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Use of *Rhodomonas salina* encapsulated in silica as an artificial feed for *Acartia tonsa* nauplii

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Abstract

Aquaculture is a growing industry that has gained importance in recent years as it ensures fish supply for human consumption. In marine aquaculture, adequate fish feed with the correct biochemical composition and size is essential for fish larvae rearing. The use of live fish feed has been well documented for first feeding of larvae in various marine fish species. In natural environments, most marine fish larvae feed on zooplankton organisms such as rotifers, brine shrimp and copepods. Copepods are small organisms with a rich lipid profile, which are the reasons they are considered a promising live feed for marine fish larvae. Throughout the years, it has become evident that the nutritional quality of the copepods feed affects its production and consequently the production of animals at higher trophic levels, including marine fish larvae. Calanoid copepods feed mainly on phytoplankton and studies have shown that the microalgae *Rhodomonas* spp. constitutes the best live feed for copepod cultivation due to its nutritional value.

In this study, an artificial feed product consisting *R. salina* encapsulated in silica was evaluated as an alternative food source for the calanoid copepod *Acartia tonsa*. The effect of the artificial feed on growth and mortality of *A. tonsa* nauplii was evaluated using three different concentrations of these artificial particles. Moreover, the carbon and nitrogen content, as well as the behavior of the artificial feed particles dissolved in seawater were examined. The results showed that the size and carbon content of the artificial particles was similar to diverse phytoplankton organisms, making them suitable for nauplii ingestion. Furthermore, the particles had a positive effect on nauplii survival but had impaired effect on their development and growth. Improvements in the nutritional composition of artificial algae encapsulated in silica need to be done before they can become a potential alternative to live feed in the cultivation of copepods.

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1. Introduction

Aquaculture and fisheries are increasing its importance as a food resource in recent years due to the demand of food high in protein as a result of the increase in human population (FAO, 2009). Human population is projected to reach 9 billion by 2050 and the food demand is predicted to increase by 60%, which means that nutritional challenges such as malnutrition could become a concern if this demands are not fulfill accordingly (FAO, 2009). On account of the environmental damage, biodiversity reduction and economic costs associated to land use for food production, attention to food and biomass obtained from aquatic environments has increased (SAPEA, 2017).

The fish supply has had a global annual increase of 3.2% between 1961 and 2013 that exceeds the human population growth (FAO, 2016). Similarly, per capita fish consumption has risen from 9.9 kg in the 1960s to 14.4 kg in the 1990s and 19.7 kg in 2013 (FAO, 2009), showing clearly the importance of meeting the growing demand for fish as food. This increase in fish consumption can be related to the presence of essential nutrients including Omega-3, vitamins and minerals within the fish meat that makes it more appealing for consumption and promotes a healthy diet (Kris-Etherton, Harris, & Appel, 2002). Meanwhile fisheries involve catching wild fish that can result in overexploitation of natural fish stocks, aquaculture or fish farming is related to the cultivation of aquatic species that have a special value to consumers in the market (Ye & Gutierrez, 2017). It has been reported that 17% of fish stocks have been overexploited, 7% have been depleted, and 1% are recovering from depletion due to poor management of marine fisheries (Beddington, Agnew, & Clark, 2007; FAO, 2009). On the other hand, aquaculture constitutes an alternative to the competition for natural resources while also representing a sustainable income for both developed and developing countries (FAO, 2009).

Aquaculture includes the cultivation of aquatic plants and animals, plus it can be done with both marine and freshwater species. FAO (Food and Agriculture Organization of the United Nations) has reported that 527 species are currently farmed worldwide, of which around 220 species are finfish and shellfish. Freshwater aquaculture consists mainly of finfish including a large production of carp while marine aquaculture comprises mostly mollusk production; nevertheless marine fish culture is expected to increase in the near future (Boyd & McNevin, 2015). Currently, aquaculture produces 1.5 million tones of aquatic biomass in Europe, of which 50% are mollusk

and crustaceans, 27% are marine fish and 23% are freshwater fish (SAPEA, 2017). Different aquaculture methods are used depending on the desired product and they can be classified according to their culture system: extensive, semi-intensive and intensive (Naylor et al., 2000). For freshwater aquaculture, extensive pond farming and recirculating systems are very common (SAPEA, 2017). Similarly, extensive brackish water farming, sea cages and recirculating systems are well used in marine fish farming (SAPEA, 2017).

One major advantage of fish farming is the possibility to grow fish in a enclosed and controlled environment where biotic and abiotic factors (such as temperature, pH, salinity, CO₂, nutrients among other) are carefully monitored (Premchand Mahalik & Kim, 2014). This ensures the production of high quality fish since conditions in the systems are well managed. Another important factor that contributes to the production of bigger and better fish in terms of its nutritional value is fishmeal. It has been proved that optimal fish growth is heavily dependent on feed biochemical composition and protein quality (Aksnes, Izquierdo, Robaina, Vergara, & Montera, 1997). The fish feed nutritional content and needs varies depending on the fish species; for instance, herbivores species require a diet enriched with vegetable oils and plant proteins while carnivore species need to have a diet with fish oil as well as proteins, minerals and vitamins (SAPEA, 2017). Both freshwater fish and marine fish have high levels of essential fatty acids and their content is attributed in a large extent to the lipid composition of their natural diet, which is mainly based on plankton. Freshwater fish are known to contain higher levels of C₁₈ polyunsaturated fatty acids (PUFA) compared to marine fish, which contained significant levels of long-chain PUFA such as eicosapentaenoic acid (EPA) and docosahexaenoic acids (DHA) (Steffens, 1997). Significant levels of linolenic acid, linoleic acid and eicosapentaenoic acid have been found in different species of algae, crustacea, and aquatic larvae of insects, and their nutritional pattern have been suggested to influence the lipid profile of both marine and freshwater fish (Aksnes et al., 1997; Houde & Roman, 1987; Steffens, 1997).

It is important to emphasize that fish larvae are very susceptible to their environment and thus require special conditions to be able to survive, develop and grow into adults (Holt, 2011). Moreover, the success of marine aquaculture is influenced in great manner by the effectiveness of larval fish rearing (Norsker, 1997). Though, the dietary requirements of larvae and juvenile fish differ from those of the adult fish. Fish larvae need to be constantly feed with high nutritional

food considering their high growth rate, which can range from 30% to 100% per day depending on the specie (Hamre et al., 2013). In addition, the digestive system of fish larvae differs functionally and structurally from adults owing to the fact that fish larvae lack a functional stomach (Drossou, Ueberschär, Rosenthal, & Herzig, 2006). In this manner, early fish stages have to release digestive enzymes in order to process the different components of their diets (Sales, 2011). For this reason, special care must be taken in the formulation of diets, where the nutritional content of feed and the digestive system morphogenesis of fish larvae should be borne in mind.

Taking into account the vulnerability and physiological aspects of larvae fish, as well as the anatomical size of the larvae mouth, live feed has been shown to be the best choice for growth purposes in aquaculture (Jonsson & Tiselius, 1990). Freshwater fish larvae fed with experimental an commercial compound diets made primarily of yeast showed 2.3-2.5 higher probability of mortality compared to the fish larvae fed with live feed composed of zooplankton organisms different from *Artemia* nauplii (Sales, 2011). Live feed is extremely important for a successful development of marine fish larvae since it is easy to ingest, digest and assimilate compared to artificial formulated feed (Abate, et al., 2016). Furthermore, live feed that are able to move actively stimulates raptorial behavior and are preferred by marine fish larvae until metamorphosis, after which it can be replaced by artificial feed (Hamre et al., 2013; Solution, 2014).

Seeing that planktonic organisms are the natural prey for many larvae of cultured fish species, their use and production has been implemented on a large scale and efforts have been made to make live feed affordable for marine aquaculture (Hamre et al., 2013). Rotifers, copepods and *Artemia* are the most common organisms used in live feed production. *Artemia* spp. commonly known as brine shrimps are harvested from natural hypersaline lakes and are preferred in the aquaculture industry thanks to its capacity of generating dormant cysts that can be stored for long periods of time (Jepsen et al., n.d.). Although, *Artemia*, as well as rotifers, do not fulfill the nutritional profile for an optimal larvae diet and they have to be enriched with marine oils in order to gain nutritional quality (Støttrup, 2000), which rises prices in the aquaculture industry. One main concern in the use of *Artemia* is their dependency on scares hypersaline lakes for the production of cysts and their large size, which hampers the first feeding of some marine fish

larvae (Abate, et al., 2016). On the contrary, copepods are rich in essential fatty acids (such as unsaturated fatty acids (HUFA)) and proteins and are numerous in species, which means that they can be used to feed a great variety of fish species (Evjemo et al., 2003). Another advantage of copepods is their small size and their swimming behavior that is attractive to marine fish larvae (Abate, et al., 2016). Also, studies have shown that fish feed based on copepods increase larval survival, reduce malpigmentation and decrease stress levels in adult fishes (Kraul et al., 1993; Shields et al., 1999; Toledo, Salvacion Golez, Doi, & Ohno, 1999).

Copepods constitute a significant part of zooplankton biomass and thus are an important component of marine ecosystems since they are a crucial element of aquatic food chains and contribute in great scale to marine secondary production (Boxshall & Halsey, 2004). Copepods are widely distributed crustaceans that generally measure less than 2 mm and can be found in marine and freshwater environments (Boxshall & Halsey, 2004). Most of the copepod species are found as free living organisms and some of them can be found at the benthic zone or drifting as plankton in marine environments. In freshwater environments, species can be found at the base of leaves, in hummus or in damp moss. Some copepod species are parasitic like the specie Sphaeronellopsis monothrix and some others serve as parasitic host such as individuals of the genus *Cyclops* that transmit the guinea worm to humans through ingestion (CDC, 2015). Copepods are classified in four main groups: Calanoida, Cyclopodia, Harpacticoida, and Misophrioida. From these orders, Calanoids are the most numerous in terms of species and Misophrioida are the least numerous, constituting of only few species. As it can be seen in figure 1, the life cycle of copepods consist of eggs that hatch into a nauplii larvae that goes through six naupliar stages followed by six copepodite larvae stages that eventually become a male or female adults (Jepsen et al., n.d.; Peterson, 1998). Adults and nauplii are increasing their importance in the aquaculture industry as they are being used as fish feed. Furthermore, copepods have been recognized as bio indicators of ocean pollution and eutrophication and, consequently, their use in laboratory experiments have increased (Gazonato et al., 2014). They are also being suggested as model organisms for ecotoxicology tests and environmental genomics (Raisuddin et al., 2007). Additionally, as noted above, copepods serve as a main source of food for a great diversity of commercially important fish species and they could be used alone or as complement to fish feed in larval cultivation of species such as tuna, flounders, turbot and halibut (Abate, et al., 2016).



Figure 1. Illustration of the life cycle of copepods and their developmental stages. Taken from National Environmental Research Council ZIMNES Project.

Cultivation of copepods is biologically and economically worthwhile. Making a comparison between the costs of fry production of turbot with a copepod diet and marine fish fed with *Artemia* feed, it becomes evident that a copepod diet has a positive profit and reduces significantly the cost of fry production, from \$1.40 to \$0.96 (Abate, et al., 2016). Also, fish farmers using *Artemia* as fish feed require highly trained personnel and more monitoring, which rises labour costs of semi-intensive cultures (Abate, et al., 2016). Another study reveals that the intensive commercial production of the copepod *Acartia tonsa* increases the production capacity of feed fish given that more outputs are produced with the same amount of inputs, which reduces the overall costs of production (Abate et al., 2015). Even though copepods cultivation is a more feasible alternative for fish feed, it also involves massive production of microalgae which constitutes copepod feed (Støttrup, 2000). For an optimal growth, reproduction and egg hatch, copepods require high food quality and quantity (Zhang et al., 2013).

Pelagic copepods feed mainly on free phytoplankton including green algae, cryptomonads, diatoms and dinoflagellates (Norsker, 1997). Because phytoplankton organisms are the primary producers of aquatic environments, they have an important role in the food chain as well as in the carbon cycle. As photosynthetic organisms, they transform the solar energy into chemical energy and consume carbon dioxide to build sugar molecules, which are then transferred to other organisms through the food chain (Descy & Gosselain, 1994). Size, edibility, toxicity and

biochemical profile (specifically HUFA and sterol content) have an important influence in the grazing and assimilation effectiveness of copepods which is later reflected in their growth efficiency (Breteler & Schouten, 1999). Successful growth has been observed in copepods fed with diatoms and stomach analyses of different species of copepods have shown that they have a marked preference for ingestion of diatoms (Ianora, Poulet, & Miralto, 2003; Lebour, 1922; Marshal, 1923). Nonetheless, studies have reported a low nutritional value in diets based on different species of diatoms (Jones & Flynn, 2005). They have also shown that these diets reduce hatching success and can have a negative effect in the copepod embryogenesis, which is attributed to aldehyde suppression (A. Ianora et al., 2004, 2003). On the contrary, other species of green algae, brown algae and dinoflagellates have not shown to be toxic for copepods.

Zhang et al. (2013) studied the effect of six monoalgal diets (*Skeletonema marinoi, Phaeodactylum tricornutum, Isochrysis galbana, Rhinomonas reticulata, Rhodomonas baltica,* and *Tetraselmis suecica*) on the productivity of the copepod *Acartia tonsa*. The results of this study showed that the cryptophytes *R. reticulate* and *R. baltica* used as monoalgal diets increase egg production and egg hatching success. Also, other species of copepods fed with the microalgae *Rhodomonas* spp present high quality of biochemical composition in terms of fatty acids and present high survival rates as well as fast naupliar development (Ianora, Vitello, & Miralto, 2009; Norsker, 1997; Sommer, 2008). Despite the fact that microalgae *Rhodomonas* spp has shown to be very effective for copepod cultivation, an important drawback of this live feed is the high cost and efforts involved in its production, which has to be covered by copepod cultivators (FAO, 2009).

The density of individuals in the production tanks, the maintenance of the culture systems where abiotic factors need to be carefully controlled, the labor work and shipping prices directly affect the costs of copepods cultivation. Besides, phytoplankton production for copepod feed adds extra costs to the production system (Figure 2). Algal quantity is an important parameter in copepod culture given that low amount of food decreases development, increases mortality and competition among individuals, and enhances cannibalism (Drillet et al., 2011). Therefore, microalgal in logarithm growth must be available at all times to ensure optimal copepod growth and density (Rayner & Hansen, 2017).



Figure 2. Illustration showing the main factor involved in the production of calanoid copepods. The copepod and phytoplankton production systems need special monitoring in order to control different abiotic factors. Algal production is necessary in order to feed and rear copepods, which are then stored and shipped worldwide. Modified from Drillet et al., 2011.

Given the fact that aquaculture is a growing industry, it is under constant pressure to enhance methods that will optimize fish production including improvements in copepod cultures. Therefore, special interest has been placed in the replacement of live feed with artificial feed for supplement or as the main source of food. This will not only reduce prices but also facilitate shipment and distribution to fish farmers worldwide. An additional advantage of using artificial feed is that it reduces the possibility of introducing contamination into copepods culture and causing a disease spread (Tacon & Metian, 2008). Dried concentrated microalgae in form of algae paste have shown positive effects on larval growth rates in the intensive rearing of mollusks (Coutteau & Sorgeloos, 1993; Robert & Trintignac, 1997). This has given the possibility of applying algae paste as feed for copepod cultivation. However, Rayner & Hansen, 2017 reported that the copepod *Acartia tonsa* had no significant growth when fed with *Isochrysis galbana* paste even though the nutritional quality of the alga paste was suitable. Therefore, the copepod behavior and perception was suggested to play an important role in the rejection of algae pasta as a potential food source (Rayner & Hansen, 2017).

The copepod *Acartia tonsa* has been widely used as fish larvae feed due to its growth facility in laboratory conditions and because its resting eggs can be stored at low temperatures for long periods of time under certain conditions (Norsker, 1997). Quiescent eggs production is well understood and can be easily produced and distributed to end customers within aquaculture industry (Drillet et al., 2011). After shipping, egg hatching can be induced and individuals can reach few naupliar stages before requiring fatty acids and nutrients for their subsequent growth (Kleppel & Burkart, 1995). Without the appropriate feed, copepods do not gain nutritional and biochemical value and become a poor quality larvae fish feed. For this reason, shipment of eggs and qualified copepod feed would be ideal to secure the effectiveness of copepods in hatcheries. Considering that live feed (microalgae) is very expensive and that alga paste does not produce the desired results, a product with freeze-dried alga transformed into diatoms could constitute a suitable solution.

The aim of this study was to evaluate the potential of freeze dried *Rhodomonas salina* algae with addition of silica to simulate diatom alga composition as an artificial feed for *A. tonsa* nauplii. The product was chosen since, as described earlier, *Rhodomonas* spp has proven to be an excellent nutrition source and diatoms are a preferred prey by copepods. The development of a high quality artificial feed will contribute to the optimization of feeding protocols resulting in the improvement of nauplii growth and copepod nutritional value. Since this is a new developed product, analysis on the artificial feed behavior in seawater as well as observations of their shape and size were made.

2. Methodology

The artificial food used in this project was pasta of the microalgae Rhodomonas salina encapsulated in silica for the purpose of imitating diatoms biochemical composition. This algae pasta was provided by Roskilde University and the silica method was developed by CISME (for details on particle production refer to CISME). This artificial food, which is referred to as particles, was stored in a closed reagent bottle at 4°C until further usage. Two different batches of particles with low and medium carbon content were used, each of which contained 6.23+0.05 $(mean\pm SD)$ and 16.05 ± 1.14 $(mean\pm SD)$ percent of carbon respectively. The first batch had a C:N ratio of 5.30 ± 0.87 (mean \pm SD) and the C:N ratio for the second batch was 5.17 ± 1.11 (mean \pm SD). The reason for obtaining a second batch of particles was the low carbon content of the first batch. A high carbon content for the particles was desired based on the carbon concentration for different algae and diatoms reported by Berggreen et al., (1988), Stottrup & Jensen (1990) and Miller & Roman (2008). The particles with low carbon content were used in the experiments comprising the evaluation of the particles properties and their behavior in filtered seawater. The medium carbon content particles were used for the growth experiment with the nauplii. The seawater used in all the experiments was filtered with a $0.2 \mu m$ filter to remove impurities and reduce the amount of contaminants that could interfere with the results of the study.

2.1 CHN analysis of particles

Given that the particles are a new product and little information about its chemical composition is available, CHN analyses of the particles were made in order to determine the appropriate concentrations to use in each of the further experiments. A fellow student carried out the analysis for the first batch of particles, the ones with low carbon content. For the second batch, five samples of 15.93 ± 1.38 (mean \pm SD) mg were taken and gently introduced into tin capsules. The tin capsules were carefully closed, introduced into a multi-well plate and placed in an exicator for one day. The Laboratory technician conducted the rest of the processing and provided the results.

2.2 Observation of particles: shape and size

To gain insight into the general shape and size of the particles, they were examined under a microscope. A solution of 80 ml containing 0.0566 g of particles and 0.2 μ m filtered seawater was prepared. Three subsamples were taken from the solution and observations were made under a microscope at 20x and 40x magnification. Since the particles presented irregular shapes, length and width measurements were taken for every particle using the NIS-Element AR software (Figure 3). A total of 48 particles were measured.



Figure 3. Example illustrating how the particles were measured. Width and length measurements were taken under a microscope at 40x magnification. The shortest measurement was taken as width and the largest as length.

Taking into account the food size spectra estimated for *A. tonsa* nauplii stages by Berggreen et al., (1988), a second solution was made due to the big size of the particles observed in the first solution. This solution was prepared in 80 ml of 0.2 μ m filtered seawater and contained 0.0566 g of particles. Subsequently, it was filtered through a 15 μ m filter to obtain particles with maximum width or length of 15 μ m. 96 particles from this solution were observed and both width and length measurements were taken under the microscope at 40x magnification using the NIS-Element AR software. A Beckman Coulter Multisizer 4e counter was also used to determine the size of the particles in the filtered solution but only a size range of 2 - 60 μ m was detected by the machine due to the diameter of the aperture tube on the coulter counter, which was of 100 μ m.

2.3 Water quality with only particles

To evaluate the behavior of the particles in seawater, 6 different concentrations of unfiltered particles were prepared and tested for oxygen, temperature, and pH during 48 hours. As this experiment was conducted before knowing the carbon content of the first batch of particles, it was assumed that the carbon content of the particles was the same as in *R. baltica* calculated by Berggreen et al., (1988), which is 47.4 ± 1.9 (mean \pm SD) pg cell⁻¹. The 6 different concentrations were selected based on the initial concentrations of food used in Berggreen et al., (1988) and the conversion to cells per milliliter was done following this same study. To ensure the correct amount of particles, all the concentrations were measured using a Beckman Coulter Multisizer 4e counter. A control without any particles was also carried out and each concentration was replicated four times, for a total of twenty-eight samples. The particles concentrations referred to as treatments are shown in table 1.

Table 1. Concentrations in cell ml⁻¹ of particles calculated for 6 different treatments. The control is stated as treatment 0.

Treatments	0	1	2	3	4	5	6
Particle concentration (cell ml^{-1})	0	1814	2595	4683	8586	17130	32679

All treatments were prepared with 0.2 μ m filtered seawater in 130 ml glass bottles. They were sealed with plastic film and closed with screw cap to ensure absence of air bubbles. To avoid sedimentation, all bottles were randomly placed on a plankton wheel at a speed of 2 rpm and a temperature of ~18°C.

The dissolved oxygen, percent of oxygen saturation, and temperature were measured using an OxyGuard Handy Polaris 2 Portable DO Meter. The pH was measured using an OxyGuard Handy pH Portable pH/Redox Meter. All parameters were measured at the beginning of the experiment and after 16h, 24h, 36h and 48h.

2.4 Water quality with particles and Acartia tonsa nauplii

An experiment with four treatments was conducted in order to establish how the interactions between nauplii and particles affected the overall conditions of the seawater environment in laboratory conditions. The treatments were prepared in 0.2 μ m filtered seawater and consisted of solutions of only particles, only nauplii, nauplii and particles, and a control. Four replicates were made for each treatment in 310 ml glass bottles, having a total of sixteen bottles. All the bottles were sealed with plastic film and closed with screw cap to avoid air bubbles. The bottles were randomly placed in a plankton wheel at a speed of 2 rpm and at a temperature of ~18°C. Measurements of dissolved oxygen, percent of oxygen saturation, and temperature were made with an OxyGuard Handy Polaris 2 Portable DO Meter. The pH was measured using an OxyGuard Handy pH Portable pH/Redox Meter. These parameters were measured at the beginning of the experiment and after 10h, 24h and 48h. An illustration of the experiment is shown below in figure 4.



Figure 4. Visual representation of the experiment set up with the different treatments and its replicates. Measurements of oxygen, pH and temperature were taken at 0h, 10h, 24h and 48h.

For the treatments that required nauplii, they were collected into a 200 ml plastic bottle from a laboratory culture of *Acartia tonsa* at Roskilde University. Filters of $150 \,\mu$ m and $200 \,\mu$ m were

used to separate early stage nauplii from adults and copepodites. A nauplii density of 3 individuals ml⁻¹ were added to the treatment with nauplii and the treatment with nauplii and particles. The nauplii density was estimated by taking five subsamples of 10 ml from the 200 ml bottle with a 10 ml kip-automat. The samples were then poured into petri dishes and 1% of acid Lugol's Iodine was added to each one. Afterwards, the counting of individuals was made under a stereoscope at 4x magnification and subsequent dilutions were made to reach the intended nauplii density.

The concentration of particles used for the treatment with only particles and the treatment with nauplii and particles was 3000 cell ml⁻¹. This concentration was chosen to evaluate the impact of the particles on the seawater quality in a medium with non-saturated food concentration (Kiorboel, Mshlenberg, & Hamburgefl, 1985), which would allow to obtained a general indication of the conditions of the medium over time. The desired concentration was obtained from a stock solution of 25 ml containing 0.1238 g of particles and 0.2 μ m filtered water. The final concentration was ensured using a Beckman Coulter Multisizer 4e counter.

2.5 Sedimentation test

To look into how fast the particles sediment in a saline environment, a quick trial with two solutions with $15-\mu$ m filtered particles were made. The first solution was a saturated concentration of particles and the second solution was approximately half the concentration of the first one (Table 2). The solutions were prepared in 50 ml beakers and were diluted from a stock solution of 200 ml containing 0.253g of particles and 0.2 μ m filtered water. Before the initial measurements were taken, the solutions were agitated to assure that the particles were evenly distributed. During the whole experiment, a Beckman Coulter Multisizer 4e counter was used to determine the initial concentrations of the two solutions and to measure the particles concentration in the water column over time. The measurements were made every 5 or 10 minutes for a period of 157 minutes.

 Table 2. Concentration of particles in the water column at time 0.

	Solution 1	Solution 2
Particles concentration (cell ml ⁻¹)	85909	46174

2.6 Acartia tonsa Nauplii growth experiment

To evaluate the effects of the particles used as artificial feed on the growth, population development and mortality of *A. tonsa* nauplii, an experiment with three different particles concentrations was conducted (Table 3). Each treatment was replicated six times, for a total of eighteen samples. The concentrations used for this experiment were based on the specific growth rate as a function of concentration of *R. baltica* calculated by Berggreen et al., (1988) and corresponded to the food levels located in the exponential phase of the growth curve. A fellow student performed the experiment with a saturated concentration of particles and a starvation control together with a positive control using *R. salina*. These results are shown in a separate study.

The particles used for this experiment had 16.05 ± 1.14 (mean \pm SD) percent of carbon content and the calculations for the concentration in cell ml⁻¹ were adjusted accordingly based on Berggreen et al., (1988). A Beckman Coulter Multisizer 4e counter with a 100 μ m aperture tube was used to determine the concentrations of the treatments. In order to assure that the concentrations remained constant during the whole experiment, sub samples of 1 ml from every beaker were taken every day and measurements of their concentrations were made with a Beckman Coulter Multisizer 4e. When decreased, the concentrations of the treatments were normalized adding particles from a stock solution of 50 ml containing 0.1 g of particles and 0.2 μ m filtered water. The stock solution was prepared every day and was filtered through a 15 μ m filter.

Table 3. Concentration of particles kept constant during the whole experiment.

Treatments	1	2	3
Particle concentration (cell ml^{-1})	3628	5190	9366

A. tonsa eggs were obtained from a cold stored culture batch at Roskilde University. The eggs were added to a 5 L container with 0.2 μ m filtered water and left aerated at a temperature of 17°C for 24 hours. After that time, it was noted that the eggs had not hatched yet; therefore they were left for another 24 hours. Thereafter, the hatched nauplii were distributed into the eighteen 1 L beakers with a density of 7831± 124 (mean±SD) individuals ml⁻¹. The nauplii density was estimated by pouring the 5 L container into two 2 L bottles. Four subsamples of 10 ml were taken from each bottle with a 10 ml kip-automat and transferred into petri dishes. 1% acid Lugol's lodine was added to the petri dishes and individuals were counted under a stereoscope at 4x magnification.

After distributing the hatched nauplii into the beakers, the particles were added according to the treatments and $0.2 \,\mu$ m filtered water was poured until a volume of 1 L was reached in each beaker. Following, the beakers were equipped with air stones connected to air pumps to ensure optimal oxygen levels and covered with plastic film to prevent contamination and reduce evaporation.



Figure 5. Life cycle and development of the copepod Acartia tonsa. This specie has six naupliar stages with a range length of $\sim 112 \ \mu m$ - 260 μm . Classification of the nauplii in the growth experiment was done based on this figure taking into account the size and the morphological features of the individuals. Taken from Roskilde University.

For the mortality and growth measurements, two sub samples of 5 ml were taken from each beaker every two days for a period of 12 days. This sub samples were taken using a Hensen piston sampler and then they were transferred to petri dishes. 1% acid Lugol's iodine was added to each petri dish and they were observed under a microscope at 13.5x magnification. The nauplii were counted, measured for total length and classified in early nauplii stages. This classification was made on the basis of nauplii morphology according to figure 5. After taking the measurements, the petri dishes were covered with aluminum foil and stored in a laboratory shelf to avoid photodecomposition. An illustration of the experimental procedure is shown in figure 6.



Figure 6. Visual representation of the experiment set up with colors representing the three treatments and its replicates in 1 L beakers. Each treatment had six replicates and 2 sub samples of 10 ml were taken from each beaker every two days for a period of 12 days.

The mortality rate of each treatment was calculated in accordance to the equations given by Breteler et al., (2004). First, the number of individuals estimated per liter was plotted as a function of time and the resulted graph corresponded to an exponential function. The exponential equation given by the graph had the form of $y = ae^{-bx}$, where *a* represents the slope of the curve and *b* represents the *y* intercept and the instantaneous rate of mortality (Z). This instantaneous rate of mortality was used in the following equation to estimate the number of individuals at a given time:

$$N_t = N_0 e^{-Zt}$$

In this equation N_t is the number of individuals per liter at a time t and N_0 is the number of individuals per liter at the beginning of the experiment. Since there were individuals killed by the sub sampling, N_t had to be corrected following Breteler et al., (2004) equation:

$$(N_t) \cdot V^{(n-1)} / [(V - v_1) \cdot (V - v_2) \cdot ... \cdot (V - v_{(n-1)})]$$

where V is the initial volume of the beakers, v is the volume of the sample and n is the ranking of the sample. The corrected N_t was plotted as a function of time in an exponential graph and a new instantaneous rate of mortality (Z) was obtained, which was finally compared among the three treatments.

2.7 Acartia tonsa Egg hatching success

The hatching success of the batch of eggs used for the nauplii growth experiment was estimated by taking a sample from the initial 5 L container into a 100 ml bottle. Then, five subsamples of 10 ml were taken from this bottle using a 10 ml kip-automat and transferred to petri dishes. The eggs were counted under a stereoscope at 10x magnification and left to hatch for 72 hours. After this time period, another five subsamples were taken from the 100 ml bottle with a 10 ml kip automat into petri dishes. Then, 1% acid Lugol's iodine was added to each sample and hatched nauplii and unhatched eggs were counted under a stereoscope at 4x magnification.

2.8 Statistical analysis

All data was tested for normal distribution and homogeneity of variance previous to the statistical analyses. Shapiro-Wilk test and Levene's test were performed for normality and equal variance, respectively. Because the oxygen measurements in the water quality with only particles experiment did not presented normal distribution, this data was subject to a logarithm transformation. When normality and variance homogeneity was verified, the data was subject to the parametric test ANOVA (Analysis of variance). A one-way ANOVA was used to test the difference in mortality rates among the different treatments in the nauplii growth experiment. To test the effect of the different treatments on the oxygen concentration over time in both experiments of water quality (particles and nauplii, and only particles) a two-way ANOVA was performed. When statistical differences were determined by the ANOVA test, Tukey's range test was performed as a post-hoc analysis to find the groups that were significantly different from each other.

The data that did not meet the requirements for the parametric test was subject to the nonparametric test PERMANOVA (multivariate permutation analysis), which was the case of the length measurements over time in the nauplii growth experiment. All the statistical analyses were performed with R 3.3.3 software, except for PERMANOVA test. PERMANOVA analysis was carried out in Primer 6 with permanova+ plugin. A significant level of α =0.05 was chosen for all the statistical tests. The graphs were generated with the package ggplot2 in R 3.3.3 software.

3. Results

3.1 Observations of particles: shape and size

The particles observed under the microscope presented irregular shapes and forms (Figure 7), as well as different sizes. The biggest particles had a lack of symmetry and their form was different for each particle, whereas the small particles had a more round like shape. A tendency to aggregate was observed in general and it was more evident in the 15 μ m filtered particles. This aggregation could contribute to the irregular shapes observed on the unfiltered particles, giving the possibility for the biggest particles to be, in reality, small particles grouped into a single set.



Figure7. Particles observed under the microscope at 40x magnification. *A.* Unfiltered particles. *B.* Filtered particles with a 15 μ m filter.

To visualize the distribution of the size of the particles, length and width measurements were plotted in histograms. In addition, Kernel density estimation was used to estimate the probability density function (Figure 8 and 9). For both filtered and unfiltered particles, length and width measurements follow a similar distribution curve. For the unfiltered particles, the mean length was 77.33 ± 37.62 (\pm SD) μ m and the median was 69.42μ m. The mean width was 50.68 ± 24.01 (\pm SD) μ m and the median was 47.01μ m. For the 15 μ m-filtered particles, the mean length was

7.61±4.99 (±SD) μ m and the median was 5.72 μ m. The mean width was 6.14±4.62 (±SD) μ m and the median was 4.61 μ m.

It is worth noting that an abundance of small particles was observed in the filtered and unfiltered particles. The majority of the particles seem to be between the ranges of 20-80 μ m for the unfiltered particles and between the ranges of 1-10 μ m for the filtered particles. Since the unfiltered particles did not exceed a width or length size of 170 μ m they could be used to feed adult copepods. Similarly, the filtered particles rarely exceeded a size of 15 μ m, thus fitting the food size spectra for nauplii (Berggreen et al., 1988).



Figure 8. Relative frequency distribution of the unfiltered particles size plotted along with the kernel density estimation, which is displayed as a black line. *A* Distribution of length measurements for the unfiltered particles. *B* Distribution of the width measurements for the unfiltered particles. *A* total of 48 particles were measured for both length and width.



Figure 9. Relative frequency distribution of the 15-um filtered particles size plotted along with the kernel density estimation, which is displayed as a black line. **A** Distribution of length measurements for the 15-um filtered particles. **B** Distribution of the width measurements for the 15-um filtered particles. A total of 96 particles were measured for both length and width.

3.2 Oxygen depletion with only particles

Temperature and pH remained constant for all treatments over the 48 hours period of the experiment; the temperature stayed at 19.70 ± 0.82 (mean \pm SD) °C and the pH was 7.70 ± 0.02 (mean \pm SD). Dissolve oxygen levels decrease constantly over time for all treatments, where a much drastic decline was seen at 48 hours for treatments 5 and 6 (Figure 10). The control (treatment 0) had a decrease of 16.6% in oxygen concentration (mg/l) and the treatment 1 had a total decrease of 23.8%. Treatment 5 and 6 had a total decrease in oxygen concentration (mg/l) of 80.2% and 80.6% respectively. The oxygen saturation also decline and it was proportional to decrease in oxygen concentration (mg/l).



Figure 10. Oxygen depletion in seawater for different concentrations of particles, which are indicated as treatments. The graph shows oxygen concentration (mg/l) as a function of time. A control without particles is stated as treatment 0 whereas treatments 1 to 6 are increasing concentrations of particles (Treatment 1: 1814 cell ml⁻¹; Treatment 2: 2595 cell ml⁻¹, Treatment 3: 4683 cell ml⁻¹, Treatment 4: 8586 cell ml⁻¹, Treatment 5: 17130 cell ml⁻¹, Treatment 6: 32679 cell ml⁻¹). Each treatment was replicated four times; giving a total of twenty-eight samples. For each treatment the mean \pm standard deviation is plotted.

The two-way ANOVA test (Table 4) revealed significant differences between the treatments (P-value<0.05) and between the hours (P-value<0.05). Moreover, it showed an interaction between the treatment and the hours (P-value<0.05) meaning that the particles concentration had an influence in the decline of oxygen concentration over time.

Table 4. A two-way analysis of variance to evaluate differences among treatments, hours and the interaction between those two factors.

	Degrees of freedom	Sum of squares	Mean square	F value	P-value
Treatments	6	25.58	4.26	20.86	5.69e-16
Time	4	162.04	40.51	198.21	<2e-16
Treatment x Time	24	71.31	2.97	14.54	<2e-16
Residuals	105	21.46	0.20		

The Tukey's test showed significant differences between treatment 5 and the control (P-value=0.001) and between treatment 6 and the control (P-value<0.001). Oxygen concentration in treatment 6 was significantly lower than in treatment 1 (P-value=0.029) at 36 and 48 hours. Similarly, oxygen concentration in treatment 5 was lower than in treatment 2 (P-value=0.013) and it was also lower in treatment 6 than in treatment 2 (P-value=0.003). However, no significant differences were found when compared treatments 5 and 6 to treatments 3 and 4 (P-values>0.05). In addition, the oxygen concentration had a significant decrease in all treatments over time except for hours 0 and 16 (P-value=0.88) and hours 24 and 36 (P-value=0.28).

3.3 Oxygen depletion with particles and Acartia tonsa nauplii

The temperature remained constant at 19.50 ± 0.63 (mean \pm SD) °C as well as the pH which was at 7.70 ± 0.04 (mean \pm SD). The dissolved oxygen concentration decreased over the 48 hour that lasted the experiment (Figure 11). The oxygen concentration (mg/l) had a total decrease of 5.7% in the control treatment and a decrease of 13.3% in oxygen concentration (mg/l) was observed in treatment with only particles. Similarly, the treatment with only nauplii has a decrease in oxygen concentration (mg/l) of 11.6% and the treatment with particles and nauplii had a decrease of 20.6% in oxygen concentration (mg/l). The decrease in dissolved oxygen (mg/l) for the treatment with particles and nauplii followed a linear pattern meanwhile for the other treatments the decrease was more stable. Following the same pattern as the experiment of

oxygen depletion with only particles, the oxygen saturation decreased at the same time as the oxygen concentration (mg/l).

The two-way ANOVA test shown in table 5 determined significant differences among treatments (P-value<0.05) and between the hours that lasted the experiment (P-value<0.05). The multiple comparisons made by the Tukey's test revealed that the control presented a higher oxygen concentration than the nauplii treatment (P-value=0.012). Also, the control had higher oxygen concentration than the nauplii and particles treatment (P-value<0.001). In the same way, the treatment with particles and nauplii had a lower oxygen concentration than the treatment with only particles (P-value=0.024). No significant differences where found between the treatment with only particles and the treatment with only nauplii (P-value=0.78).



Figure 11. Oxygen depletion for 4 treatments in 0.2 um-filtered seawater medium is shown as oxygen concentration (mg/l) as a function of time. A particle concentration of 3000 cell ml^{-1} was used for the treatment with only particles and for the treatment with particles and nauplii. A nauplii density of 3

individuals ml^{-1} was added to the treatment with only nauplii and the treatment with particles and nauplii. The control treatment consisted of only 0.2-um filtered seawater. Four replicates were made for each treatment; giving a total of sixteen samples. The mean \pm standard deviation is plotted for each treatment.

The Tukey's test also showed differences between the all hours where the measurements were taken except for hour 0 and 10 (P-value=0.18), and hour 10 and 24 (P-value=0.09). As a result, the marked decline of oxygen concentration occurred after 24 hours in the treatment with particles and nauplii. A less drastic decline occurred in the treatment with only particles and the treatment with only nauplii but it was also significant after 24 hours.

Table 5. Results from a two-way analysis of variance stating differences among tratments, hours and the interaction between these two factors.

	Degrees of freedom	Sum of squares	Mean square	F value	P-value
Treatments	3	2.39	0.79	15.59	4.73e-07
Time	3	6.48	2.16	42.26	5.15e-13
Treatments x Time	9	2.00	0.22	4.35	4.28e-04
Residuals	44	2.25	0.05		

3.4 Sedimentation test

The number of particles in suspension for both solutions 1 and 2 had a substantial decline over time (Figure 12A). The solution 1 had a decrease of 47.4% in the number of particles in the water column. Likewise, the solution 2 had a decrease of 59.9% in the number of particles suspended in seawater at the end of the experiment. Regardless of the initial concentration of particles in the solutions, the number of suspended particles is reduced by half after 157 minutes.

The mean size of the particles in suspension over time for solution 1 and 2 is shown in Figure 12B and 12C. The mean size of the particles in the water column in solution 1 at time 0 was 11.09 ± 7.28 (\pm SD) and for the particles in suspension in solution 2 at time 0 was 7.56 ± 5.14

(\pm SD). At the end of the experiment the mean size of the particles in solution 1 was 5.04 \pm 1.12 (\pm SD) and in solution 2 was 5.18 \pm 1.37 (\pm SD). These results suggest that the particles sediment at the same rate independent of their size since no apparent change in size of the particles in suspension was observed during the sedimentation test. Additionally, sedimentation of the particles occurred very fast in a short period of time.



Figure 12. A Number of particles over time in the water column of two solutions with different concentration of unfiltered particles (refer to table 2). **B** Mean \pm standard deviation of particles size in suspension over time for solution 1. **C** Mean \pm standard deviation of particles size in suspension over time for solution 2. The same particles counted in **A** for both solutions were measured in **B** and **C** using a coulter counter.

3.5 Acartia tonsa Nauplii growth

The hatching success of the batch of eggs used in this experiment was 71.19 ± 10.95 (mean \pm SD). Figure 13 shows the increase in size of nauplii during the 12 days of the experiment. For treatment 1 a linear and constant increase in length was observed until day 10, where it had an important decrease. A similar tendency was observed for treatment 2 which remained constant until day 6 where it had a marked increase and then it started decreasing till day 12. Treatment 3 had few fluctuations, however, an overall increase in length was observed over time.



Figure 13. Nauplii growth for 3 different concentrations of particles indicated as treatments (Treatment 1: 3628 cell ml^{-1} , Treatment 2: 5190 cell ml^{-1} , Treatment 3: 9366 cell ml^{-1}). The graph shows average of total length of nauplii over time for the 3 treatments. Six replicates were carried out for each treatment; giving a total of eighteen samples. The mean length \pm standard deviation is plotted for all measurements. **A.** Total nauplii length for treatment 1 in 12 days. **B.** Total nauplii length for treatment 2 in 12 days. **C.** Total nauplii

length for treatment 3 in 12 days. **D.** Significant length differences found in day 12 between treatment 3 and treatments 1 and 2.

Low nauplii growth was observed in treatments 1 and 2: for treatment 1 the total increase in length was 4.62% and for treatment 2 was 2.96%. Treatment 3 had a little more growth with 11.48% of total length increase. Since there was not a considerably increase in length of the individuals over time, growth rate was not calculated.

PERMANOVA analysis (Table 6) revealed significant differences among the treatments on day 12 (Figure 9, D). Nauplii length in treatment 3 was notably higher than in treatment 1 (P-value=0.0198) and than in treatment 2 (P-value=0.0297). The average length for treatments 1 at day 12 was 124.63 ± 15.84 (\pm SD) μ m, for treatment 2 was 123.67 ± 15.78 (\pm SD) μ m, and for treatment 3 was 135.27 ± 11.89 (\pm SD) μ m. For the rest of the days of the experiment no significant differences in length were found between the treatments (P-value>0.05).

Table 6. Results from PERMANOVA of difference in length between treatments and days. Significantdifferences were found among days and in the interaction between treatments and days.

	Degrees of freedom	Sum of squares	Mean square	Pseudo-F	P (perm)
Treatments	2	55.479	27.739	0.17315	0.8416
Time	6	8568.1	1428	8.9138	0.0099
Treatments x Time	12	4216.4	351.36	2.1932	0.0099
Residuals	497	70621	160.2		
Total	517		92461		

3.6 Acartia tonsa Nauplii mortality

For all treatments the instantaneous nauplii mortality was low and fairly constant. An estimated decrease of 21.67 ± 10.20 (mean \pm SD) % of individuals per day was calculated for treatment 1. For treatment 2 the decrease of individuals per day was 19.04 ± 7.94 (mean \pm SD) % and for treatment 3 the decrease of individuals per day was 24.35 ± 7.89 (mean \pm SD) %.

Mortality rates for each treatment are displayed in figure 14, where subtle differences in mean values are evident despite the overlapping of the data's distribution. However, no significant differences were found in mortality rates when comparing them among treatments (P-value>0.05) (Table 7).



Figure 14. Boxplot of the mortality rates for 3 Treatments with different concentration of particles (Treatment 1: 3628 cell ml⁻¹, Treatment 2: 5190 cell ml⁻¹, Treatment 3: 9366 cell ml⁻¹). Six replicates were made for each treatment for a total of eighteen samples. The line in the middle of the boxes represents the median, which is the mid-point of the data. The lower and upper hinges illustrate the first and third quartile, whereas the whiskers show the 95% confidence interval of the data.

Table 7. Results from a one-way analysis of variance performed to compare mortality rates betweentreatments.

	Degrees of	Sum of	Mean square	Evalue	P-value
	freedom	squares	Mean Square	I value	1 -value
Treatments	2	0.001215	0.0006077	1.367	0.285
Residuals	5	0.006670	0.0004446		

3.7 Population development

Figure 15 shows the nauplii stages at the beginning of the experiment and at the end of the experiment for each treatment. No gut content indicating ingestion of particles was apparent in nauplii for any of the treatments at day 12. Moreover, after 10 days all individuals were observed to be very transparent in all treatments, which suggest low lipid storage.



Figure 15. Nauplii illustrating stages at the beginning and at the end of the growth experiment. *A.* Hatched nauplii after 72 hours, consisting of the nauplii used at day 0. *B.* Nauplii after 12 days in treatment 1. *C.* Nauplii after 12 days in treatment 2. *D.* Nauplii after 12 days in treatment 3.

A similar nauplii stage progression was followed by all the treatments and no differences were found among them (Figure 16). Nauplii stages III-IV started to appear at day 4 and became predominant at day 6. At the end of the experiment all nauplii were at stage III-IV. The duration of the first naupliar stages (I-II) was shorter than nauplii at stages III-IV, still no further growth was observed.



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Figure 16. Percentage of nauplii in the initial stages of development according to the treatments with different concentration of particles. Six replicates were made for each treatment for a total of eighteen samples **A.** Percentage of individuals over time in Treatment 1 with a particle concentration of 3628 cell ml⁻¹. **B.** Percentage of individuals over time in Treatment 2 with a particle concentration of 5190 cell ml⁻¹. **C.** Percentage of individuals over time in Treatment 3 with a particle concentration of 9366 cell ml⁻¹. No differences were found among the treatments and they seem to follow the same pattern.

In addition, the time of stage switch from nauplii stage I-II to nauplii stage III-IV of 50% of the population was calculated for each treatment. The time calculation was made from the intersection between the two lines showing the population abundance of the nauplii stages: stage I-II and stage III-IV (Figure 17). A one-way analysis of variance was performed to determined differences in time transition among treatments (Table 8), however no significant differences were found (P-value >0.05). For treatment 1 the transition time of half of the population from stage I-II to stage III-IV was 5.30 ± 0.68 (mean \pm SD) days, for treatment 2 was 5.08 ± 0.18 (mean \pm SD) days and for treatment 3 was 5.14 ± 0.49 (mean \pm SD) days.

Table 8. Results from a one-way ANOVA comparing the time that if takes for 50 percent of the population to
transitioned form one naupliar stage to the next one in nauplii development between treatments.

	Degrees of	Sum of	Moon square	F value	P-value
	freedom	squares	wean square		
Treatments	2	0.15	0.07477	0.306	0.741
Residuals	15	3.67	0.24469		



Figure 17. Abundance graph illustrating the number of individuals as a function of time for 3 treatments with different concentration of particles (Treatment 1: 3628 cell ml⁻¹, Treatment 2: 5190 cell ml⁻¹, Treatment 3: 9366 cell ml⁻¹). Six replicates were made for each treatment; giving a total of eighteen samples. The black line represents the number of nauplii in stage I-II over time and the gray line represents the number of nauplii in stage III-IV over time. The mean length \pm standard deviation is plotted for all data **A** Population abundance for Treatment 1. The red line represents the total number of individuals over time. **B** Population abundance for Treatment 2 where the green line represents the number of individuals over time. **C** Population abundance for Treatment 3. The purple line represents the total number of individuals over time.

4. Discussion

Copepods require food rich in fatty acids and PUFAs in order to have a successful development, which allows them to become a high-quality feed for fish larvae. Calanoid copepods like Acartia tonsa produce quiescent eggs that can be stored at low temperatures and distributed to hatcheries around the world. Nonetheless, copepod feed such as live microalgae is expensive to produce and difficult to transport since it is susceptible to degradation if stored for a prolonged time. Alga paste, on the other hand, can be preserved but does not have positive effects on copepod production. Thus, a product (referred to as particles) containing dried Rhodomonas *salina* encapsulated in silica to mimic diatoms can constitute a high quality food source that can be stored for long periods of time and easily shipped along with copepods eggs. The microalgae *Rhodomonas* spp biochemical composition meet the nutritional need of copepods and it has been reported that copepods have a preference for diatoms in their diets. In this study, the growth performance and mortality of A. tona nauplii fed with particles was evaluated. Likewise, the particles efficiency as an artificial feed for individuals in early stages of development was determined. Also, the behavior of the particles in seawater as well as the abiotic factors of the seawater when mixed with particles was monitored to establish its influence in the overall conditions of the medium where copepods are commercially produced.

4.1 Analysis of particles composition and morphology

The physical characteristics of the particles are important because they need to be appealing and detectable by copepods. It has been reported that many species of calanoid copepods have mechanoreceptors that allow them to locate their prey (Bollens, Frost, & Cordell, 1994). Also, it has been shown that visual cues are important for predatory behavior in calanoid copepods as they respond to slightly changes in light intensity (Bollens et al., 1994). Since the particles mimic diatoms and had a red color similar to *R. salina*, they could be playing a favorable effect that attracts copepods towards them.

Measurements of the unfiltered particle show that they have a wide distribution of sizes in both length and width, as it is shown in figure 8. This could be an advantage given that the same batch of particles can be used to feed all stages of *A. tonsa* according to the particle size of maximum

clearance and the upper size limit reported by Berggreen et al, (1988) for all developmental stages, which are 15 μ m for nauplii and 250 μ m for adults. Similarly, as shown in figure 9, the 15 μ m filtered particles sizes for both length and width were also within the retention spectra range reported for *A. tonsa* nauplii (Berggreen, Hansen, & Kiorboe, 1988). Few particles above 15 μ m in length and width were observed for the 15 μ m filtered particles, but their presence does not constitute a problem for nauplii feed since their abundance was not significant. When observed under the microscope, the particles presented irregular shapes with the smaller ones having a more spherical-like shape. It is expected that the shape of the particles may not constitute an obstacle for its ingestion by copepods given that many phytoplankton organisms that are part of natural diets of copepods have morphological variability, with some microalgae presenting protuberances while others having a oval or round shape (Naselli-Flores, Padisák, & Albay, 2007). Also, it has been shown that copepods have an adaptive response to changes in abundance (Swadling & Marcus, 1994; Ogle, Nicholson, & Lotz, 2002).

Nonetheless, aggregations observed among small particles in the 15 μ m filtered solution could constitute a problem given that nauplii would not be able to eat these accumulations as they increase in size. Besides, gravitational sedimentation has an influence in elements of big dimensions that leads them to sediment faster (Laidlaw & Steinmetz, 2005). If that happened, aggregations could result in less food availability in the water column and starvation of individuals after a long period of time. However, this might not be the case for the particles since the sedimentation test showed that there was not a significant change in size of suspended particles over time (Figure 12 B and C). In addition, sedimentation was evident over a short period of time where c.a half of the number of particles in the water column was reduced after 157 minutes regardless of the initial concentration of the solutions (Figure 12 A). For this reason, a gentle agitation of the seawater medium must be done in order to secure an homogeneous mixture. Finally, it is likely for very small particles to be present in the solution ($<1\mu$ m), in which case they will not settle by gravity (Laidlaw & Steinmetz, 2005) and therefore stay in suspension and be available at any time. Though, this cannot be assured because it was not possible to determine the size of the smaller particles since the coulter counter used for the measurements only detected particles above 2 μ m.

With regard to the carbon content of the particles, the batch used in the nauplii growth experiment had less percent of carbon content $(16.05\pm1.14 \text{ (mean}\pm\text{SD}) \%)$ of what it is reported for *Rhodomonas baltica* (Berggreen et al., 1988) but it was within the range of what it has been found for other species of green algae and diatoms (Stottrup & Jensen, 1990; Miller & Roman, 2008). Additionally, the carbon and nitrogen ratio (C:N) of the particles , which was of 5.17 ± 1.11 (mean \pm SD), was very close to the ratios found in long-phase diatoms and ciliates (C. A. Miller & Roman, 2008). As with carbon, nitrogen content food is also important for synthesis of proteins in copepods and because it has been demonstrated that it influences the composition of fatty acids as it can increase lipid content (Rasdi & Qin, 2016). Given the results of the CHN analysis, the particles seem to have an appropriate concentration of carbon and C:N ratio for rearing of copepods. Yet, for a better understanding of the nutritional value of the particles, further analysis on the levels of fatty acids such as PUFA, EPA and DHA must be carried out in both the particles and fed nauplii.

The water quality experiments demonstrated that the particles did not have an effect in the pH or temperature on seawater due that these variables remained constant and within the range of seawater natural values. The results on water quality with only particles shown in figure 10 exhibited a decrease in oxygen concentration proportional to the particles concentration, which could be attributed to microbial degradation. After 36 hours, the oxygen concentration decreased to 1.4 ± 0.74 (mean \pm SD) mg/l and 1.35 ± 1.70 (mean \pm SD) mg/l for the highest concentrations of particles, which were 17130 cell/ml and 32679 cell/ml respectively. In contrast, the results of the experiment on water quality with particles and nauplii showed that even though the oxygen concentration decreased over time, its levels did not dropped below 5.5 mg/l (Figure 11). The lowest oxygen concentration $(5.60\pm0.08 \text{ (mean}\pm\text{SD}) \text{ mg/l})$ was reported at 48 hours for the treatment containing 3000 cell/ml particles and a nauplii abundance of 3 ind/ml. Here, an additive effect of nauplii respiration and possible microbial degradation was observed. This oxygen concentration was similar to the one obtained for treatment 2 $(5.77\pm0.08 \text{ (mean}\pm\text{SD}))$ mg/l) in the water quality experiment shown in figure 10 where the particle concentration was 2595 cell/ml. Hypoxia, defined as low oxygen concentrations dissolved in water, constitutes an stress factor that can induce quiescence in the eggs of *A. tonsa* and can have a negative impact on copepod survival (Invidia, Sei, & Gorbi, 2004). Concentration of oxygen below 2 mg/l have

negative effect in population growth rate, abundance of naupliar stages and fertility of copepods (Invidia et al., 2004; Marcus, Richmond, Sedlacek, Miller, & Oppert, 2004). Since the experiment with particles and nauplii did not exhibit levels of dissolved oxygen lower that 5 mg/l, the particles at medium and low concentrations are not expected to cause stress on nauplii. Nevertheless, it was demonstrated that higher concentration of particles depletes oxygen at harmful levels for nauplii development (< 2.1 mg/l). In this regard, is important to replace the water of closed bottles at least every two days to avoid hypoxia if air bubbles cannot be provided when saturated concentrations of particles are used.

4.2 Acartia tonsa nauplii growth and mortality

Total length of nauplii measured for treatments with three different concentrations of particles (3628 cell/ml, 5190 cell/ml, and 9366 cell/ml) showed that there was a slight increase in length during the period of time that lasted the experiment (Figure 13). As seen in figure 13 D, significant differences in length were evident on day 12 between the treatment with the highest concentration and the other two treatments, suggesting that a high concentrations of particles could have an effect on nauplii growth. The first stages of nauplii development (specifically stage I), are considered non feeding stages since they use the energy reserves transferred from their mother to survive (Jónasdóttir, 1994). Thus, nauplii growth on the first days of the experiment could be a reflection of the diet composition of the previous generation of copepods. This is given that adult females invest most of their energy in the production of eggs and in the build up of nutritional adequate egg yolk (Jónasdóttir, 1994). This could explain the similarity in growth pattern observed for all three treatments where no increase in nauplii length was detectable for the first 4 days of the experiment. After that time, individuals had a minor increase in length with no significant differences among treatments except for day 12.

After the 12 days that lasted the growth experiment, it was observed that nauplii did not reached development beyond stages III-IV regardless of particles concentration. It was also evident that the population development followed a similar pattern for the three treatments, as shown in figure 16 and 17. In addition, the time that took for half of the population to transition from nauplii stage I-II to nauplii stage III-IV was similar for all treatment and was about 5 days. This suggest that the copepod time development was delayed compared to the developmental time

that it took for the copepod *A. tonsa* to reach an adult state when fed with *Rhodomonas spp.* at 18 °C, which was reported to be of around 13 days (Berggreen et al., 1988). Miller & Johnson (1977) indicated that when *A. tonsa* is present in an environment with excess of food, the time progression for all stages is constant and its development is linear over time, whereas its growth is exponential. On the other hand, it has also been shown that *A. tonsa* present an isochronal development and a prolonged duration of nauplii stage II when fed with saturated concentrations at 18 °C (Miguel & Peter, 2006). The weight loss and the inability to catch food has been suggested as the principal cause of a prolonged duration of nauplii in stage II (Miguel & Peter, 2006). In addition, it has been stated that the long duration of this naupliar stage can be attributed to the fact that when *A. tonsa* produces resting eggs, it has to arrest development and slow down metabolic activity of the quiescent eggs. As a consequence, these eggs take longer to hatch and the development time for the first naupliar stages can also be longer (Miguel & Peter, 2006). This could be a reason for the slow development observed in this study, since the batch of eggs used for the growth experiment was cold stored before hatching was induces, meaning that they were in a resting state.

Another explanation for the delayed in nauplii development and the slow growth of individuals could be a consequence of reduced particle retention due to the small size of the particles available for ingestion. As mentioned before, it is possible that particles < 1 μ m were always present in the water column and after sedimentation of some particles, individuals fed from them. This could have lead to a low clearance rate given that these small particles are below the low size limit for particle capture in nauplii stages which is 1.9-3.7 μ m (Berggreen et al., 1988). In addition, nauplii would have to process high amount of food in order to obtain the necessary amount of nutrients for their successful development (Jonasdottir, 1994). If nauplii are proven to be nutritionally valuable, a delay in their development could constitute an advantage for hatcheries that need to feed species of marine fish larvae with small mouth dimensions.

Observations of nauplii after 12 days of feeding with particles (Figure 15) did not show evidence of ingestion and gut content was difficult to visualize. Moreover, individuals were very transparent, indicating low lipid content. It has been reported that compared to adults, nauplii stages are less sensitive to food abundance and instead, their development and growth is heavily dependent on the quality of the food in terms of chemical composition (Rasdi & Qin, 2016). For this reason, it is important to conduct further research on the content of fatty acids and amino acids of the particles and how much of those the nauplii assimilate after ingestion. On the other hand, results illustrated in figure 13 D showed that nauplii fed with treatment 3, which was the highest concentration of particles (9366 cell/ml), presented an increase in length compared to the other two treatments, meaning that the abundance of particles can have an impact in nauplii growth.

All three concentrations of particles showed a similar tendency in the decline on the number of individuals over time (figure 17). Furthermore, all treatments had a low mortality rate compared to the results obtained by Kioski & Breteler (2003) on copepods fed under laboratory conditions with *Rhodomonas* spp. Differences in mortality rates among treatments were not significant, suggesting that the different concentrations of particles did not have an influence in nauplii mortality. Even though, the particles in it selves had a positive effect on nauplii survival when compared to starvation and diets based on other microalgae species reported by other studies (Kioski & Breteler, 2003; Knuckey, Semmens, Mayer, & Rimmer, 2005; Stottrup & Jensen, 1990).

5. Conclusion

The results of this study showed that the particles, composed by dried Rhodomonas salina encapsulated in silica, can sustain Acartia tonsa nauplii population but are not appropriate for rapid growth and development of copepods. The morphology and sizes of the particles was appropriate for *A. tonsa* nauplii consumption when filtered through a 15 μ m filter. The particles decreased dissolved oxygen concentration but did not drop it at hypoxia levels when mixed with seawater at medium concentrations. Even though mortality was low, nauplii showed signs of nutrient deficiency, as they did not had a significant growth in total length and their development was impaired. Analyses on the lipids and amino acids profile of the particles must be done despite the fact that the CHN analysis provided evidence that the they had a carbon content and a C:N ratio similar to some species of microalgae present in natural diets of calanoid copepods. In conclusion, the particles seem to work as artificial feed for *A. tonsa* nauplii since they are able to eat them; nevertheless, improvements in their biochemical composition must be readjusted in order to obtain positive results in growth and nauplii development. Finally, special care must be taken regarding aggregation and sedimentation of the particles as they can have a negative impact in A. tonsa nauplii rearing. Further enhancement of the chemical and physical properties of the particles can provide a suitable alternative for copepod feed if improved particles are proven to have favorable nutritional characteristics and are successfully ingested by copepods.

6. Bibliography

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