Palmitoyl-protein thioesterase 1	880	*Caenorhabditis elegans*	0.9	1.8	**3.0**	＜0.1	0.1
Desaturase							
Fatty acyl-CoA desaturase	1162	*Eimeria praecox*	0.1	0.1	0.1	3.7	0.1
Fatty acid desaturase: rubrerythrin	1471-1521	*Nematostella vectensis*	**6.5**	**4.3**	**12.6**	**6.3**	**28**
Fatty acid desaturase	1470	*Nematostella vectensis*	0.6	0.3	0.9	0.5	**2.6**
Putative fatty acyl-CoA desaturase	654	*Toxoplasma gondii*	＜0.1	＜0.1	＜0.1	**3.5**	0.5
Delta(6)-fatty-acid desaturase fat-3	615	*Fonticula alba*	＜0.1	＜0.1	＜0.1	2.0	0.3
Omega-6 fatty acid desaturase	546	*Perkinsus marinus*	＜0.1	＜0.1	＜0.1	**2.8**	＜0.1
Delta(12) fatty acid desaturase fat-2	584-1289	*Caenorhabditis elegans*	0.2	**1.8**	**2.4**	＜0.1	0.2
Elongase							
Elongation of very long chain fatty acids protein	1345	*Bombyx mori*	**2.2**	0.8	1.7	**2.6**	**2.7**

Table 5.6 Expression levels of predicted genes invovled in the lipid biosynthesis and heat shock protein family regulation for immunization of *A. tonsa*. Gene expression level is calculated by using fragments per kilobase of the transcript per million mapped reads (FPKM).

Other proteins, ferritin was also found to have key roles in immunization process regulation in organisms (Harrison & Arosio, 1996; Reif, 1992; Tarrant et al., 2008), and JH also played an important function in reproductive process regulation in organisms (Dubrovsky, 2005). However, these proteins were not differentially expressed among different groups. We will pay close attention to these genes in future expression profile analyses of groups.

Real-time PCR

We validated differentially expressed genes in HSP family (HSP70) involved with immunization process by real-time PCR. The samples of *A. tonsa* used for RT-PCR were collected after 4 days, and processed as the methods used in the transcriptome sequencing. The sequences of the primers in HSP70 gene were shown in the materials and methods (above). Our results showed that expression quantity of HSP70 exposed to different toxicants all kept in a low level, which were 0.26 ± 0.04 (NiNP 8.5 mg/L), 0.09 ± 0.02 (NiNP 17 mg/L), 0.11 ± 0.03 ($NiCl_2$ 0.065 mg/L), 0.3 ClNiClllectedM), respectively (all cessed as the methods used in the transcriptome sequencing. The

sequences of the primers in HSP70 gene were shown in the materials and methods (above). Our results showed that expression quantity of HSP7g.

SNP discovery

SNP is a variation in a single nucleotide that occurs at a specific position in the genome, where each variation is present to some appreciable degree within a population (e.g. >1%). We used SOAPsnp software to detect potential SNPs (Li et al., 2009). 547,979, 581,485, 816,933, 486,052 and 525,844 high-quality SNPs were identified in control vs NiNPs 8.5 mg/L, control vs NiNPs 17 mg/L, NiNPs 8.5 mg/L vs NiNPs 17 mg/L, control vs $NiCl_2$ 0.065 mg/L and control vs 0.5nM QDs groups, respectively. We found that the data of SNPs showed an extensive set of genetic markers for *A. tonsa*. These data will help to facilitate researches of genetic connectivity and genetic mapping at a previously unprecedented level of detail. *A. tonsa* was widely distributed species and many populations can be discovered in most coastal waters. As the key link between primary producers and secondary producers, it is very important to study this population. Researchers usually used the way of exploring population structure to understanding population recruitment mechanism. Compared with other gene markers, SNP gene markers had more information in population studies of copepod (Wang, 2010). The abundance of SNPs from transcriptome will take a effective way for studying the population structure of *A. tonsa*.

5.3 Conclusions

In this thesis, we are starting to study the effects of different toxicants on its productivity and their potential impact on the marine ecosystem. Transcriptome sequencing and gene expression analysis open new perspective in studying copepod physiology and allow to select organisms with a good reproductive fitness.

We completed the transcriptome sequencing (control and toxic groups) of *A. tonsa* using the Illumina HiSeq 2500 platform. Illumina sequencing of *A. tonsa* provided a transcriptome for the model species and new insights into specific expression patterns under stress conditions. Of the data from transcriptome sequencing, we filtrated and identified a lot of candidate genes that were potentially involved with reproductive process and immunization process, which are valuable for further researches. Analysis of expression pattern focused on transcripts involved in lipid biosynthesis suggest that transcripts with similar annotations may exhibit different expression patterns during control and toxic groups in reproductive process. This is not surprising given the importance of lipids in reproductive processes.

As high throughput sequencing technology progress, most researchers focused on the different developmental stages of copepod in transcriptome levels or explore interpopulation patterns of

divergence (Barreto et al., 2011; Ning et al., 2013). In this thesis, the differentially gene expression of copepod exposed to contaminants in transcriptome level was firstly studied. In toxic risk assessment, the responses of transcriptome, as other assessment ways, just provide an indication to show the imperceptible changes in organisms, but from molecular level. Our results revealed the subtle changes in transcript level, which may not via post-translational modification (showing changes in protein level) (Taylor et al., 2013). The comparative sequencing of *A. tonsa* between control and toxic groups has made a database about the gene variations involved with some pathways, which might potentially reflect stress response exposed to toxicants. In the Lipid biosynthesis pathway, there were no significant differentially gene regulation under slightly toxic level exposure. This conclusion was also supported by results of fecundity of *A. tonsa*, which were also not significantly effected by toxic exposure. This is the first study which investigate on the transcriptome and gene expression of *A. tonsa* copepod.

5.4 References

Ahameda M, Posgaia R, Goreya TJ, Nielsena M, Hussainb SM, Rowea JJ (2010) Silver nanoparticles induced heat shock protein 70, oxidative stress and apoptosis in *Drosophila melanogaster*. Toxicology and Applied Pharmacology 242 (3): 263-269.

Anders S, Huber W (2010) Differential expression analysis for sequence count data. Genome Biology. 11: R106.

Barreto FS, Moy GW, Burton RS (2011) Interpopulation patterns of divergence and selection across the transcriptome of the copepod *Tigriopus californicus*. Molecular Ecology 20: 560-572.

Cao Y, Ohwatari N, Matsumoto T, Kosaka M, Ohtsuru A, Yamashita S (1999). TGF-β11 mediates 70-kDa heat shock protein induction due to ultraviolet irradiation in human skin fibroblasts. Pflugers Arch 438 (3): 239-44.

Cuzin-Roudy J, Amsler MO (1991) Ovarian development and sexual maturity staging in Antarctic krill, *Euphausia superba* Dana (Euphausiacea). J Crust Biol 11: 236-249.

Dubrovsky EB (2005) Hormonal cross talk in insect development. Trends in Endocrinology and Metabolism 16: 6-11.

Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, et al. (2011) Full length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol 29: 644-652.

Harrison PM, Arosio P (1996) The ferritins: molecular properties, iron storage function and cellular regulation. Biochimica et Biophysica Acta 1275: 161–203.

Ianora A, Santella L (1991) Diapause embryos in the neustonic copepod *Anomalocera patersoni*. Mar Biol 108: 387-394.

Irigoien X (2004) Some ideas about the role of lipids in the life cycle of *Calanus finmarchicus*. Journal of Plankton Research 26: 259-263.

Johnson CL, Runge JA, Curtis KA, Durbin EG, Hare JA, et al. (2011) Biodiversity and ecosystem function in the Gulf of Maine: Pattern and role of zooplankton and pelagic nekton. Plos One 6: e16491.

Jung H, Lyons RE, Dinh H, Hurwood DA, McWilliam S, Mather PB (2011). Transcriptomics of a giant freshwater prawn (*Macrobrachium rosenbergii*): de novo assembly, annotation and marker discovery. PloS one 6(12): e27938.

Laabir M, Buttino I, Ianora A, Kattner G, Poulet SA, Romano G, Carotenuto Y, Miralto A (2001) Effect of specific dinoflagellate and diatom diets on gamete ultrastructure and fatty acid profiles of the copepod *Temora stylifera*. Mar Biol 138: 1241-1250.

Langmead B, Trapnell C, Pop M, Salzberg SL (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology* 10(3): R25.

Lee RF, Hagen W, Kattner G (2006) Lipid storage in marine zooplankton. Marine Ecology Progress Series 307: 273-306.

Lee RF, Walker A (1995) Lipovitellin and lipid droplet accumulation in oocytes during ovarian maturation in the blue crab, *Callinectes sapidus*. J Exp Zool 272: 401-412.

Lenz PH, Roncalli V, Hassett RP, Wu LS, Cieslak MC, Hartline DK, Christie AE (2014) De Novo Assembly of a Transcriptome for *Calanus finmarchicus* (Crustacea, Copepoda) – The Dominant Zooplankter of the North Atlantic Ocean. PLOS one 9(2): e88589.

Li B, Colin ND (2011) RSEM: accurate transcript quantification from RNA Seq data with or without a reference genome. BMC Bioinformatics 12: 323.

Li Y, Fang X, Yang H, Wang J, Kristiansen K, Wang J (2009) SNP detection for massively parallel whole genome resequencing. Genome Research 19: 1124-1132.

Matz JM, Blake MJ, Tatelman HM, Lavoi KP, Holbrook NJ (1995). Characterization and regulation of cold-induced heat shock protein expression in mouse brown adipose tissue. Am. J. Physiol. 269 (1): R38-R47.

Nilsson B, Jepsen PM, Rewitz KF, Hansen BW (2014) Expression of hsp70 and ferritin in embryos of the copepod *Acartia tonsa* (Dana) during transition between subitaneous and quiescent state. Journal of Plankton Research 36 (2): 513-522.

Ning J, Wang M, Li C, Sun S (2013) Transcriptome Sequencing and De Novo Analysis of the Copepod *Calanus sinicus* Using 454 GS FLX. PLoS ONE 8(5): e63741.

Reif DW (1992) Ferritin as a source of iron for oxidative damage. Free Radical Biology & Medicine 12: 17-427.

Riddiford LM (2008) Juvenile hormone action: a 2007 perspective. Journal of Insect Physiology 54: 895-901.

Riesgo A, Andrade SC, Sharma PP, Novo M, Pérez-Porro AR, Vahtera V, González, VL, Kawauchi GY, Giribet G (2012). Comparative description of ten transcriptomes of newly sequenced invertebrates and efficiency estimation of genomic sampling in non-model taxa. Frontiers in zoology 9(1): 1-24.

Ritossa F (1962). A new puffing pattern induced by temperature shock and DNP in drosophila. Experientia 18 (12): 571-573.

Sargent JR, Henderson RJ. Lipids. In: Corner EDS, O'Hara SCM, editors. The Biological Chemistry of Marine Copepods. Oxford: Clarendon Press 1986, PP. 59-108.

Tarrant AM, Baumgartner MF, Verslycke T, Johnson CL (2008) Differential gene expression in diapausing and active *Calanus finmarchicus* (Copepoda). Marine Ecology Progress Series 355: 193-207.

Taylor RC, Webb Robertson BJM, Markillie LM, Serres MH, Linggi BE, Aldrich JT, Hill EA, Romine MF, Lipton MS, Wiley HS (2013) Changes in trans-lational efficiency is a dominant regulatory mechanism in the environmental response of bacteria. Integr. Biol 5: 1393-1406.

Vos MJ, Hageman J, Carra S, Kampinga HH (2008) Structural and Functional Diversities between Members of the Human HSPB, HSPH, HSPA, and DNAJ Chaperone Families. Biochemistry 47: 7001-7011.

Vrablik TL, Watts JL (2012) Emerging roles for specific fatty acids in developmental processes. Gene Dev 26: 631-637.

Wang M (2010) Application of molecular markers to the researches on pelagic copepods in the Chinese coastal regions (Dissertation, in Chinese with English abstract). Qingdao: Institute of Oceanology, Chinese Academy of Sciences.

Zheng Y, Zhao L, Gao J, Fei Z (2011) iAssembler: a package for de novo assembly of Roche-454/Sanger transcriptome sequences. BMC bioinformatics 12(1): 1.

6 Acknowledgments

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7. Glossary

Productivity (eggs/female): reproductive success is measured as eggs female^{-1} day^{-1} (E r).

Egg hatching success: the number of nauplii out of total number of eggs, which are produced by female. **Naupliar mortality** is the opposite.

Naupliar mortality: after 72h, to check the dead nauplii out of hatched naupli.

EC_{50}: effective concentration of toxicants for inducing 50% naupliar mortality and/or immobilization.

s.e.: standard error

NM: nanomaterials.

Ni: Nickel.

NiNPs: Ni nanoparticles.

QDs: Lumidot orange quantum dots.

DLS: Dynamic Light Scattering.

ICP-MS: Induced Coupled Plasma–Mass Spectroscopy.

Acute test: eggs were incubated for 48h, by toxicant exposure, then to check the egg hatching success and nauplii dead or immobilized.

Semichronic test: eggs were incubated continually for 7 days, by toxicant exposure, after 48h, 5 days and 7 days, hatched eggs and swimming nauplii were calculated and transferred in new medium.

Chronic test: toxic exposure of copepod adults in 4 days, and to test egg production and egg hatching success everyday.

Swiss-Prot is the manually annotated and reviewed section of the UniProt Knowledgebase (UniProtKB). It is a high quality annotated and non-redundant protein sequence database, which brings together experimental results, computed features and scientific conclusions.

Gene ontology (GO) is a major bioinformatics initiative to unify the representation of gene and gene product attributes across all species.

Clusters of Orthologous Groups (COG) is generated by comparing predicted and known proteins in all completely sequenced microbial genomes to infer sets of orthologs.

EuKaryotic Orthologous Groups (KOG) is a eukaryote-specific version of the Clusters of Orthologous Groups (COG) tool for identifying ortholog and paralog proteins.

Kyoto Encyclopedia of Genes and Genomes (KEGG) is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies.

FPKM: fragments per kilobase of transcript per million mapped reads.

RT-PCR: Real-time Polymerase Chain Reaction.

UTRs: untranslated sequence regions of gene.

HSP: heat shock protein.

SNP: Single Nucleotide polymorphism.

8. Appendix

UNIVERSITÀ DEGLI STUDI DI NAPOLI FEDERICO II

DOTTORATO DI RICERCA IN
SCIENZE E TECNOLOGIE DELLE PRODUZIONI AGRO-ALIMENTARI

VERBALE DEL 07/03/2016

Il giorno 07/03/2016, alle ore 12.00, si è riunito nella Sala Giunta del Dipartimento del Dipartimento di Agraria il Collegio dei Docenti del Dottorato di Ricerca in "Scienze e tecnologie delle produzioni agro-alimentari" per discutere del seguente o.d.g.:

1) Dottorandi 28° e 27° ciclo: approvazione relazione finale e lavoro di tesi
2) Commissioni Esami finali ed afferenza dottorandi alle varie commissioni
3) Eventuale proposta di rilasciare il titolo di dottore di ricerca con o senza la denominazione dell'indirizzo
4) Varie ed eventuali

Sono presenti i proff.: Barbieri Giancarlo, Cavella Silvana, Villani Francesco, Barone Carmela, Sansone Giovanni
Sono assenti giustificati i proff.: Fogliano Vincenzo, Moio Luigi, Di Vaio Claudio, Masi Paolo
Sono, inoltre, assenti i proff.: Ferranti Pasquale, Sacchi Raffaele
Presiede il prof. Barbieri e funge da segretario la prof. Cavella.

1) Dottorandi 28° e 27° ciclo: approvazione relazione finale e lavoro di tesi
Il prof. Barbieri comunica che all'inizio di febbraio 2016 aveva invitato a produrre la necessaria documentazione e che sono pervenute le relazioni conclusive, riportanti anche una significativa sintesi della tesi, dei seguenti dottorandi:

XXVIII CICLO	Tutor	Indirizzo
1. BIONDI LOREDANA	Raffaele Romano	Scienze e tecnologie prod. alimentari
2. CECERE BIANCA	Barbieri, IZSM	Scienze e tecnologie prod. alimentari
3. ESPOSITO FRANCESCO	T. Cirillo	Scienze e tecnologie prod. alimentari
4. IACCARINO DORIANA	Barone	Acquacoltura
5. KONG XIANGHUI	Fogliano, Vitaglione	Scienze e tecnologie prod. alimentari
6. LI JIJI	Fogliano, Dott.ssa Manzo Enea	Acquacoltura
7. MASSA MARINA	Fogliano	Acquacoltura
8. SCHIAVO SIMONA	Fogliano, Dott.ssa Manzo Enea	Scienze e tecnologie prod. alimentari
9. STELLATO GIUSEPPINA	Ercolini	Scienze e tecnologie prod. alimentari
10. VOLPE STEFANIA	Cavella, Torrieri	Scienze e tecnologie prod. alimentari
11. ZHOU CHAO	Fogliano, Buttino – ISPRA Livorno	Acquacoltura
XXVII CICLO		
12. CAPORASO NICOLA	Sacchi	Scienze e tecnologie prod. alimentari
13. D'ANTUONI ISABELLA	Sacchi	Scienze e tecnologie prod. alimentari
14. DI MARTINO VERONICA	Villani	Scienze e tecnologie prod. alimentari
15. MENNELLA ILARIO	Fogliano, Vitaglione	Scienze e tecnologie prod. alimentari
16. TROISE ANTONIO DARIO	Fogliano, Vitaglione	Scienze e tecnologie prod. alimentari

1.11 dott. ZHOU CHAO
Il dott. Zhou Chao presenta una tesi dal titolo "Cultivation of the marine calanoid copepod *Acartia tonsa* (Dana) as alternative food in aquaculture: evaluation of reproductive performance (fecundity and larval survival) and gene expression in different stress conditions." (Tutor Prof. Fogliano, co-tutor Buttino – ISPRA Livorno).

Oltre alle previste attività formative del dottorato ed alla partecipazione a progetti di ricerca dipartimentali, il dott. Zhou ha preso parte ad alcune manifestazioni scientifiche ed alle seguenti attività di formazione:
Apertura dei seminari, Prof. Roberto Bargagli, Seminario presso l'Università di Siena, 04/03/2014
Isolation and identification of environmental organic substance, Prof. Hongyu Luo, September 2014
Biological response to environmental stresses (Marine species), Prof. Changwen Wu, September 2014
Modeling of biological systems, Prof. Sheng Zhao, October 2014
Experimental management and rationality, Prof. Aiyi Zhu, November 2014
Data processing and statistical analysis, Prof. Yibing Deng, March 2015
Academic paper writing and contribution, Prof. Changwen Wu, March 2015
Applying for scientific research project, Prof. Changwen Wu, May 2015

Il Segretario
(Prof. Silvana Cavella)

Il Coordinatore
(prof. Giancarlo Barbieri)

Control technology of harmful algal blooms, based on allelopathy, Professor Yinghua Chen, 21/10/2014
Physiological functions of the pituitary prolactin of fish, Professor Zhan Yin, 13/11/2014
Function description and operating procedures of BIOBANK (biological germplasm banks), Professor Ángel F. González, 28/11/2014
Development of biological sciences and biotechnology revolution, Professor Wen Wang, 15/12/2014
The impact of food on physiological response of *Acartia spinicauda* and its molecular mechanism, Prof. Changwen Wu, Zhejiang Ocean University
3rd World Conference on Marine Biodiversity WCMB, Qingdao (PRC), 12th-16th October 2014
6th Biannual ECO toxicology MEeting (BECOME 2014), Livorno, Italy, 11th-13th November 2014
Center Academic Conference, National Engineering Research Center For Marine Aquaculture (PRC), 3th September 2014

Periodi formativi all'estero
06/2014. -04/2015. National Engineering Research Center of Marine Facilities Aquaculture, Zhejiang Ocean University, per Transcriptome research of *Acartia tonsa* e The impact of food on physiological response of *Acartia spinicauda* and its molecular mechanism.

E' autore di 7 lavori di cui 4 su rivista e 3 comunicazioni a convegni, come risulta dall'elenco seguente

Pubblicazioni in riviste
1. Zhou, C., Wu, C.W., Guo, B.Y., Cheng, Y.J. 2015. Genetic diversity in ribosomal 18S rRNA and mitochondrial COIII genes in Chinese cultured populations of mussel *Mytilus galloprovincialis*. Biochemical Systematics and Ecology. 59: 135-143. ISSN: 0305-1978
2. Zhou, C., Vitiello, V., Pellegrini, D., Wu, C.W., Morelli, E., Buttino, I. 2016. Toxicological effects of CdSe/ZnS quantum dots on marine planktonic organisms. Ecotoxicology and Environmental Safety. 123: 26-31. ISSN:0147-6513
3. Zhou, C., Vitiello, V., Casals, E., Puntes, V.F., Iamunno, F., Pellegrini, D., Wu, C.W., Benvenuto, G., Buttino, I. 2016. Toxicity of nickel in the marine calanoid copepod *Acartia tonsa*: Nickel chloride versus nanoparticles. Aquatic Toxicology. 170: 1-12. ISSN 0166-445X
4. Vitiello, V., Zhou, C., Scuderi, A., Pellegrini, D., Buttino, I. 2016. Cold storage of *Acartia tonsa* eggs: a practical use in ecotoxicological studies. Ecotoxicology. (Accepted). ISSN: 0963-9292 (print version), ISSN: 1573-3017 (electronic version)

Comunicazioni a Convegni
1. Zhou, C., Wu, C.W., Vitiello, V., Pellegrini, D., Buttino, I. 2014. Effects of Nickel nanoparticles on the calanoid copepod *Acartia tonsa*. 3rd World Conference on Marine Biodiversity WCMB, Qingdao (PRC).
2. Buttino I., Zhou, C., Vitiello, V., Pellegrini, D. 2014. Cold storage of *Acartia tonsa* embryos for a practical use in ecotoxicological studies. 6th Biannual ECO toxicology MEeting (BECOME 2014) - Environmental emergencies: ecotoxicology as a management tool, Fondazione Livorno Euro Mediterranea (LEM), Livorno, Italy.
3. Zhou, C., Wu, C.W., Vitiello, V., Pellegrini, D., Buttino, I. 2014. Acute and chronic effects of Nickel nanoparticles on the calanoid copepod *Acartia tonsa* (Dana): a comparion with the soluble nickel chloride toxicant. "Center Academic Conference", National Engineering Research Center For Marine Aquaculture (PRC).

Giudizio sulla tesi
La tesi presentata ha per obiettivi di valutare la possibilità di allevamento del copepode *Acartia tonsa* e di studiare la sua performance riproduttiva e l'espressione genica prestazioni al fine di comprendere l'impatto dei fattori di stress chimici sulla sua biologia.
La tesi è valutata positivamente.

Giudizio complessivo
Il Collegio, considerati l'attività scientifica del dott. Chao Zhou, i risultati ottenuti e l'attività di formazione svolta e valutato positivamente il lavoro di tesi presentato, approva la relazione sull'attività svolta durante il corso e ritiene il candidato meritevole dell'ammissione all'esame finale per il conseguimento del titolo di Dottore di Ricerca in Scienze e Tecnologie delle Produzioni Agro-Alimentari 28° ciclo.

OMISSIS

Non essendovi altro da deliberare, la seduta è sciolta alle ore 14.00.
Letto e approvato

Il Segretario
(Prof. Silvana Cavella)

Il Coordinatore
(prof. Giancarlo Barbieri)

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Toxicity of nickel in the marine calanoid copepod *Acartia tonsa*: Nickel chloride versus nanoparticles

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ABSTRACT

Nickel compounds are widely used in industries and have been massively introduced in the environment in different chemical forms. Here we report the effect of two different chemical forms of nickel, $NiCl_2$ and nickel nanoparticles (NiNPs), on the reproduction of the marine calanoid copepod *Acartia tonsa*. The behavior of nickel nanoparticles was analyzed with different techniques and with two protocols. In the "sonicated experiment" (SON) NiNP solution was sonicated while in the "non-sonicated experiment" (NON-SON) the solution was vigorously shaken by hand. Final nominal concentrations of 5, 10 and 50 mg L^{-1} and 1, 5 and 10 mg L^{-1} NiNPs were used for the acute and semichronic tests, respectively. Nanoparticle size did not change over time except for the highest concentration of 50 mg L^{-1} NiNPs, in which the diameter increased up to 843 nm after 48 h. The concentration of Ni dissolved in the water increased with NP concentration and was similar for SON and NON-SON solutions. Our results indicate that sonication does not modify toxicity for the copepod *A. tonsa*. Mean EC_{50} values were similar for NON-SON (20.2 mg L^{-1}) and SON experiments (22.14 mg L^{-1}) in the acute test. Similarly, no differences occurred between the two different protocols in the semichronic test, with an EC_{50} of 7.45 mg L^{-1} and 6.97 mg L^{-1} for NON-SON and SON experiments, respectively. Acute and semichronic tests, conducted exposing *A. tonsa* embryos to $NiCl_2$ concentrations from 0.025 to 0.63 mg L^{-1}, showed EC_{50} of 0.164 and 0.039 mg L^{-1}, respectively. Overall, *A. tonsa* is more sensitive to $NiCl_2$ than NiNPs with EC_{50} being one order of magnitude higher for NiNPs. Finally, we exposed adult copepods for 4 days to $NiCl_2$ and NiNPs (chronic exposure) to study the effect on fecundity in terms of daily egg production and naupliar viability. Egg production is not affected by either form of nickel, whereas egg viability is significantly reduced by 0.025 mg L^{-1} $NiCl_2$ and by 8.5 mg L^{-1} NiNPs. At NiNP concentration below the acute EC_{50} (17 mg L^{-1}) only 9% of embryos hatched after 4 days. Interestingly, the percentage of naupliar mortality (>82%) observed in the semichronic test at the nominal concentration of 10 mg L^{-1} NiNPs corresponding to almost 0.10 mg L^{-1} of dissolved Ni, was similar to that recorded at the same Ni salt concentration. Electron microscopical analyses revealed that *A. tonsa* adults ingest NiNPs and excrete them through fecal pellets. To the best of our knowledge, this is the first study investigating the toxicity of two different forms of Ni on the reproductive physiology of the copepod *A. tonsa* and showing the ability of the calanoid copepod to ingest nanoparticles from seawater.

1. Introduction

Nickel (Ni) is an ubiquitous element naturally present in the biosphere and in the water and is considered an essential trace component for living organisms (USEPA, 1986). However, different chemical forms of Ni are introduced in the environment by industries (alloys, electroplating, batteries, coins, stainless-steels), oil

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Toxicological effects of CdSe/ZnS quantum dots on marine planktonic organisms

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Gene expression

ABSTRACT

Quantum dot nanoparticles (QDs) are proposed as novel materials for photovoltaic technologies, light emitting devices, and biomedical applications. In this study we investigated the effect of CdSe/ZnS QDs on the growth rate of four microalgae: the diatom *Phaeodactylum tricornutum*, the cryptophyte *Rhinomonas reticulata*, the prymnesiophyte *Isochrysis galbana* and the green alga *Dunaliella tertiolecta*. In addition we analyzed the effect of QDs on the copepod *Acartia tonsa*. A classical acute test (48-h) with embryos was carried out to evaluate naupliar survival. Moreover, a 4-day chronic test with adult copepods was conducted to evaluate their fecundity (embryos $f^{-1}day^{-1}$) and egg hatching success. QDs in the range from 1 to 4 nM gradually inhibited the growth rate of *P. tricornutum*, *I. galbana*, *R. reticulata* and *D. tertiolecta* with an EC_{50} of 1.5, 2.4, 2.5 and 4.2 nM, respectively. Acute tests with *A. tonsa* (QD concentration tested from 0.15 to 1.5 nM) showed an increased naupliar mortality in response to QD treatment, exhibiting an EC_{50} of 0.7 nM. Chronic test showed no negative effect on egg production, except on the last two days at the highest QD concentration (2.5 nM). No significant reduction of the percentage of egg hatching success was recorded during the exposure. Toxicity assessment of QDs was also investigated at the molecular level, studying heat shock protein 70 gene expression (*hsp 70*). Our results indicate that *hsp70* was upregulated in adults exposed 3 days to 0.5 nM QDs. Overall, these results suggest that species unable to swim along the water column, like *P. tricornutum* and early hatched copepods, could be more exposed to toxic effects of QDs which tend to aggregate and settle in seawater.

1. Introduction

Recent developments in the field of nanotechnology have led to an increased interest to understand the effect of nanomaterials on the human health and on natural environment (Handy et al., 2008; Corsi et al., 2014). Nanoparticles can reach estuarine and coastal waters and undergo physicochemical transformation affecting their fate, behaviour and toxicity towards aquatic organisms at different levels of the food chain (Matranga and Corsi, 2012). In particular, in the marine environment the high ionic strength of sea water facilitates aggregation of nanoparticles, thereby favouring settlement on the sediment or mixing with the water column, following marine movements (Klaine et al., 2008). In this scenario, marine organisms will be exposed to nanoparticles through different routes of exposure, depending on the physiology of the marine organisms. As a consequence, the extent to which benthic rather than pelagic organisms can be exposed to nanoparticles needs to be investigated.

Quantum dots (QDs) are semiconductor nanocrystals, exhibiting unique photophysical properties, very attractive for biomedical applications as well as for manufactured products, including image sensors, emitting materials in LEDs and solar cells (Winnik and Maysinger, 2013). Due to their growing industrial production, increasing amounts of QDs are expected to enter the aquatic ecosystems where their effects on biological communities are poorly understood (Hardman, 2006). There is a growing body of literature regarding the effects of QDs on aquatic organisms, such as bacteria, microalgae, invertebrates and fishes (Gagnè et al., 2008; Domingos et al., 2011; Zhang et al., 2012; Yang et al., 2012; Ambrosone et al., 2012; Leigh et al., 2012). Although it has been reported that QDs can partially degrade in aqueous environments

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Genetic diversity in ribosomal 18S rRNA and mitochondrial COIII genes in Chinese cultured populations of mussel *Mytilus galloprovincialis*

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ABSTRACT

To analyze the genetic diversity and genetic structure of cultured populations of *Mytilus galloprovincialis*, 44 individuals were sampled from three localities, Yantai, Rushan of Shandong province and Dongji of Zhejiang province. Sequence analysis of ribosomal (18S rRNA gene) and mitochondrial DNA (COIII gene) revealed 23 haplotypes of 18S rRNA and 30 haplotypes of COIII. Haplotype diversities (*Hd*), nucleotide diversities (*Pi*) and average nucleotide differences (*K*) were 0.712 (18S rRNA) and 0.946 (COIII), 0.0044 (18S rRNA) and 0.0207 (COIII), and 0.703 (18S rRNA) and 15.316 (COIII), respectively. Fixation indices (Φ_{ST}) of the three cultured populations showed no genetic divergence between Yantai and Rushan populations but significant genetic divergence between Dongji and the other two populations. In the present study, we found no evidence for decline in genetic polymorphism of cultural *M. galloprovincialis* populations. The findings of the present study will be useful in the management and conservation of *M. galloprovincialis* resources.

1. Introduction

Mussels are important bivalves in Chinese aquaculture and market, including many ecologically and environmentally important species that are widely distributed among the dominant macrofauna in littoral and shallow sub-littoral systems (Distel, 2000). *Mytilus galloprovincialis*, belonging to Mollusca, Bivalvia, Anisomyaria, Mytilidae, *Mytilus*, is distributed mainly along the coast of the Yellow Sea and Bohai Sea in China. In the 1950s, *M. galloprovincialis* was only found along the coast of Dalian, China, however, at present, its range expanded southward, along the coast of Shandong, Zhejiang, Fujian and Guangdong provinces, China (Wang, 1997). Due to its strong fecundity, fast growing, high nutritional values as well as good commercial prices, it has been cultivated in large-scale in the past decades. In the aquaculture industry, hatchery cultured stock was frequently used as original stock to produce the next generation and repeated every year. Such closed cycle might lead to reproductive isolation from the wild populations and making a reduction in genetic diversity due to low efficiency of population size and inbreeding (Hedgecock and Sly, 1990).

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