NTNU – Trondheim
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Marine Coastal Development
Submission date: May 2014
Supervisor: Elin Kjørsvik, IBI
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Anh, Phan Hung

Cover picture by Anh Phan Hung

Abstract

Acartia tonsa is a pelagic calanoid copepod with a diverse distribution, a wide tolerance of temperature and salinities, has short generation time, can produce resting eggs, and has been considered as an excellent feed for marine fish larvae. The current study included two experiments: nauplii production (NP) and egg production (EP). In the NP experiment, the nauplii copepod *Acartia tonsa* Dana were reared for 14 days in a flow-through aquaculture system (FTAS), a conventional recirculating aquaculture system (cRAS) and a recirculating system including a membrane filtration unit (mRAS) to study the potential microbial influences on nauplii production (NP). Each system had four replicates (100L tank) and the initial density of copepods in each rearing tank was 75000 nauplii L^{-1}. After finishing the NP experiment, the visually biggest copepods were transferred from the 12 rearing tanks of NP experiment to 36 1L beakers with stagnant water (15 copepods per beaker and 3 beakers per tank) where the egg production (EP) was studied for 14 days. The unicellular algae *Rhodomonas baltica* was fed to the copepods in the study at saturated food concentration.

The results showed that the fraction of opportunistic bacteria in the rearing tanks was probably more important and influential on the copepod (*A. tonsa*) performance than the fluctuation of total number of bacteria. The low fraction of opportunistic bacteria in FTAS rearing tanks (below approximately 50%) during the whole NP experiment probably had positive effects on the survival rate and development of *A. tonsa* in NP experiment, and probably led to the later positive effects on the adult performance (number of eggs $female^{-1}$ day^{-1}, survival of adults) in the EP experiment. In contrast, the high fraction of opportunistic bacteria in the rearing tanks of cRAS & mRAS (above 50%) in NP experiment probably had adverse effects on the copepod performance in both NP and EP experiment.

Abbreviations

cRAS: Conventional Recirculation Aquaculture System

mRAS: Recirculation Aquaculture System with Membrane filtration

FTAS: Flow-Through Aquaculture System

DO: Dissolved Oxygen

CFU: Colony Forming Units

SGR: Specific Growth Rate

WER: Water Exchange Rate

TAN: Total ammonia nitrogen

LC_{50}: Lethal Concentration to kill 50% of a population after a period of time

NP experiment: Nauplii Production experiment

EP experiment: Egg Production experiment

per. comm.: personal comments (e.g. Andreas Hagemann (SINTEF) comments on copepod's length)

min(s): minute(s)

CC: carrying capacity

µg C: microgram Carbon

ng C: nanogram Carbon

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1. Introduction

1.1 Why copepods in aquaculture?

The application and cultivation of copepods as live feed in marine larviculture has been developed since 1980s (Støttrup et al., 1986, Schipp, 2006). In nature, copepods is probably the most important factor in the diet of marine fish larvae, so it could generally be concluded that the nutritional composition of copepods satisfy the requirements of fish larvae (Evjemo et al., 2003). Many studies showed that using copepods alone or in combination with rotifers or *Artemia*, improved the development, survival rate and frequency of normal pigmentation of fish larvae when compared to using only rotifers or *Artemia*, as reported for *Atlantic halibut (Hippoglossus hippoglossus)* (McEvoy et al., 1998, Evjemo et al., 2003) golden snapper *(Lutjanus johnii) (Schipp et al., 1999),* grouper *(Epinephelus coioides)(Toledo et al., 1999, Toledo et al., 2005),* and Australian seahorse (*Hippocampus subelongatus*) (Payne and Rippingale, 2000a). In addition, using the calanoid copepods (*Acartia tonsa*) during larval first feeding period resulted in a better survival, growth, and quality of Atlantic cod (*Gadus morhua*) larvae (Hansen, 2011), and a better growth & stress tolerance, more effective predator, earlier onset of ossification of the axial and fin ray skeleton, less skeletal anomalies, higher organ volume growth rates, higher proportion of musculature, more mature and developed intestine on Ballan wrasse (*Labrus bergylta*) larvae (Sørøy, 2012, Almli, 2012, Gagnat, 2012, Berg, 2012, Stavrakaki, 2013). This can be due to their superior nutritional value and higher digestibility in comparison to enriched rotifer and *Artemia* (Schipp, 2006). Furthermore, small sizes (<100 μm) of nauplii and/or first copepodite stages can make them become a first feed for small larvae with small mouth gape (Schipp, 2006). With jerky or zigzagging swimming pattern, most copepod nauplii and copepodites strongly stimulated the feeding behavior of fish larvae (Marcus, 2005). Due to the mentioned benefits of copepods in aquaculture, especially larviculture, researches on fundamental fields (phylogeny, physiology and ecology), and on reliable continuous cultures (applying automation and recirculation technology) should be considered (Drillet et al., 2011).

1.1.1 Copepod cultivation systems

Wild zooplankton is a major source for harvesting and collecting copepods to supply for larviculture (Conceição et al., 2010). Moreover, wild copepods are produced in extensive and semi-intensive systems in natural outdoor ponds or lagoons (Conceição et al., 2010, Ajiboye et al., 2011). In the extensive and semi-intensive production the culture can be started by adding fertilizer to increase biomass of phytoplankton since this is the main food source for

copepods (Støttrup, 2003, Conceição et al., 2010). Fish larvae can then be cultured directly in such enclosures and ponds (Støttrup, 2003) or the copepods can be concentrated by using a filter with a specific mesh sizes and then fed to the fish larvae in the tanks (van der Meeren and Naas, 1997). Different copepod species have been observed in these cultures including calanoid generas (*Acartia spp.*, *Centropages spp.*, and *Temora spp.*,), hapactoid (*Tisbe spp.*,) and cyclopoid (*Oithona spp.*) (van der Meeren and Naas, 1997, Støttrup, 2003). However, seasonal variations in species abundance, nutritional values, as well as possible infections of parasites and pathogens when using wild copepods directly as live feed can limit their application in aquaculture (Helland et al., 2003, Ajiboye et al., 2011).

In fact, the copepods from extensive and semi-intensive production can become intermediate hosts for several teleost parasites, which can cause negative effects on survival and growth of fish larvae (Marcogliese, 1995, Støttrup, 2000). Lahnsteiner et al. (2009) used chemical methods (sodium chloride 3.5%, hydrogen peroxide 0.7%, citric acid 2.0%) to eradicate procercoids from copepods in order to prevent parasite infestation of juvenile fish, but all of the copepods died after 5-10 minutes exposure, while procercoids were still attached on the dead copepods even after 30-60 minutes exposure to chemicals. This led to very low survival of juvenile fish because a high amount of chemicals was absorbed by the copepods and subsequently ingested by the fish (Lahnsteiner et al., 2009). A report of Støttrup (2003) stated that in order to reduce the risk of parasitic infection, the adult copepods were cultivated over one generation and then the nauplii were used as live food for fish larvae. Støttrup (2003) and Ajiboye et al. (2011) also discussed that intensive production method/system of copepod cultivation can be a solution to the problem of parasitic infection.

Many attempts to develop intensive systems for mass culture of copepods have been made with different species and with various successes since the early 1990s (Støttrup, 2003). Not only due to the problems of using harvested copepods, but because of a global shortage of *Artemia* cysts (Støttrup, 2003) and a mismatch between the size of traditional live feed in aquaculture (enriched rotifer and *Artemia*) and the mouth gape of very small fish larvae species (Payne and Rippingale, 2000b). Copepod species with short life cycle at ambient temperature were regarded as probably the best candidates for intensive mass culture production (Støttrup, 2003). The three most common copepod orders in aquaculture are Calanoida, Harpacticoida and Cyclopoida (Støttrup, 2003).

The calanoid copepods *Acartia spp.*, *Eurytemora spp.*, *Centropages spp.*, and *Gladioferens imparipes* are planktonic during the whole life cycle, and are more readily available to pelagic

fish larvae (Støttrup, 2003). However, many difficulties such as decrease in fecundity and hatching success, and increase in mortality when cultivating *Centropages typicus* and *Acartia tonsa* at high densities has been reported by Miralto et al. (1996) and Peck and Holste (2006). Due to planktonic distribution in the water column, cultivation of calanoid species need large volume, and the density for adult calanoids should be less than 100 individuals L^{-1} (Støttrup, 2003). In fact, Peck and Holste (2006) reported that adult stocking density for maximum egg production and 48 hours egg hatching success of *Acartia tonsa* in intensive cultures was only about 50 individuals L^{-1}, at a salinity of 14-20 ppt and photoperiods between 16 and 20 hours.

On the other hand, with high tolerance to salinity and temperature as well as high fecundity, and a wide range of feed (live and inert diets), the harpacticoids can be considered as the best candidate for mass culture production (Støttrup, 2003, Ajiboye et al., 2011). The harpacticoid copepods, e.g. *Tisbe sp.*, *Euterpina acutifrons*, *Tigriopus japonicas* and *Nitokra spp.*, are easier to cultivate at high density (from 10000 to 400000 individuals L^{-1}) than the calanoids (Støttrup, 2003), but they are less available to pelagic fish larvae due to a mainly benthic distribution (Støttrup, 2003, Fleeger, 2005, Schipp, 2006). A requirement for surface area rather than volume makes their mass culture complicated which can lead to a decline in an output production (nauplii or eggs) (Støttrup and Norsker, 1997, Støttrup, 2000, Fleeger, 2005).

The cyclopoid copepods have only been used in very few studies because some species (e.g. *Apocyclops dengizicus*) cannot be digested by the fish larvae (Ajiboye et al., 2011). Furthermore, the cyclopoids could predate on freshwater fish larvae and the magnitude of predation depended on the size and density of cyclopoids and the size of fish larva (Frimpong and Lochmann, 2005).

The intensive culture systems can be started with natural collected copepods (Schipp et al., 1999, Payne and Rippingale, 2001, VanderLugt, 2005) or sediments containing resting eggs (Næss, 1996). The copepod intensive cultivation can be as batch (Støttrup and Norsker, 1997, Payne and Rippingale, 2001), semi-continuous (Payne and Rippingale, 2001), and continuous cultures (Støttrup and Norsker, 1997), with system configuration as a flow-through system (Schipp et al., 1999, Vu Thi Thuy, 2011) or a recirculating system (Zillioux, 1969, Vu Thi Thuy, 2011, Buttino et al., 2012, Carotenuto et al., 2012, Holan et al., 2013b). A supply of at least two different microalga species to ensure a high n-3 PUFA (polyunsaturated fatty acid) content in diets for the copepods have been applied (Støttrup, 2000, Knuckey et al., 2005). To ensure better suspension or distribution of the algae, and to avoid anoxic areas in the tanks,

continuous aeration is applied (Støttrup, 2003). The output production (copepod nauplii or eggs) can be collected manually or automatically by siphoning, plankton nets or light traps (Støttrup et al., 1986, Støttrup and Norsker, 1997, Fleeger, 2005, Støttrup, 2006). After harvesting, the copepod nauplii can be supplied directly to the fish larvae at any given time, while the copepod eggs can be stored for later uses (Drillet et al., 2011).

1.1.2 *Acartia tonsa* Dana

Acartia tonsa is a pelagic calanoid copepod species (Crustacea/ Copepoda/ Calanoida/ Acartiidae) with a diverse distribution in coastal water (Blaxter et al., 1998, Razouls et al., 2005). *Acartia tonsa* is tolerant to a wide temperature range (0-30^{0}C) (Blaxter et al., 1998) and salinity range (1-72ppt) (Cervetto et al., 1999), and has a small size (total length of nauplii stages: 0.1-0.26 mm; prosome length of copepodite stages: 0.31-0.69 mm and prosome length of adults: > 0.7 mm) (Corkett, 1981, Schipp et al., 1999, Leandro et al., 2006, Alver et al., 2011), and short generation time (13 days at 16-18^{0}C) (Berggreen et al., 1988). With the ability to produce subitaneous and diapause eggs (Castro-Longoria, 2001), *Acartia tonsa* can maintain their population in outdoor extensive systems (Marcus, 2007). Moreover, Drillet et al. (2006) reported that when cultivated in intensive systems, *Acartia tonsa* eggs can be harvested, concentrated and stored for future use up to 12 months at 2-3^{0}C, without altering their viability and nutritional values. With the mentioned advantages, the species is one of the most researched candidates for many different aquaculture purposes (Drillet, 2010). Therefore, developing cultivation systems by using automation and recirculation technology to scale-up cultures of *A. tonsa* is probably necessary (Drillet et al., 2011).

1.2 Microbial characterization and recirculating aquaculture system

1.2.1 Microbial characterization in intensive aquaculture systems

Microbial communities in aquatic hatcheries include pathogenic, innocuous and beneficial bacteria (Schulze et al., 2006). Most fish diseases are caused by facultative pathogens or opportunistic bacteria, which can turn into pathogens when the resistance mechanism of the host is lessening by environmental stress factors (Salvesen et al., 1999). The opportunistic bacteria exhibit relatively high and fast growth rate (r-strategists, shown in **Figure 1.1**), which are favored by high substrate availability per bacterium (Odum et al., 1971, Morita, 1982, Li and Dickie, 1985, Andrews and Harris, 1986, Vadstein et al., 1993) or by high loads of organic material in rearing tanks from feces and uneaten feed (Vadstein et al., 1993, Skjermo et al., 1997). In contrast, non-opportunistic bacteria express a low and slow growth rate (K-strategists, shown in **Figure 1.1**), which are preferred by low substrate supply per capita (i.e.

per bacterium) or low loads of organic material in the rearing tanks (Odum et al., 1971, Atlas and Bartha, 1981, Andrews and Harris, 1986, Skjermo et al., 1997).

Carrying capacity (CC)
= Maximum sustainable biomass
= # bacteria that can be maintained in a system over time

Determined by:
physical/density dependent restrictions
= Supply of nutrients
= Dissolved organic matter supply (heterotrophic bacteria)

Characteristics	Environment	Substrate supply per capita	Favoured ability
r-selection	Unstable or unpredictable, empty niches	High	Reproduce quickly, fast growing
K-selection	Stable or predictable, crowded	Low, close to CC	Compete for limited resources

Figure 1.1 Key characteristics of r- and K-selected organisms (provided by Kari Attramadal pers. comm. , summarized from MacArthur (1967)).

On the other hand, Attramadal et al. (2012a) proposed that the trasition from low microbial carrying capacity (CC) of inlet water to higher substrate levels in rearing tanks probably opened a risk for opportunistic proliferation in a flow-through system. Meanwhile, Salvesen et al. (1999) used a recirulating aquaculture system (RAS) to produce microbial maturation of inlet water at a CC closer to the CC in rearing tanks, which could maintain a higher microbial stability and then induced a higher K-selective pressure (**Figure 1.1**). This can be achieved through the biofilters of RAS, where a large surface area is available for growth of K-selected bacteria (Attramadal et al., 2012a). Microbial maturation selecting for non-opportunistic bacteria (K-selection) can inhibit proliferation of opportunistic bacteria, and resulted in a significantly higher growth and survival rate of marine fish larvae (Vadstein et al., 1993, Skjermo et al., 1997, Salvesen et al., 1999).

1.2.2 Recirculating aquaculture system (RAS)

A RAS is an aquaculture system that incorporates treatment and reuse of the water, with under 10% replacement of the total water volume per day (Timmons and Ebeling, 2007). RAS has some major advantages such as low requirements for water and land, the ability to control the water quality and temperature (Tetzlaff, 1990). RAS can capture 96% - 100% of the waste emission (depending on water exchange rate), so it can minimize the risk of disease and pollution (Timmons and Ebeling, 2007). Moreover, in RAS the microbial community can be controlled through biofilter units (Blancheton, 2000, Michaud et al., 2006) where successively

matured water with dominance and selection of slow growing bacteria (K-strategist) can be established (Blancheton, 2000, Attramadal et al., 2012a)

Two of the most important things to control in RAS are the content of solids, and the amount of nitrogen compounds such as nitrite (NO_2) and ammonia (NH_3) (Timmons and Ebeling, 2007). A rapid increase in organic solids in the system can trigger the fast growing bacteria, which can be considered as an increase in opportunistic bacteria (Vadstein et al., 1993, Skjermo et al., 1997, Salvesen et al., 1999), while the nitrogenous wastes such as ammonia (NH_3) can be toxic to cultivated organisms (Rogers and Klemetson, 1985, Handy and Poxton, 1993, Parra and Yúfera, 1999, Foss et al., 2004).

Ammonia is the final product of protein catabolism, consisting of two forms: un-ionized ammonia (NH_3-N) and ionized ammonia (NH_4^+-N) (Timmons and Ebeling, 2007). The sum of NH_3-N and NH_4^+-N is considered as total ammonia nitrogen (TAN) (Timmons and Ebeling, 2007), where the NH_3-N is reported to be more toxic than the NH_4^+-N because it is non-polar and soluble in the lipids of biological membranes (Timmons and Ebeling, 2007, Holan et al., 2013a). In general, the concentration of NH_3-N should be under 0.1 to 0.05 mg L^{-1} (Timmons and Ebeling, 2007). For example, a lethal concentration (48-h LC50) for calanoid copepod *Acartia tonsa* (nauplii) when exposed to ammonia (NH_3) was between 0.179 and 0.224 mg L^{-1} (Sullivan and Ritacco, 1985). The fraction of un-ionized ammonia (NH_3-N) of the TAN is depending on pH, temperature and salinity(Timmons and Ebeling, 2007). An increase in pH and temperature, or decrease in salinity results in an increase in the fraction of un-ionized form of the TAN (Bower and Bidwell, 1978). The **Table 1.1** showed a conversion of inorganic nitrogen compound in the term of nitrogen they contain (Timmons and Ebeling, 2007).

Table 1.1 Concentrations of Nitrogenous compounds when normalized to the molecular weight of nitrogen (Timmons and Ebeling, 2007)

Nitrogen based name	Nitrogen based concentration of 1 mg/L	Equivalent concentration of compound in mg/L
Ammonia-nitrogen	NH_3-N	1.21 NH_3
Ammonium-nitrogen	NH_4^+-N	1.29 NH_4^+
Total Ammonia-nitrogen (TAN)	TAN	1.21 NH_3 or 1.29 NH_4^+
Nitrite-nitrogen	NO_2^--N	3.29 NO_2^-
Nitrate-nitrogen	NO_3^--N	4.43 NO_3^-

The biofilters in RAS that can be designed as a moving-bed-bioreactor (MBBR), is the place for removal of ammonia through nitrification process (Gutierrez-Wing and Malone, 2006, Rusten et al., 2006). Ammonia is converted into less toxic nitrate nitrogen (NO_3^--N) via the intermediate compound nitrite nitrogen (NO_2^--N) by a two-step biological nitrification process, and which is carried out sequentially by autotrophic bacteria. The first step is performed by ammonia oxidizing bacteria (*Nitrosomonas, Nitrosococcus, Nitrososprira, Nitrosolobus* and *Nitrosovibrio*) and the second step is carried out by nitrifying bacteria (*Nitrobacter, Nitrococcus, Nitrospira* and *Nitrospina*) (Haynes et al., 1986, Zehr and Kudela, 2011).

The equation for the oxidation of ammonia to nitrite by *Nitrosomonas* and the reaction for the oxidation of nitrite to nitrate by *Nitrobacter is* shown as equation 1.1 and 1.2 respectively (EPA, 1993).

$NH_4^+ + 1.5\ O_2 \Rightarrow NO_2^- + H_2O + 2H^+$ (1.1)

$NO_2^- + 0.5\ O_2 \Rightarrow NO_3^-$ (1.2)

The NO_2^--N is also toxic to aquaculture organisms by oxidizing the iron in the hemoglobin molecule from ferrous state to ferric state, referred to brown-blood disease (Tomasso et al., 1979). The NO_3^--N is the least toxic of nitrogen compounds (Masser et al., 1999).

The solids in RAS are generated from feces, bioflocs (dead and living bacteria) and uneaten food (Chiam and Sarbatly, 2011). In fact, depending on species and stages, 26-46% of the ingested feed finished up as faecal pellets (Beveridge et al., 1991), while 11-38% of the applied feed finished up as uneaten feed and waste excretion (McLaughlin, 1981, Mudrak, 1981, Chen et al., 1993). RAS with conventional filtration techniques can only remove particles that are >60 μm in diameter while about 90% of the particles by weight are 30 μm in diameter or less (Lekang et al., 2000, Cripps and Bergheim, 2000, Timmons and Ebeling, 2007, Chiam and Sarbatly, 2011, Lekang, 2013). Therefore, this can lead to an accumulation of fine suspended solids in the conventional RAS, which can further dissolve into minor components such as ammonia-nitrogen, dissolved organic material and phosphorous (Martins et al., 2010, Chiam and Sarbatly, 2011). The accumulation of colloids can also lead to some problems such as clogging the biofilters (Eding et al., 2006), increasing biofouling and oxygen demand (Timmons and Ebeling, 2007, Chiam and Sarbatly, 2011), triggering growth of opportunistic bacteria by dissolving into microbial substrates and increasing the amount of nitrogen by mineralization (Holan et al., 2013a).

However, the problems from the accumulation of colloids can be avoided by integrating a membrane bioreactor (MBR) in the recirculating aquaculture system (mRAS). In conventional MBR, activated sludge (AS) and membrane filtration are commonly combined for removal of organic material, ammonia and suspended solids (AS-MBR) (Leiknes and Ødegaard, 2007, Ivanovic et al., 2008). A hybrid biofilm membrane bioreactor (BF-MBR) consisting of biofilters (designed as the MBBR) and a submerged membrane reactor has recently been developed (Leiknes and Ødegaard, 2007, Ivanovic et al., 2008, Sun et al., 2010), which probably improved removal efficiency of particles in the system (Holan et al., 2013b). Nevertheless, membrane filtration in RAS can be a challenge because of membrane fouling resulting from the accumulation of particles on the surface of or inside the membrane that will reduce the filtration flux over time. Membrane fouling and clogging can be manipulated by running the system below critical flux, back flushing or backwashing periodically, relaxation techniques, and air-scouring (Wu et al., 2008, Judd, 2010).

An attachment of membrane filtration units (50 nm pore size) between two biofilters (designed as MBBR) into a RAS, referred to mRAS, possibly resulted in positive effects on turbidity, number of colloidal particles and bacterial stability & concentrations, then probably led to higher survival and growth of cod larvae (*Gadus morhua*) (Holan et al., 2013a). In constrast, a RAS without installing the membrane filtration units, referred to conventional RAS (cRAS), showed a lower survival and growth rates on cod larvae (*Gadus morhua*), compared to mRAS (Holan et al., 2013a). However, the BF-MBR in mRAS could not possibly remove the number of particles in the live feed (*Acartia tonsa* and *Brachionus ibericus*) cultivation tanks due to an excess of amount of suspended solids being added during feeding in comparison with the removed amount in the recycle system (Holan et al., 2013b) . Therefore, the suspended solids might dissolve into microbe substrate which could possibly trigger the growth of opportunistic bacteria; as a result, negatively affecting to the growth and survival of cultivated species (Holan et al., 2013b, Wold et al., 2014). Therefore, different operating regimes of membrane filtration and/or a higher recirculation rate should be investigated in further experiments to take advantage of the enhanced particle removal of the membrane filtration units so that the performance of live feed (e.g. *Acartia tonsa*) could then be improved (Holan et al., 2013b).

The current experiment was to analyze the microbial and particle characterization and influence on copepod (*Acartia tonsa*) nauplii and egg production in water treatment with and

without membrane filtration. This master thesis analyzed the microbial part while the particle part was analyzed by Day (*2014*).

1.3 Aim of study

The main aim of the thesis work is to investigate microbial influence on copepod (*Acartia tonsa*) nauplii and egg production in a flow-through aquaculture system (FTAS), a conventional RAS (cRAS) and a RAS with membrane filtration (mRAS). The ultimate objective was to determine which system can be used to improve copepod (*A. tonsa*) production to supply for intensive production of marine fish larvae as live feed in aquaculture.

The following main questions were established for the present study:

With the advanced particle removal of the membrane filtration units, can mRAS have a higher stability of the total number of bacteria and a lower fraction of opportunistic bacteria compared to cRAS and FTAS?

And if so, can the positive effects of microbial community result in a higher performance of copepods (survival, growth, development and fecundity) in mRAS, compared to cRAS and FTAS?

2. Materials and Methods

The study was conducted at NTNU Centre of Fisheries and Aquaculture (NTNU Sealab), Trondheim, Norway. The experiments were done during the autumn 2013.

2.1 Experimental setup

The study included 2 periods which lasted for 30 days in total (**Figure 2.1**). In the first part of the experiment, nauplii copepods (*Acartia tonsa*) were reared for 14 days in a flow-through aquaculture system (FTAS), a conventional (cRAS) and a RAS with membrane filtration (mRAS) to investigate microbial influences on nauplii production (NP). In the second part of the experiment, the copepods from the NP experiment were transferred to beakers (stagnant water) where egg production (EP) was studied for 14 days (**Figure 2.1**).

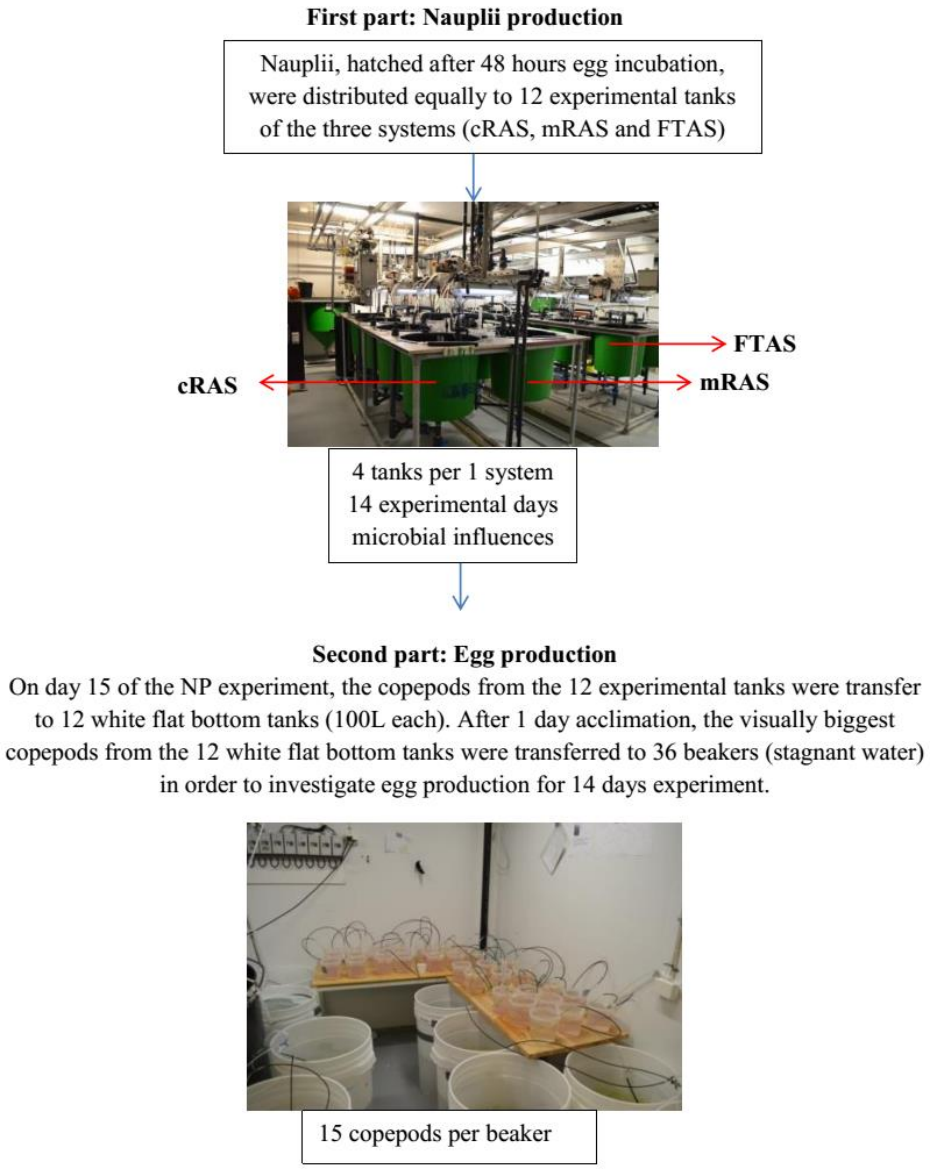

Figure 2.1 Two parts of the experiment including the first part (Nauplii Production) and the second part (Egg Production). Photo: Anh Phan Hung, 2014.

2.1.1 First part of the experiment (Nauplii Production)

2.1.1.1 Experimental system for cRAS, mRAS and FTAS

The copepod nauplii were reared at density of 75 inds mL^{-1} in a cRAS, an mRAS and a FTAS (**Figure 2.2**). The water exchange rate (WER) was set at 3 times per day from day 0 to day 3, and increased to 5 times per day from day 4 to day 7, and then to 8 times per day from day 8 to day 14 for the three systems.

The cRAS (**Figure 2.2**) included 4 cultivation tanks (100 L) connected to a water reservoir (160 L), a protein skimmer (80L), two biofilters connected in series (267L each), and a degasser (50 L, vacuum operated) for removal of N_2 and CO_2 (Holan et al., 2013a). The mRAS was constructed in the same way, however, in this system a membrane filtration unit was connected between biofilter 1 and biofilter 2. The membrane filtration unit consisted of two low pressure ultrafiltration modules that were submerged in water from the heterotrophic biofilter. The membrane was operated in an outside-in mode, discharging the treated clean water (permeate) into the nitrification biofilter. The biofilters were designed as moving-bed-biofilm reactors (MBBR) filled with biofilm carriers type K1 (Anox Kaldnes) (60L of biofilm carriers in the nitrification biofilter and 40L of biofilm carriers in the heterotrophic biofilter). The filling fraction in the nitrification biofilter and heterotrophic biofilter was 22.5% and 15 % of the reactor volume respectively, giving an area for biofilm growth of 112.5 $m^2/m^3_{\text{reactor volume}}$ and 75 $m^2/m^3_{\text{reactor volume}}$ respectively.

The design of each membrane module (PURON® polymer membranes from Koch Membrane Systems) was similar to those studied by Holan et al. (2013a), in which two membrane modules (each with an area of 0.97 m^2 and with the pore size of 50 nm) was placed in two separate 30L tanks. A stable filtration flux of 33 L m^{-2} h^{-1} was set, and a regular backwashing (10 min two times day^{-1}) followed by completely emptying of the membrane tank, and continuous air-scouring (17 L/min) was applied to reduce problems regarding fouling (Holan et al., 2013a). This design treated the whole water volume 2.0 times day^{-1} or 8.5% of the flow (12.7 L/min) at any time (Holan et al., 2013a). The performance of the membrane system was regulated by measuring the trans-membrane pressure (TMP), observed by a pressure transducer (Standard Genspec, 4 – 20mA, ESI Technology). The precautious point[1] for chemical cleaning of the membrane was set at a TMP of 0.3 bars. Before starting the NP experiment, chemical cleaning was conducted. The chemical cleaning of the membrane was accomplished by adding 40 g citric acid in the 30L membrane tanks (4 hours), then by adding

[1] The precautious point (TMP = 0.3 bars) is probably a signal of membrane fouling or clogging.

hypochlorite to a concentration of 0.5% (8 hours), and finally by adding 20 g of citric acid in the membrane tanks (3 hours) (Holan et al., 2013a).

The flow-through aquaculture system (FTAS) included 4 cultivation tanks receiving seawater from a reservoir (6 m^3) filled with sandfiltered seawater (>40µm, 34-35 ppt) from the main inlet at 70 m depth. The seawater was kept at 21-22^{0}C in the reservoir, and then aerated and vacuumed to avoid supersaturating of nitrogen gas.

Stainless steel outlet filters with mesh size of 40 µm were placed in the center of the copepod cultivation tanks from day 0 to day 7, to make sure the copepod nauplii could not escape from the rearing tanks. This filter was replaced by nylon outlet filter with mesh size of 64 µm from day 8 to day 14 (the end of the NP experiment). The purpose of this replacement was to increase the transport of accumulated solids from the rearing tank.

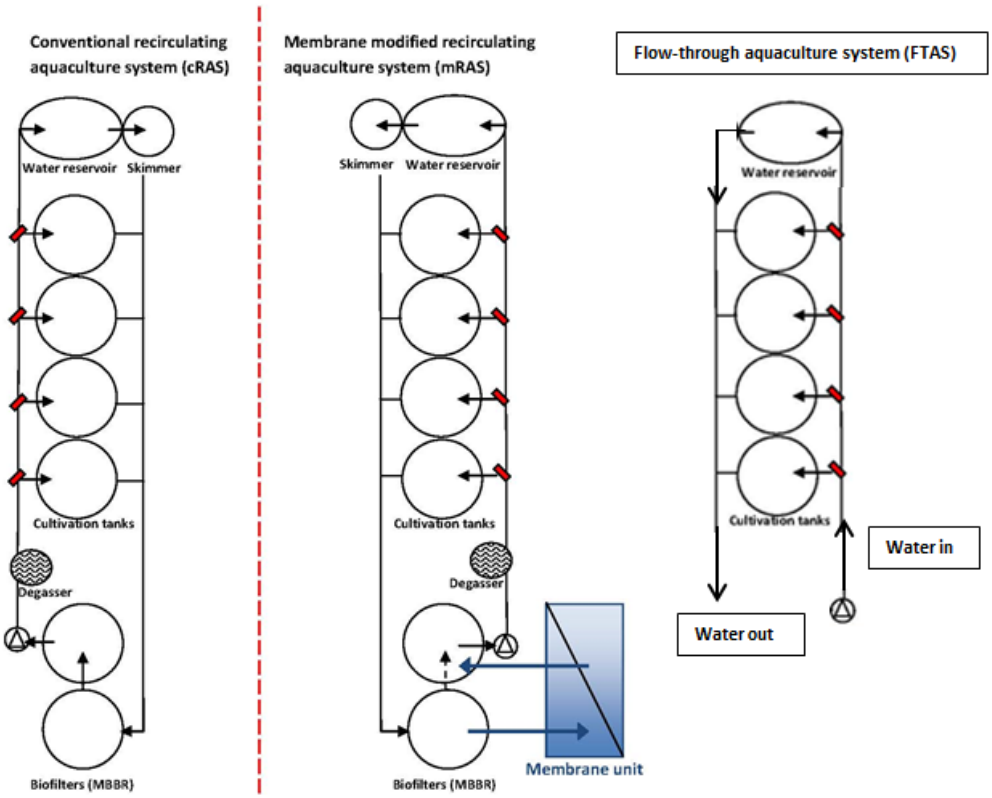

Figure 2.2 System configuration (Holan et al., 2013a) including a conventional RAS (cRAS), a membrane modified RAS (mRAS) and a flow-through aquaculture system (FTAS).

2.1.1.2 Light, aeration and cleaning procedure in the NP experiment

The light system of the rig was set to 24 hours light (Philips Master TL-D 90 Graphica G13, med El.nr. 3821501). All rearing tanks received aeration from a rubber tube, attached to the outlet filter at the bottom to ensure sufficient oxygen and to avoid clogging of the outlet filter.

The biofilter tanks and a feeding tank[2] received high aeration to ensure enough oxygen for the bacteria (heterotrophic and autotrophic oxidation) and to avoid sedimentation of algae, respectively.

2.1.2 Second part of the experiment (Egg Production)

Since it was difficult to collect copepod eggs in the 12 cultivation tanks of the three systems (cRAS, mRAS, FTAS), the copepods were transferred to 12 white polycarbonate flat bottom tanks (100L each) for back-up case at day 15 of the NP experiment with the conditions as described in **Table 2.1**. After 1 day acclimation, the visually biggest copepods were transferred from the 12 white flat bottom tanks to 36 1L beakers (15 copepods per beaker and 3 beakers per tank) (**Figure 2.1**). From this day, the number of eggs and newly hatched nauplii and survival rate of adults had been daily recorded during 14 days EP experiment. The experimental conditions for the copepods in the beakers (stagnant water) were described in **Table 2.1.**

Table 2.1 Experimental conditions during the 14 days EP experiment.

	Temperature (^{0}C)	DO (mg O_2 L^{-1})	Salinity (ppt)	pH	Light	Manually supplying algae		Algae density in the white tanks or beakers (cells mL^{-1})
						Density (cells mL^{-1})	Times per day	
12 white tanks	22-24	7-8	32-35	7.5-8.5	24 hours	1.5 – 2.0 x 10^6	4	> 10^4
36 beakers	22-24	7-8	32-35	7.5-8.5	24 hours	1.5 – 2.0 x 10^6	2	> 5 x 10^4

The method for determination of egg production in the EP experiment is illustrated in **Figure 2.3** and **Appendix 4**.

The sex of dead copepods was daily recorded while the number of live female copepods in the beakers was determined at the end of the EP experiment. Based on the number of eggs and newly hatched nauplii and the number of dead and live females, the egg production was calculated according to the equation **2.1**.

Egg production (EP) = (number of eggs + number newly hatched nauplii)/ number of adult females (eggs female^{-1} day^{-1}) (2.1)

Due to the problem of loosing unknown sex copepods, the sex of the adult copepods in all of beakers was determined in day 7 of the EP experiment. Therefore, the data of egg production was calculated from day 7 until day 14 of the EP experiment.

[2] A feeding tank is a reservoir containing 200L of live *Rhodomonas baltica* supplied to the 12 cultivation tanks through a feeding robot every day.

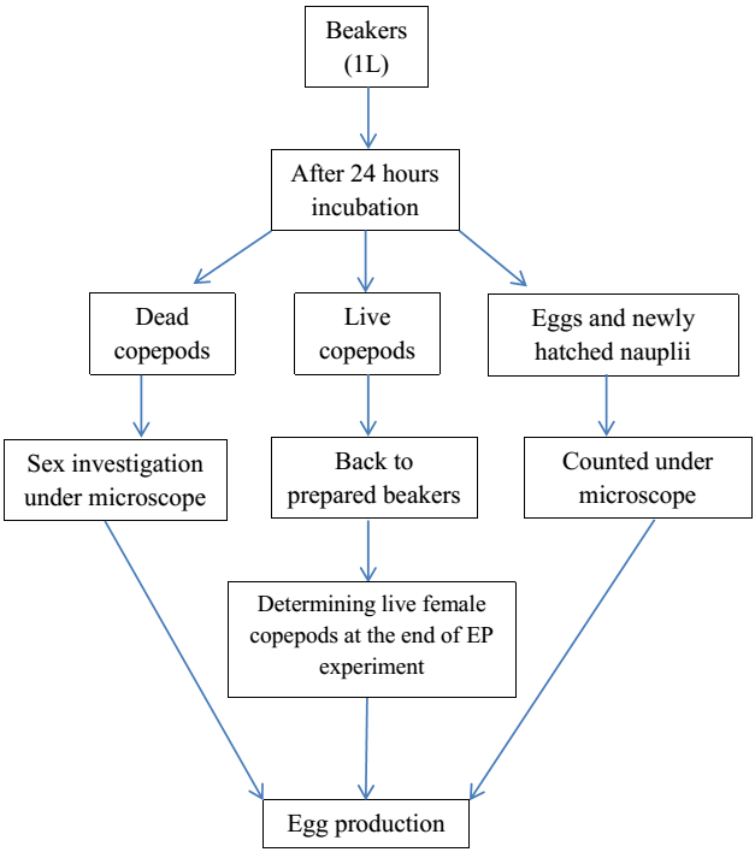

Figure 2.3 Method of determination of egg production in the second part of the experiment.

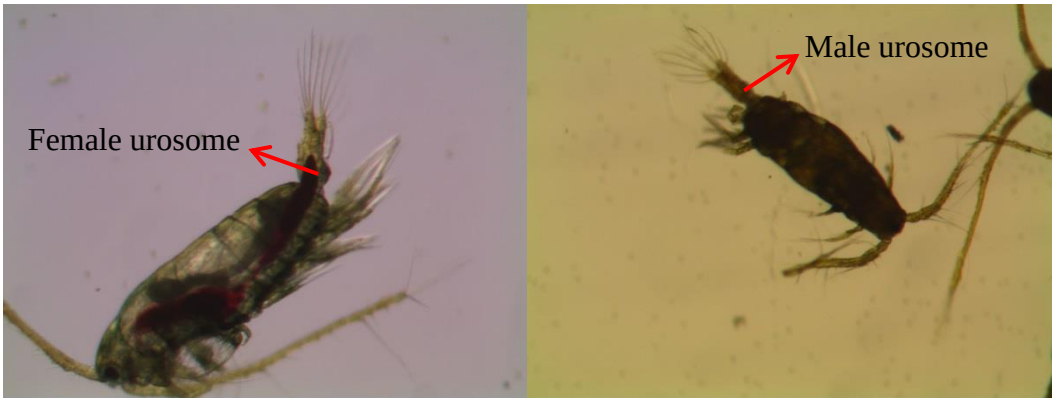

Figure 2.4 Difference in urosome of adult male and female. Photo: Anh Phan Hung, 2014. The first segment in urosome of adult females is bigger than males (described in Figure 2.5)

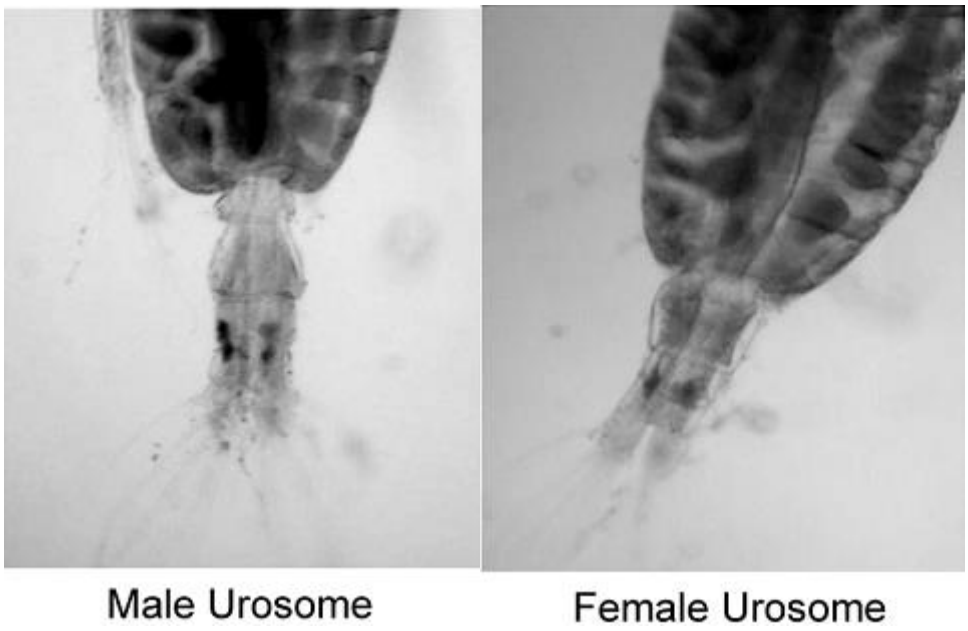

Figure 2.5 Different urosome conformation between adult males and females (Marchus and Wilcox, 2007).

2.2 Production of *Rhodomonas baltica* for the experiment

2.2.1 Calculation of necessary amount of algae volume

To ensure maximum growth rate for the copepods , the algal density in the rearing tanks should be above 10^4 cells mL^{-1} at all time (Skogstad, 2010). A calculation of the expected algae density and volume for the experiment is illustrated in **Appendix 2**. Supplying enough live algae for the copepods could either be done by increasing the volume of live algae supplying the rearing tanks or by increase the density of the live algae. However, if the volume of live algae was increased, it would affect the recirculation rate (the degree of re-use) of cRAS and mRAS. The recirculation rate was calculated according to equation **2.2** (Lekang, 2007)

$$\mathbf{R = (1-(Q_N/Q_T)) \times 100} \qquad \mathbf{(2.2)}$$

Where: R: degree of re-use or recirculation rate (%)

Q_N: new incoming water (algae and freshwater[3])

Q_T: total water supply to the cultivation tanks

The total volume of each system was 1275 L (including 400L (4 cultivation tanks), 160L (water reservoir), 80L (skimmer), 267L x2 (biofilters), 50L (degasser), and 51L (pipes)). In order to ensure the high recirculation rate (above 90%), a trial test[4] to increase the density of live algae was conducted to reduce the volume of algae supplied to the rearing tanks.

[3] Freshwater was added into Heterotrophic biofilter to stabilize the salinity of the RAS (10 L day^{-1}) in this study.
[4] A trial test was performed by using normal sedimentation and mechanical centrifugation to concentrate the algal density.

However, this trial test was not successful, so a new procedure to increase the algal density was applied before starting experiment. In fact, the algal density at SINTEF with previous procedure was about 1.4 – 1.5 x 10^6 cells mL^{-1}. This was then increased to 2.7 – 3.1 x 10^6 cells mL^{-1} after 2 days without harvesting by using 5% daily dilution of sterilized seawater and daily addition of Conwy medium (100 mL per 160 L algae and 140 mL per 200 L algae).

The details of procedure for starting algae culture and increasing the algae density is illustrated and summarized in **Appendix 3**.

R. baltica (200L, 2.7 – 3.1 x 10^6 cells mL^{-1}) was harvested daily and transferred to the feeding tank (200L). The algae was then automatically pumped from the feeding tank to a feeding robot (Storvik Robman – Storvik AS – www.storvik.no), which supplied 15L of *Rhodomonas baltica* to each rearing tank in each system every day.

2.2.2 Measurement of *Rhodomonas baltica* for feeding

The algal density in the production culture was measured every morning using a Beckman Multisizer^{TM}3 Coulter Counter, based on particles concentration (cells mL^{-1}) with the diameter range from 5.333 µm to 9.561 µm, which was considered as the cell size of *Rhodomonas baltica*. The algal density in rearing tanks was measured daily to assess whether the algae concentration in the rearing tanks was high enough to ensure maximum growth rate for the copepods.

2.3 The copepod *Acartia tonsa*

2.3.1 Hatching copepod eggs, concentrating and distributing nauplii of *A. tonsa* in the NP experiment

Copepod eggs were harvested from a flow-through system consisting of 4 production tanks (3 1.6 m^3 tanks and 1 1m^3 tank) and stored in 200 mL EasyFlasks (NUNC^{TM}) fully filled with sterilized seawater (32-35 ppt). The eggs were stored in the dark at 2^0C in a refrigerator (SANYO Pharmaceutical Refrigerator MPR-311D (H), Japan). 450 million 1 - 4 months old copepod eggs were hatched in 25 white polypropylene flat bottom tanks (100 L) filled with seawater (33-34 ppt, 22 ^{0}C), with aeration supplied through an airstone under continuous lighting. The copepod eggs were incubated at densities between 175 – 185 eggs mL^{-1} seawater during 48 hours. Feed was not added during the hatching period to prevent the copepods from developing beyond naupliar stage 2. After 48 hours, aeration was turned off for 30 minutes to allow for sedimentation of unhatched eggs and eggshells, which was removed by siphoning. A nauplii concentrator (**Figure 2.6**) was used to concentrate and collect all of the copepod nauplii and then transferred into a holding tank (100L) with heavy

aeration which was then distributed equally to the experimental rearing tanks in the three systems. The initial density in the rearing tanks was 75 000 nauplii L^{-1} (naupliar stage 1 and 2).

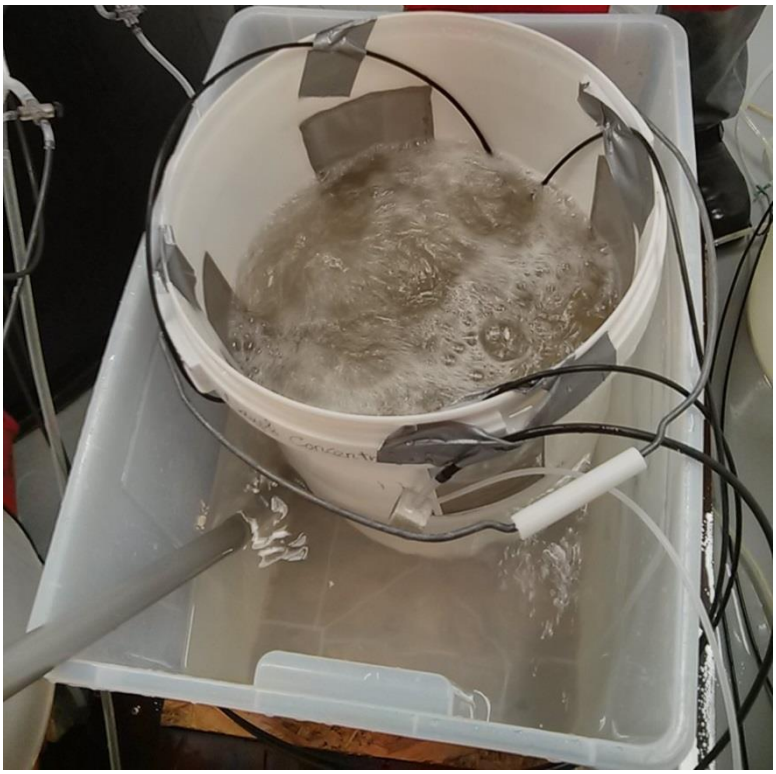

Figure 2.6 Nauplii concentrator consisting of 4 filter frames (40 µm mesh size iron sieves) on the side wall and attaching with strong aeration on each frame. Photo: Anh Phan Hung, 2014.

2.3.2 Daily density measurement of *Acartia tonsa* in the NP experiment

A polycarbonate rod (6 mm inner diameter) was used to sample copepods at 4 positions in each rearing tank in each system. The rods were disinfected with 70% ethanol and rinsed with hot and cold freshwater between samplings. The copepod samples were collected in a plastic cup and fixed with 2-3 droplets of Lugol fixation, then mixed thoroughly and transferred into a Petri dish by an Eppendorf pipette (100 µL in each droplet) before counting under stereomicroscope (Nikon SMZ 1000, Japan; connected with computer by a sensor adapter FC Infinity 1 - 3C Model 0189646, Canada). The determination of density, survival rate and daily mortality rate is illustrated in **Figure 2.7**.

The schedule for measuring density of the copepods for the three systems during the NP experiment is illustrated **Appendix 1**.

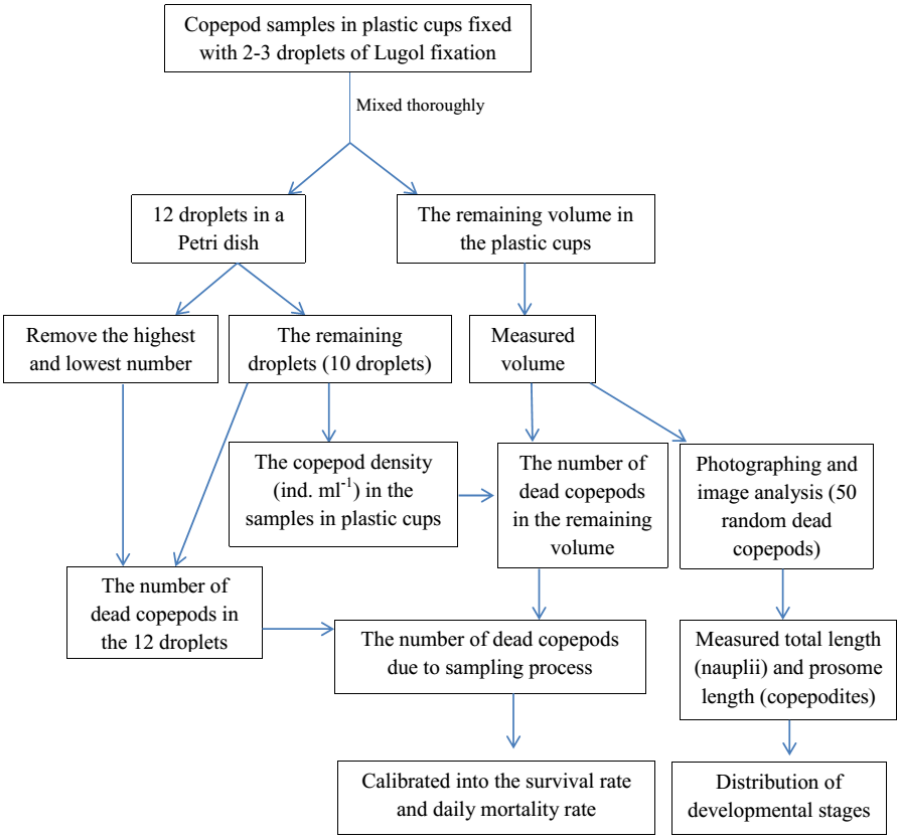

Figure 2.7 The process of daily density measurement and determination of distribution of developmental stages in the NP experiment

The instantaneous rates of mortality (z day^{-1}) was calculated according to Klein Breteler et al. (2004) using equation **2.3.**

$$N_t = N_0 e^{-zt} \qquad (2.3)$$

Where t is the time in days; N_0 and N_t is the number of individuals L^{-1} at time 0 and at time t, respectively.

The instantaneous rates of mortality (z day^{-1}) was used in equation **2.4** to calculate daily percentage mortality of a specific time interval (Klein Breteler et al., 2004)

$$\% \text{ mortality } day^{-1} = (e^{z}-1) \times 100\% \qquad (2.4)$$

2.3.3 Photographing, image analysis and stage development of *Acartia tonsa* in the NP experiment

50 random dead copepods from the remaining volume in the plastic cups after measured volume (**Figure 2.7**) were photographed to measure the total length of nauplii stages and prosome length of copepodite stages by using INIFINITY ANALYZE Camera software (http://www.lumenera.com) compatible with the microscope.

The schedule for measuring developmental stages of the copepods for the three systems during the NP experiment is illustrated **Appendix 1**.

The total length (nauplii stages) and prosome length (copepodite stages) were used for determining the developmental stages of *A. tonsa* (**Figure 2.8**). The developmental stages were grouped as N1-N2-N3, N4-N5-N6 for nauplii stages; C1-C2-C3, C4-C5 for copepodite stages, and adult stage to describe the developmental stage distribution (**Table 2.2**).

Table 2.2: Developmental stages of *A. tonsa* were grouped basing on the total length of nauplii stages and prosome length of copepodite and adult stages, according to Corkett (1981), Schipp et al. (1999), Støttrup et al. (1999), Leandro et al. (2006), Alver et al. (2011), A. Hagemann, SINTEF, pers. comm., (2014).

Grouped stages	I (N1-N2-N3)	II (N4-N5-N6)	III (C1-C2-C3)	IV (C4-C5)	V (Adults)
Total length or Prosome length (mm)	0.09-0.175	0.185-0.260	0.31-0.51	0.55-0.69	≥ 0.7

Figure 2.8 Total length of nauplii (A) and prosome length of copepodite (B) of *Acartia tonsa* in the NP experiment. Photo: Anh Phan Hung, 2014

The mean measured length of 50 random nauplii (total length, µm) and copepodites (prosome length, µm) was converted to body weight (W, ng C) according to the equations **2.5** and **2.6** (Berggreen et al., 1988).

Nauplii:

$$W = 3.18 \times 10^{-6} L^{3.31} \quad (2.5)$$

Copepodites:

$$W = 1.11 \times 10^{-5} L^{2.92} \quad (2.6)$$

Estimate of specific growth rate (***g***) was calculated according to the regression of body weight (μg C) vs time (t) **2.7** (Berggreen et al., 1988).

$$\ln W_t = \ln W_0 + gt \qquad (2.7)$$

Where W_0 is the initial mean body weight (μg C) and W_t is the final mean body weight (μg C) at time t; t is the time interval (days) between the measurements.

In the NP experiment, specific growth rate (SGR or ***g***) was calculated separately for nauplii and copepodite stages as well as for the whole period (total). The SGR of nauplii stages was calculated from the mean body weight of nauplii (μg C) from day 0 to day 7 of the experiment because all of tanks in the three systems were sampled at day 7 for measuring survival and developmental stages. At day 7, the fraction of nauplii and copepodites was determined according to the length (**Table 2.2**) (< 310 μm and ≥ 310 μm, respectively). Based on the fraction of nauplii and copepodites, the mean body weight of nauplii and copepodites (μg C) in each rearing tank at day 7 was calculated from the equation **2.5** and **2.6**. The SGR of copepodite stages was calculated from the mean body weight of copepodites (μg C) from day 7 to day 14 (end of the NP experiment). The SGR total was calculated from the mean body weight of copepods (both nauplii and copepodite stages) from day 0 to day 14.

2.4 Water quality measurement in the NP experiment

The schedule for taking samples to measure temperature, salinity, pH, dissolved oxygen and nitrogenous waste products (TAN, NO_2-N, NO_3-N) in the three systems is described in **Appendix 1**.

2.4.1 Physiochemical water quality parameter in the NP experiment

The dissolved oxygen (DO, mg L^{-1}) and water temperature (^{0}C) was measured in each tank by using a handheld optical dissolved oxygen meter (ProODO – Digital Professional series YSI – model 12A100403 – assembled in USA). The pH in each tank was measured by using VWR international - pH10 (serial model: JC000397 – Lot Code: 11J2). The pH, temperature and DO in each rearing tank were measured by submerging the sensors at the position between the outlet filter and the wall of the tank for 1 min. The salinity (ppt) was measured with a Refractometer (ATC-S/Mil-E Range 0-100%, Japan). All equipment was disinfected with 70% ethanol between measurements to prevent the spreading of microbes from one tank to the next.

2.4.2 Nitrogen waste products (TAN, NO_2-N, NO_3-N) in the NP experiment

Seawater from the three systems was sampled on day 0, 3, 7, 11, 14 for analyses of nitrogen waste products (**Table 2.3**).

Table 2.3 The number of samples analysed for TAN, NO_2-N, and NO_3-N in the three systems during experimental days. The numbers in the last row of the three systems presented for the number of samples per day.

Sampling days for TAN, NO_2-N, NO_3-N	cRAS		mRAS			FTAS	
	Inlet	Tank	Inlet	Tank	Permeate	Inlet	Tank
Day 0	1	1	1	1	2	1	1
Day 3, 7, 11, 14	3	3	3	3	2	3	3

The total ammonia nitrogen (TAN[5]), nitrite nitrogen (NO_2-N) and nitrate nitrogen (NO_3-N) were measured by a colorimeter (DR/890 HACH Datalogging Colorimeter HACH, USA), using HACH reagents[6] in 10 ml samples according to the instrument manual procedure. The results for TAN, NO_2-N, and NO3-N were recorded in mg L^{-1}.

Based on the pH and temperatures during the NP experiment, the un-ionized ammonia nitrogen (NH_3-N) was calculated from the measured concentration of TAN. Since temperatures and pH in cRAS and mRAS was significantly higher than in the FTAS, 2.20 % was selected for the cRAS and mRAS while 1.63% was selected for the FTAS (**Table 2.4**).

[5] Total Ammonia Nitrogen is the sum of un-ionized ammonia (NH_3) and ionized ammonia (NH_4^+)

[6]The HACH reagents were consisted of 26532-99 Ammonia Salicylate Reagent & 26531-99 Ammonia Cyanurate Reagent for TAN, 21071-69 NitriVer 3 Nitrite Reagent for NO_2-N, and 21061-69 NitraVer 5 Nitrate Reagent for NO_3-N, ordered from http://www.hach.com/

Table 2.4 Percentage of un-ionized ammonia nitrogen (NH_3-N) of TAN in seawater (32-40ppt) at different temperature and pH values (Bower and Bidwell, 1978)

Temp (°C)	pK_a^s	pH										
		7.5	7.6	7.7	7.8	7.9	8.0	8.1	8.2	8.3	8.4	8.5
0	10.16	0.218	0.275	0.346	0.435	0.547	0.687	0.863	1.09	1.36	1.71	2.14
1	10.13	0.235	0.296	0.372	0.468	0.589	0.740	0.930	1.17	1.47	1.84	2.30
2	10.10	0.253	0.319	0.401	0.504	0.634	0.797	1.00	1.26	1.58	1.98	2.48
3	10.06	0.273	0.343	0.432	0.543	0.683	0.858	1.08	1.35	1.70	2.13	2.66
4	10.03	0.294	0.370	0.465	0.585	0.735	0.924	1.16	1.46	1.83	2.29	2.86
5	10.00	0.317	0.398	0.501	0.630	0.792	0.995	1.25	1.57	1.97	2.46	3.08
6	9.97	0.341	0.429	0.540	0.678	0.852	1.07	1.34	1.69	2.11	2.65	3.31
7	9.93	0.367	0.462	0.581	0.730	0.918	1.15	1.45	1.82	2.27	2.85	3.56
8	9.90	0.396	0.498	0.626	0.787	0.988	1.24	1.56	1.95	2.45	3.06	3.82
9	9.87	0.426	0.536	0.674	0.847	1.06	1.34	1.68	2.10	2.63	3.29	4.11
10	9.84	0.459	0.577	0.726	0.912	1.15	1.44	1.80	2.26	2.83	3.54	4.41
11	9.80	0.495	0.622	0.782	0.982	1.23	1.55	1.94	2.43	3.04	3.80	4.74
12	9.77	0.533	0.670	0.842	1.06	1.33	1.67	2.09	2.61	3.27	4.08	5.08
13	9.74	0.574	0.721	0.906	1.14	1.43	1.79	2.25	2.81	3.51	4.38	5.46
14	9.71	0.618	0.777	0.976	1.23	1.54	1.93	2.42	3.02	3.78	4.71	5.85
15	9.67	0.665	0.836	1.05	1.32	1.66	2.07	2.60	3.25	4.06	5.05	6.28
16	9.64	0.717	0.900	1.13	1.42	1.78	2.23	2.79	3.49	4.36	5.42	6.73
17	9.61	0.772	0.970	1.22	1.53	1.92	2.40	3.00	3.75	4.68	5.82	7.22
18	9.58	0.831	1.04	1.31	1.64	2.06	2.58	3.23	4.03	5.02	6.24	7.73
19	9.54	0.895	1.12	1.41	1.77	2.22	2.78	3.47	4.33	5.39	6.69	8.28
20	9.51	0.963	1.21	1.52	1.90	2.39	2.98	3.73	4.65	5.78	7.17	8.87
21	9.48	1.04	1.30	1.63	2.05	2.57	3.21	4.01	4.99	6.20	7.69	9.49
22	9.45	1.12	1.40	1.76	2.20	2.76	3.45	4.30	5.36	6.65	8.23	10.1
23	9.41	1.20	1.51	1.89	2.37	2.97	3.71	4.62	5.75	7.13	8.81	10.8
24	9.38	1.29	1.62	2.04	2.55	3.19	3.98	4.96	6.17	7.64	9.43	11.6
25	9.35	1.39	1.75	2.19	2.74	3.43	4.28	5.32	6.61	8.18	10.1	12.4

2.5 Analysis of the microbial community in the NP experiment

The sampling schedule for analyses of colony forming units (CFU) and Flow cytometry in the three systems is described in **Table 2.5** and **Appendix 1.**

Table 2.5 The number of samples analysed for CFU and Flow cytometry in the three systems during experimental days. CFU samples were taken on day 0, 3, 6, 12, while Flow cytometry samples were taken on day 0, 3, 6, 12, 14. The last row denoted the number of samples of each system at each sampling day.

Sampling days for CFU and Flow cytometry	**cRAS**		**mRAS**			**FTAS**	
	Inlet	Tank	Inlet	Tank	Permeate	Inlet	Tank
Day 0	1	1	1	1	2	1	1
Day 3, 6, 12, 14	3	3	3	3	2	3	3

2.5.1 Colony Forming Unit (CFU)

The colony forming unit (CFU) method was applied as a tool to investigate the presence of fast and slow growing bacteria in the systems. Marine agar plates were prepared in a sterilized cabinet by mixing thoroughly 55.1 g Difco™ Marine agar 2216 with 1000 mL purified water,

heated with frequent agitation and boiled for one minute for completely dissolving the powder and then autoclaving at 121 ^{0}C in 15 minutes before pouring into plastic plates. The plates were stored upside down in a 10^{0}C fridge.

The seawater samples were stored in 50ml brown glass bottles (sterilized at 121^{0}C, 20 minutes before use) and then analyzed at the same day. The water samples were diluted in series from 10^{-1} to 10^{-3} for samples taken on day 0 and from 10^{-1} to 10^{-4} for samples taken on day 3, 6, 12 by diluting 0.1 mL of the sample with 0.9 mL of sterilize filtered 80% seawater and diluting 0.1 mL of this diluted sample for the next dilution. The method for plating the seawater samples on the marine agar plates as well as incubating the agar plates was conducted according to Vu Thi Thuy (2011).

The total number of visible colonies on the agar plates after 2 days incubation time (CFU_{0-2}) was registered as the number of fast growing bacteria according to Salvesen and Vadstein (2000) and Attramadal et al. (2012b). The number of slow growing bacteria was the visible colonies that appeared in the same plates from day 2 to day 14 of incubation (CFU_{2-14}). When the number of colonies on the plates was above 300, the plates were not counted to the final results (Attramadal et al., 2012b). The fraction of fast growing bacteria (CFU_{0-2}) of the total CFU_{0-14}[7] was considered as the percentage (%) of opportunistic bacteria in each system (Salvesen and Vadstein, 2000).

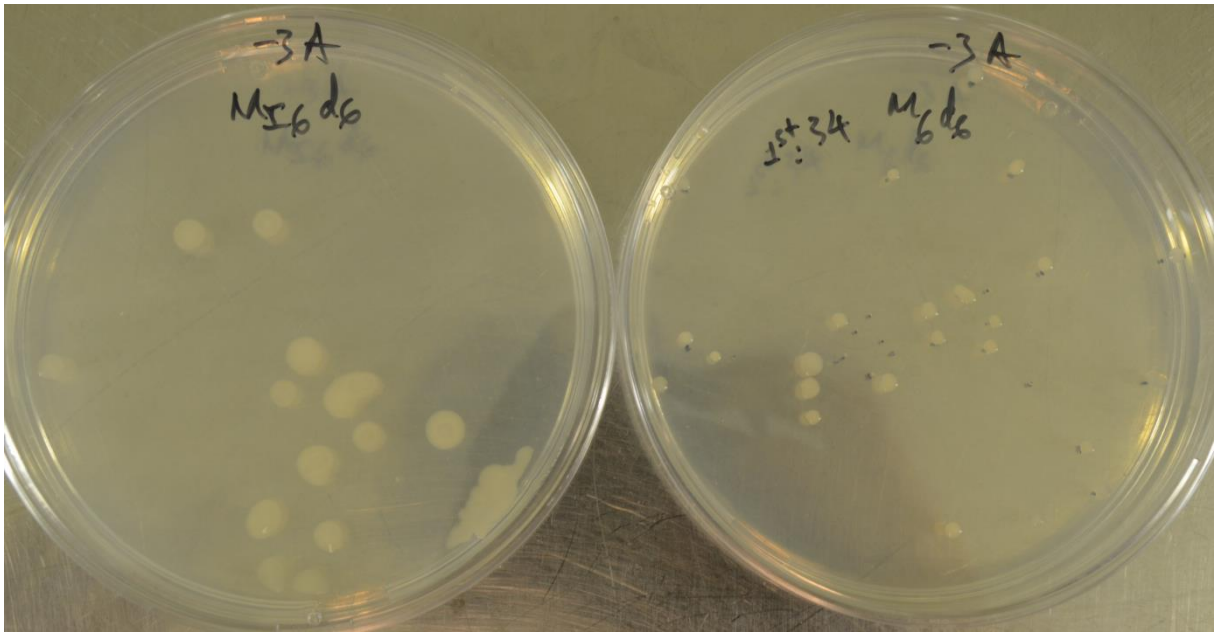

Figure 2.9 Visible culturable bacteria colonies on Marine Agar after 2 days of incubation at dilution 10^{-3}. Photo: Anh Phan Hung, 2014.

[7] The sum of the number of fast growing and slow growing bacteria in the same plates after 14 days of incubation

2.5.2 Flow Cytometry

The seawater samples of the three systems were taken at day 0, 3, 6, 12, 14 (**Table 2.5**) for flow cytometry measurement by using Sybr Green I, a nucleic-acid gel stain, as a fluorescent dye to enumerate the number of bacteria in marine samples (Noble and Fuhrman, 1998, Marie et al., 2005).

At each sampling time 23 mL seawater was stored in a 50ml brown glass bottle and then fixed with 2 mL paraformaldehyde[8], to a final concentration of 2%, and stored in a dark room at 4 ^{0}C. The samples were then diluted and analyzed by using the **BD Accuri™ C6 Flow Cytometer** following the process in **Figure 2.10** which was modified from the study of Marie et al. (2005) and Noble and Fuhrman (1998) to measure the number of bacteria.

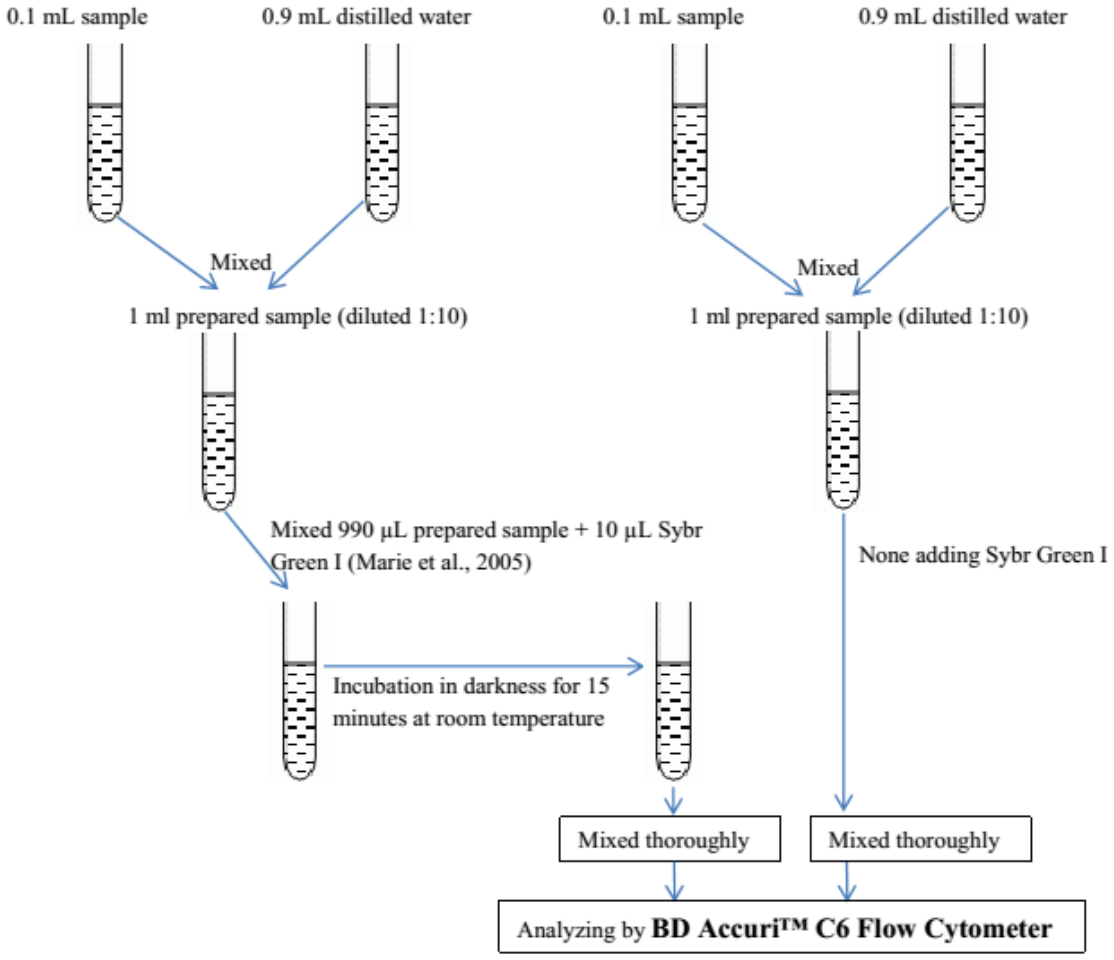

Figure 2.10 The process of preparing and analyzing the Flow cytometry samples. Using Sybr Green I (nucleic-acid gel stain, Molecular Probes Invitrogen) as a fluorescent dye (Noble and Fuhrman, 1998, Marie et al., 2005).

[8] Paraformaldehyde with stock concentration (25%) made according to KARNOVSKY, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *J. Cell Biol.*, 27**,** 137A-138A. The standard procedure to make this paraformaldehyde solution is based on a laboratory manual instruction from the provider of this fixative (http://www.carlroth.com/media/_de-de/sdpdf/0335.PDF)

Moreover, based on an instruction of the provider of Sybr Green I in this experiment (http://bio.lonza.com/uploads/tx_mwaxmarketingmaterial/Lonza_ManualsProductInstructions_SYBR_Green_I_Nucleic_Acid_Gel_Stain_-_Protocol.pdf), the emission of DNA stained with SYBR® Green I Stain is centered at 521 nm, which is matched with an optical filter detector (530/30 nm BP, **Figure 2.12**) of the **BD Accuri™ C6 Flow Cytometer**.

Based on a result from a test of adding Sybr Green I (**Figure 2.11B**) and non-adding Sybr Green I (**Figure 2.11A**), the difference in number of black dots between the **Figure 2.11** (**B**) and the **Figure 2.11** (**A**) are the number of bacteria in the seawater sample. The black dots outside the area **P1** in **Figure 2.11** are self-fluorescing microorganisms.

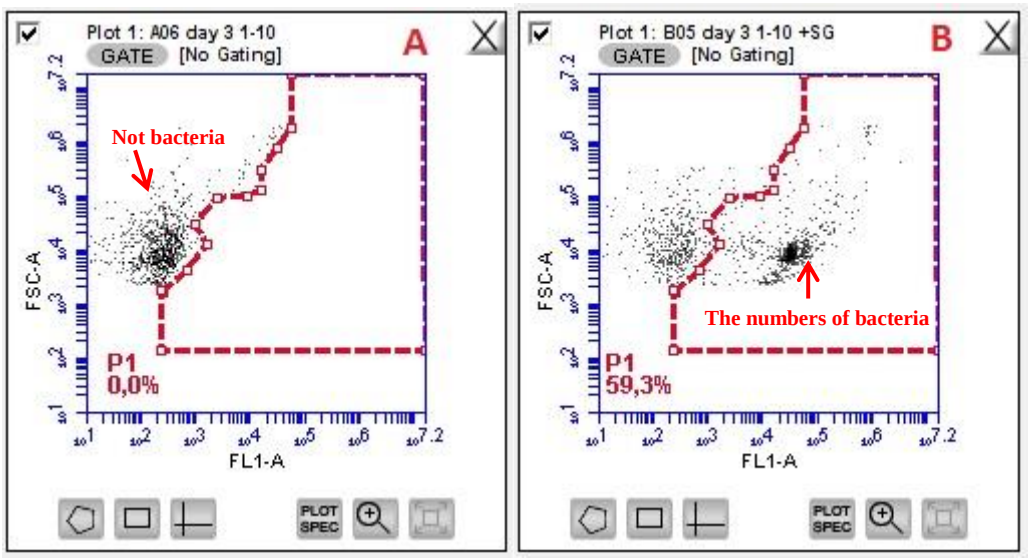

Figure 2.11 The results from BD Accuri™ C6 Flow Cytometer for the sample without adding Sybr Green I (A) and with adding Sybr Green I (B). FSC-A refers to Forward Scatter Size, FL1-A refers to optical filter detector at 530/30 nm (Figure 2.12) when using Sybr Green I as the fluorescence dye.

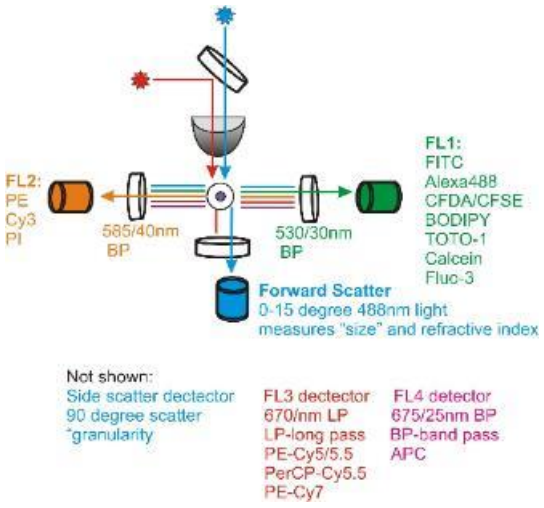

Figure 2.12 The protocol of BD Accuri™ C6 Flow Cytometer (http://www.ljidi.org/services.html, 2014).

2.6 Statistical Analysis

The scientific graphs and data analyses software Sigmaplot 12.3 (Systat Software, Inc. SigmaPlot for Windows, USA/Canada) was used for all statistical analysis.

Data for environmental parameters (temperature, pH, salinity, dissolved oxygen), nitrogenous wastes (NH_3-N, NO_2-N, NO_3-N), density (individuals mL^{-1}), survival rate (%), daily mortality rate (%), specific growth rate (SGR day^{-1}), microbiology (CFU and Flow cytometry), fraction (%) of nauplii, copepodite and adults in the NP experiment were tested for significant differences between treatments ($p < 0.05$) by using One-way ANOVAs, Kruskal-Wallis one-way ANOVAs on ranks, and post hoc Student-Newman-Keuls.

Data for egg production and survival rate (%) of adults in the EP experiment were tested for significant differences between treatments ($p<0.05$) with One-way ANOVA, Kruskal-Wallis one-way ANOVAs on ranks, and post hoc Student-Newman-Keuls.

In the NP experiment, each tank had each corresponding data of % opportunistic bacteria and survival rate at each sampling day. The % opportunistic bacteria of such tanks of the three systems in all sampling days was classified into ≥50% and <50% (referred as A and B, respectively, in **Appendix 12**) by using Microsoft Office Excel for Windows (Microsoft Inc.). After classification, the two grouped tanks (A and B) had the two corresponding data of survival rate which was tested for significant difference ($p < 0.05$) by using t-test in the software Sigmaplot 12.3.

3. Results

3.1 Water quality

3.1.1 Physiochemical water quality parameter in the NP experiment

Temperature, salinity, dissolved oxygen and pH showed only slight variations in culture tanks of the cRAS, mRAS and FTAS during the nauplii production (NP) experiment, as shown in **Table 3.1**. The temperature and pH in cRAS and mRAS was significantly higher than in FTAS, but there were no significant differences between cRAS and mRAS. The salinity in the culture tanks of mRAS was highest, followed by cRAS and FTAS. There were no significant differences in dissolved oxygen in the culture tanks among the three systems.

Table 3.1 Water quality parameters (Mean±SE) in cultivation tanks of the cRAS, mRAS and FTAS during the NP experiment. Different superscripts in a row denote significant differences between systems.

	cRAS	mRAS	FTAS
Temperature (^{0}C)	22.72±0.05^a	22.65±0.05^a	21.77±0.04^b
Salinity (ppt)	37.15±0.18^a	38.30±0.11^b	35.98±0.15^c
Oxygen (mg O_2 L^{-1})	6.93±0.06^a	7.08±0.05^a	6.95±0.09^a
pH	7.84±0.02^a	7.85±0.02^a	7.78±0.02^b

3.1.2 Nitrogenous wastes

Un-ionized ammonia nitrogen (NH_3-N)

The calculated concentration of un-ionized ammonia nitrogen (NH_3-N) in the cultivation tanks and in the inlet water of three systems (**Figure 3.1A** and **Figure 3.1B**, respectively) was calculated from the measured concentration of TAN (**Appendix 5**).

In the rearing tanks: There were no significant differences of the NH_3 concentration among the three systems at day 3, day 7 and day 11, while at day 14, the NH_3 concentration in cRAS was significantly higher than in mRAS and FTAS.

In the inlet water: The NH_3 concentration was not significantly different among the three systems at day 3, day 11 and day 14, but at day 7, the NH_3 concentration in cRAS was

significantly higher than in mRAS and FTAS at day 7. The NH_3 concentration of the membrane permeates[9] was relatively stable at low level during all sampling days.

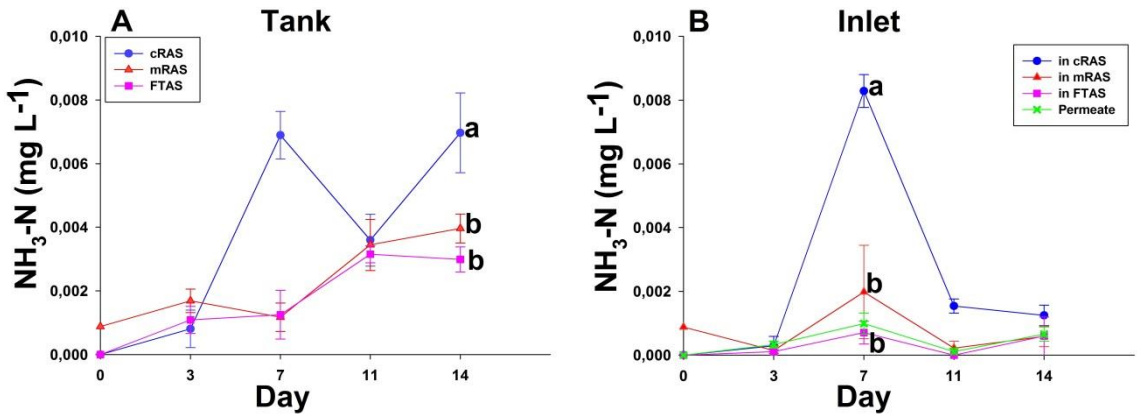

Figure 3.1 Mean (±SE) calculated values of un-ionized ammonia nitrogen (NH_3-N) in cultivation tanks (A) and in inlet water (B) during the NP experiment. Different letters in the graph at sampling days denote significant differences between systems.

Nitrite nitrogen (NO_2-N)

The NO_2-N concentration in both the rearing tanks (**Figure 3.2A**) and inlet water (**Figure 3.2B**) of the three systems had the same trend during the NP experiment.

In the rearing tanks and inlet water: The NO_2-N concentration in cRAS and mRAS was significantly higher than in FTAS during all sampling days. The NO_2-N concentration of cRAS and mRAS slightly fluctuated below 0.5 mg L^{-1} from day 0 to day 11. However, from day 11 to day 14, the NO_2-N concentration sharply increased in cRAS (about 1.0 mg L^{-1}), but was relatively stable in mRAS.

The NO_2-N concentration in the membrane permeates slightly increased during the whole NP experiment.

[9] Seawater after passing through the membrane filtration units

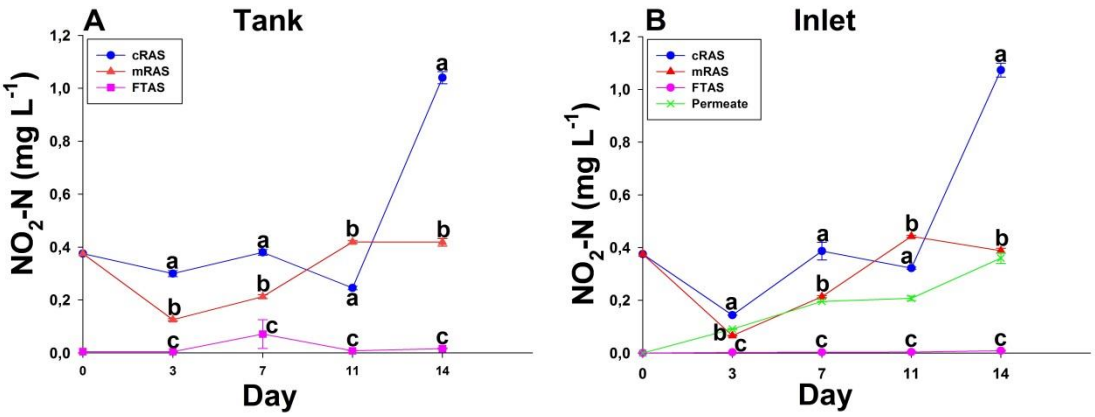

Figure 3.2 Mean (±SE) measured values of Nitrite nitrogen (NO_2-N) in cultivation tanks (A) and in inlet water (B) during the NP experiment. Different letters in the graph at sampling days denote significant differences between systems.

Nitrate nitrogen (NO_3-N)

The NO_3-N concentration in both the rearing tanks (**Figure 3.3A**) and inlet water (**Figure 3.3B**) of the three systems had the same trend during the NP experiment.

In the rearing tanks and inlet water: The NO_3-N concentration of cRAS and mRAS were considerably reduced from day 0 to day 3, they were relatively stable between day 3 and day 11, and increased considerably during the last three days. In contrast, the NO_3-N concentration of FTAS was nearly constant from day 0 to day 11, and increased considerably between day 11 and day 14.

The NO_3-N concentration in the membrane permeates slightly increased from day 0 to day 3, was stable between day 3 and day 7, and increased considerably from day 7 to day 14.

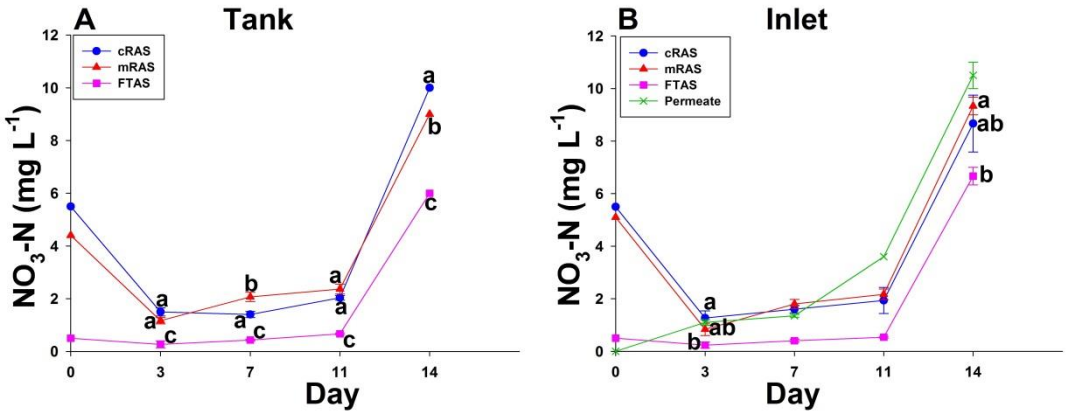

Figure 3.3 Mean (±SE) measured values of Nitrate nitrogen (NO_3-N) in cultivation tanks (A) and in inlet water (B) during the NP experiment. Different letters in the graph at sampling days denote significant differences between systems.

3.2 Microbial characterization

3.2.1 Colony forming units (CFU)

Fast growing bacteria

The total number of colony forming units of fast growing bacteria ($CFU_{0\text{-}2}$) in the rearing tanks and in the inlet water is shown in **Figure 3.4A** and **Figure 3.4B** respectively.

The $CFU_{0\text{-}2}$ in the rearing tanks and inlet water of mRAS and FTAS was relatively stable at low level during all sampling days; in contrast, that of cRAS was relatively stable from day 0 to day 6, and increased considerably from day 6 to day 12, which resulted in significant differences between cRAS and the mRAS & FTAS at day 12. The $CFU_{0\text{-}2}$ in membrane permeates was probably stable at very low level during all sampling days.

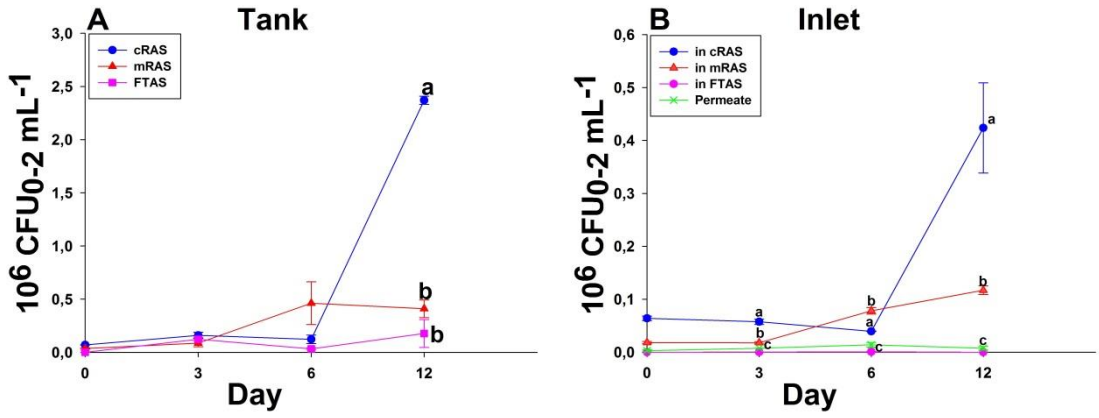

Figure 3.4 The total number of fast growing bacteria (Mean ±SE) in cultivation tanks (A) and in inlet water (B) during the NP experiment. $CFU_{0\text{-}2}$ is the number of colonies counted on Marine agar plates after 2 days of incubation. Different letters in the graph at sampling days denote significant differences between systems.

Slow growing bacteria

The total number of colony forming units of slow growing bacteria ($CFU_{2\text{-}14}$) in the rearing tanks and in the inlet water is shown in **Figure 3.5A** and **Figure 3.5B** respectively.

In the rearing tanks: There were no significant differences in the $CFU_{2\text{-}14}$ among the three systems during all sampling days.

In the inlet water: The $CFU_{2\text{-}14}$ of cRAS was probably constant from day 0 to day 3, and increased considerably from day 3 to day 12, while the $CFU_{2\text{-}14}$ of mRAS increased slightly during the whole experiment. The $CFU_{2\text{-}14}$ of FTAS and membrane permeates fluctuated very slightly at low level during all sampling days.

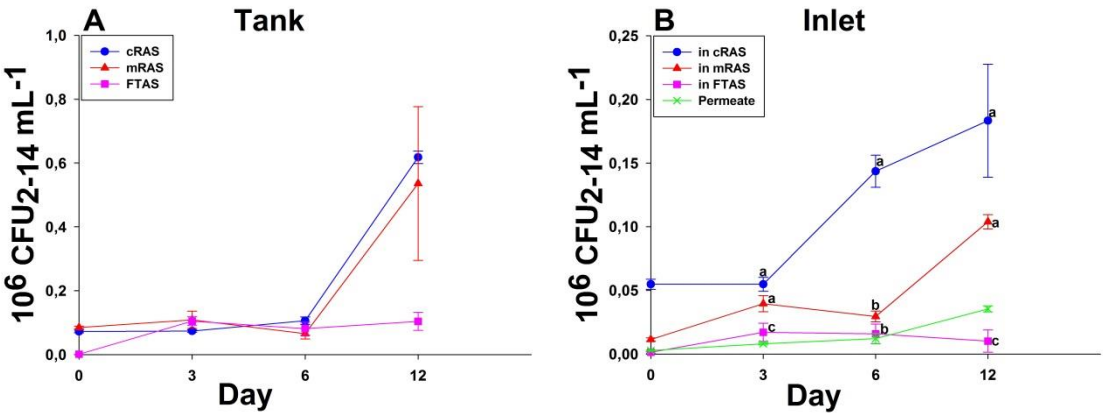

Figure 3.5 The total number of slow growing bacteria (Mean ±SE) in cultivation tanks (A) and in inlet water (B) during the NP experiment. $CFU_{2\text{-}14}$ is the number of colonies counted on Marine agar plates from day 2 to day 14 of incubation. Different letters in the graph at sampling days denote significant differences between systems.

Fraction (%) of opportunistic bacteria (referred as opportunists (%) in Attramadal et al. (2012a):

The opportunists (%) are the ratio between the $CFU_{0\text{-}2}$ and the sum of $CFU_{0\text{-}2}$ and $CFU_{2\text{-}14}$ in each system. The opportunists (%) of the three systems in the rearing tanks and in the inlet water are shown in **Figure 3.6A** and **Figure 3.6B** respectively.

In the rearing tanks: The opportunists (%) of the three systems were relatively fluctuating throughout the experimental period. The fluctuating tendency in cRAS and FTAS was similar with the top at day 3 and bottom at day 6, but the range of fluctuation was above 50% in cRAS and approximately below 50% in FTAS during the whole NP experiment. In contrast, the mRAS showed a considerable up and down with the peak of opportunists (%) at day 6 (85.8%) that was significantly different with the cRAS and FTAS.

In the inlet water: The opportunists (%) in FTAS were relatively stable and much lower than in cRAS and mRAS, which were considerably fluctuating, throughout the experimental period. The opportunists (%) of membrane permeates slightly increased from day 0 to day 6 and decreased considerably from day 6 to day 12.

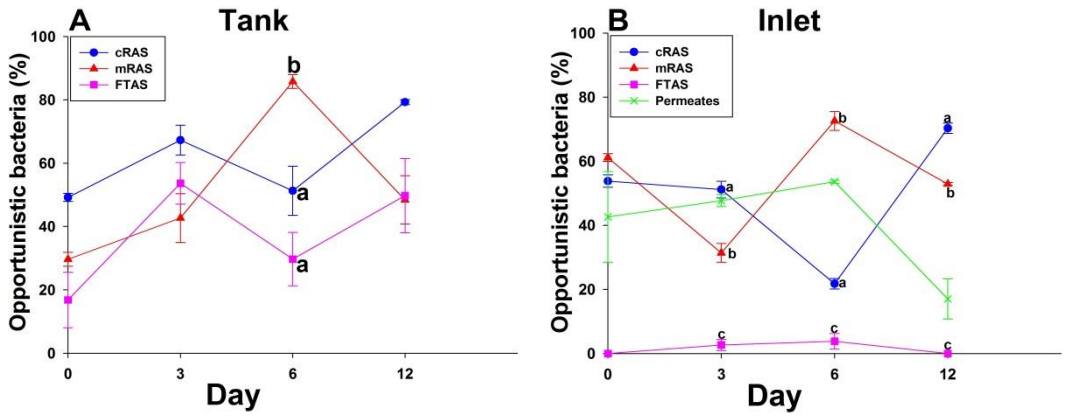

Figure 3.6 The percentage (%) of opportunistic bacteria (Mean ±SE) in the in cultivation tanks (A) and in inlet water (B) during the NP experiment. Different letters in the graph at sampling days denote significant differences between systems.

3.2.2 Total number of bacteria (Flow cytometry)

The total number of bacteria of the three systems in the rearing tanks and in the inlet water is shown in **Figure 3.7A** and **Figure 3.7B** respectively.

In the rearing tanks: The total number of bacteria of the three systems was relatively fluctuating throughout the experimental period. From day 3 to day 6, the total number of bacteria was decreased considerably in cRAS, but decreased slightly in mRAS and FTAS which led to significant differences between the cRAS and the two other systems at day 6.

In the inlet water: The total number of bacteria of cRAS & FTAS and membrane permeates was relatively stable during all sampling days, whereas the total number of bacteria of mRAS was considerably fluctuating during the whole NP experiment with a sharp down and up between day 6 and day 14.

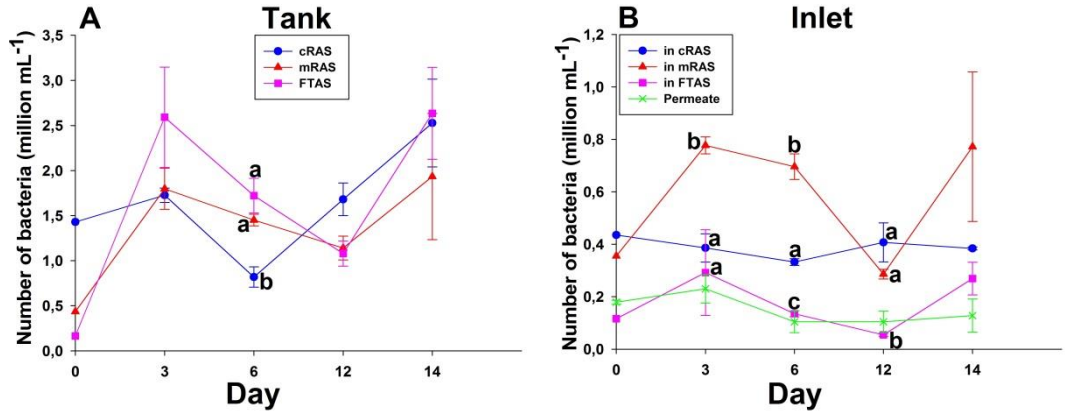

Figure 3.7 The total number of bacteria (Mean ±SE) in cultivation tanks (A) and in inlet water (B) during the NP experiment. Different letters in the graph at sampling days denote significant differences between systems.

3.3 Growth of copepods (*Acartia tonsa*)

3.3.1 Density and survival of *Acartia tonsa* in the NP experiment

Density (**Figure 3.8A**) and survival rate (**Figure 3.8B**) of *Acartia tonsa* had the same tendency in all treatments during the whole NP experiment. The initial density of the copepods at day 0 was 75000 individuals L^{-1}, corresponding to 100% survival rate in the three systems. From day 0 to day 7, the density and survival rate of the three systems were considerably reduced, but there were no significant differences among the three systems at day 1 and day 7. From day 7 to day 14, the FTAS showed a relatively stable tendency in the density and survival rate, whereas the cRAS and mRAS showed a continuously considerable decreased trend, which led to significantly differences between FTAS and mRAS at day 11 as well as between FTAS and cRAS & mRAS at day 14.

Detailed data for density and survival rate is also shown in **Appendix 8**.

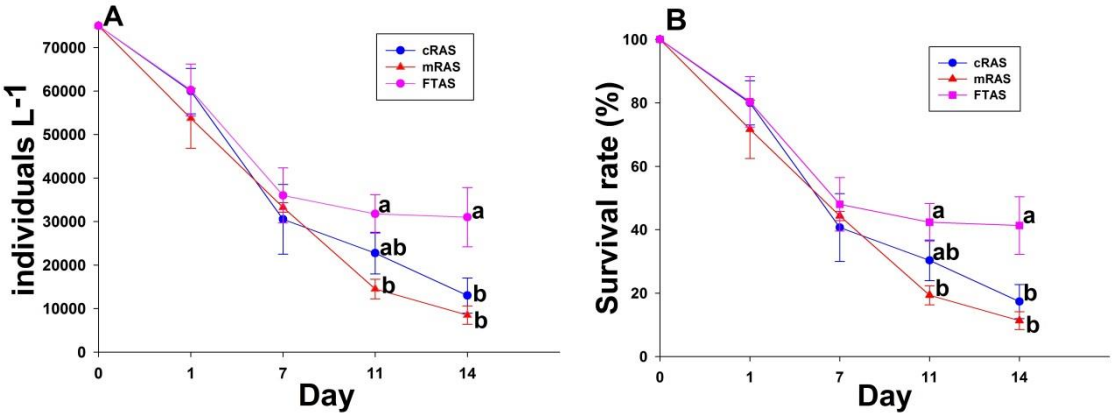

Figure 3.8 Density of *Acartia tonsa* (Mean ± SE) in three system (A) and Survival rate (Mean ± SE) in three systems (B) during the NP experiment. Different letters in the graph at sampling days denote significant differences between systems.

3.3.2 Daily mortality of *Acartia tonsa*

The average daily mortality of copepods in the mRAS was significantly higher than in the FTAS, while there were no significant differences between in the cRAS and the FTAS as well as the cRAS and the mRAS (**Table 3.2**).

Table 3.2 Daily mortality (Mean ± SE) of *Acartia tonsa* during the NP experiment. Different superscripts in a row denote significant differences between systems.

Mortality (% day^{-1})	cRAS	mRAS	FTAS
Day 0 - Day 14	13.57 ± 2.48^{bc}	17.52 ± 2.85^{b}	6.60 ± 1.58^{c}

3.3.3 Distribution of developmental stages of *A. tonsa*

Distribution of developmental stages of *A. tonsa* according to grouped nauplii, copepodite and adult stages during experimental days is shown in **Figure 3.9.**

During the first six days of the NP experiment (from day 0 to day 5), the nauplii stages (Group I and Group II) dominated in the three systems, while a dominance of copepodite stages (Group III and Group IV) was observed from day 6 until day 14 (**Figure 3.9**). Copepodite stages were first observed at day 4 in the FTAS (**Figure 3.9C**), and at day 5 in both the cRAS and the mRAS (**Figure 3.9A** and **Figure 3.9B**, respectively). The first adult individuals appeared at day 9 in the FTAS (**Figure 3.9C**), and at day 10 in the mRAS (**Figure 3.9B**) and at day 11 in the cRAS (**Figure 3.9A**).

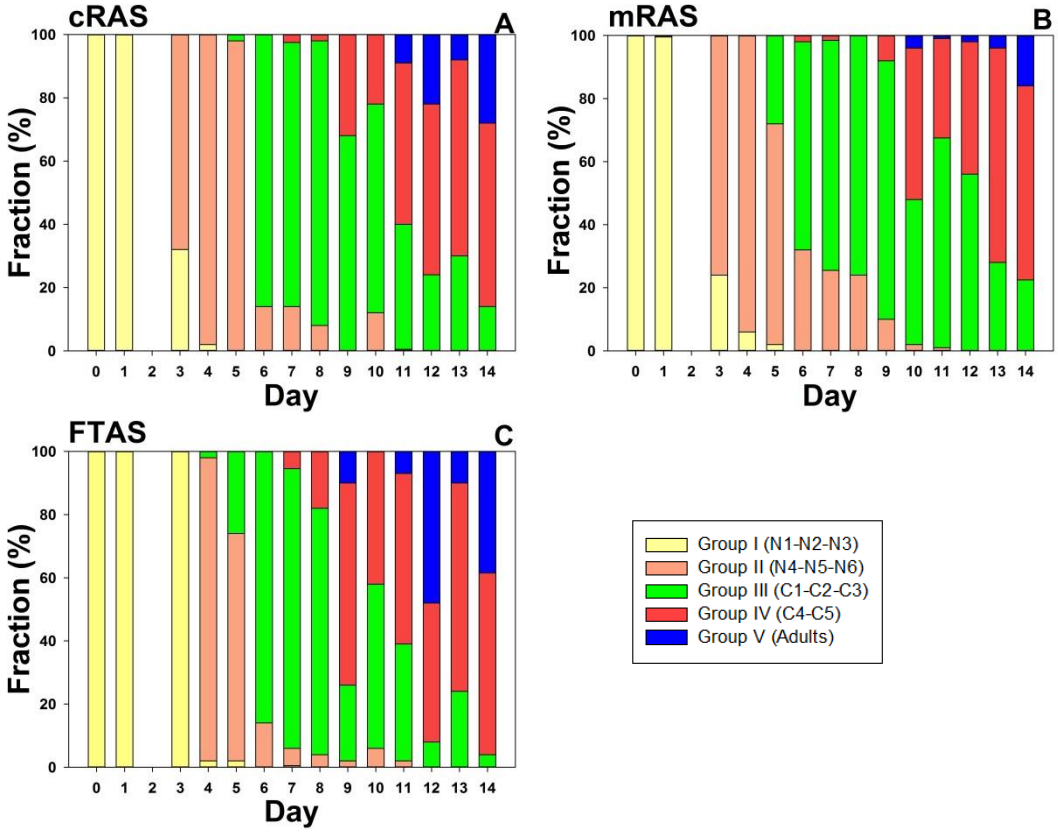

Figure 3.9 Fraction of nauplii, copepodite and adults in the cRAS (A), the mRAS (B), and the FTAS (C) during the NP experiment.

Fraction of nauplii, copepodite and adult stages of the three systems at day 1, day 7, day 11, and day 14, is shown in **Table 3.3**.

For nauplii stages: The mRAS had a significantly higher fraction of nauplii than cRAS and FTAS at day 7.

For copepodite stages: The mRAS had a significantly lower fraction of copepodites than cRAS and FTAS at day 7.

For adult stage: There were no significant differences among the three systems at day 11 and day 14.

Table 3.3 Fraction (%) of nauplii, copepodite and adults (Mean ± SE) in the three systems at specific sampling days during the NP experiment. Different superscripts at a row in the same grouped stages (Nauplii, Copepodite, and Adults) denote significant differences between systems.

Sampling days	Nauplii			Copepodites			Adults		
	cRAS	mRAS	FTAS	cRAS	mRAS	FTAS	cRAS	mRAS	FTAS
Day 1	100	100	100	0	0	0	0	0	0
Day 7	14 ± 3.36^{a}	25.5 ± 2.75^{b}	6 ± 2.82^{a}	86 ± 3.36^{x}	74.5 ± 2.75^{y}	94 ± 2.82^{x}	0	0	0
Day 11	0.5 ± 0.5^{a}	1 ± 0.5^{a}	2 ± 1.41^{a}	90.5 ± 3.5^{x}	98.0 ± 0.0^{x}	91.0 ± 5.74^{x}	9.0 ± 3.70^{e}	1.0 ± 0.58^{e}	7.0 ± 6.35^{e}
Day 14	0	0	0	72.0 ± 7.65^{x}	84.0 ± 7.52^{x}	61.5 ± 8.95^{x}	28.0 ± 7.66^{e}	16.0 ± 7.53^{e}	38.5 ± 8.96^{e}

3.3.4 Growth rate of *Acartia tonsa*

The specific growth rate (SGR day^{-1}) were calculated for each developmental phase (nauplii and copepodites) as well as for the whole period (total) during the NP experiment (**Figure 3.10** and **Appendix 9**). There were no significant differences in SGR between the three systems for nauplii and copepodite stages, nor the whole period (total). The copepods in the three systems grew faster in nauplii stages (0.29 - 0.32 day^{-1}) than in copepodite stages (0.18 – 0.20 day^{-1}).

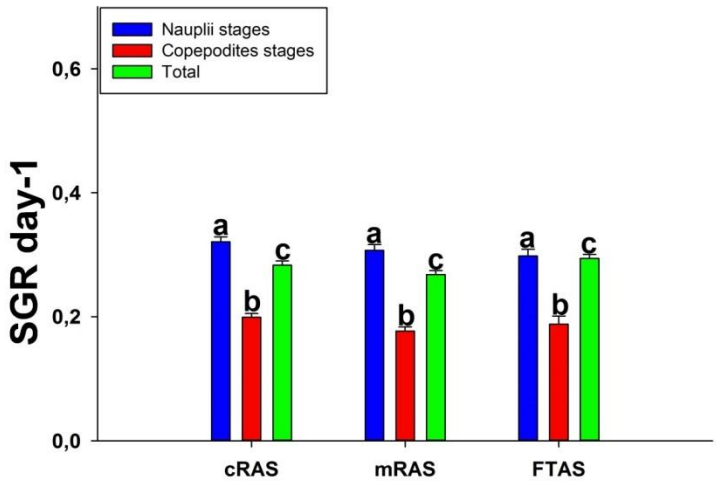

Figure 3.10 Specific growth rate (SGR day^{-1}) of *Acartia tonsa* in different developmental stages in the three systems (Mean ± SE). Different letters in the graph denote significant differences between systems.

3.3.5 *R. baltica* concentration in the three systems in the NP experiment

The concentration of algae in rearing tanks of the three systems (**Figure 3.11**) was relatively fluctuating during the whole NP experiment, except a sharp increase in cRAS from day 13 to day 14. The mean algal density of three systems was higher than the saturated level of food concentration for the copepods (10^4 cells ml^{-1}) (Skogstad, 2010) during all sampling days.

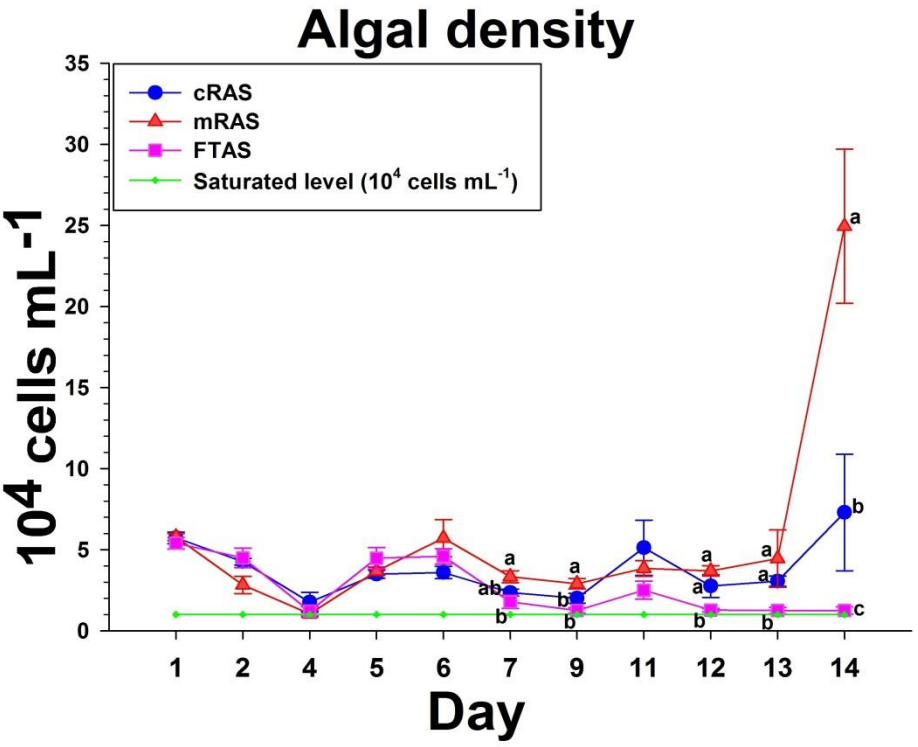

Figure 3.11 Concentration of R. baltica (Mean ± SE) in the rearing tanks of three systems during the NP experiment. Different letters in the graph denote significant differences between systems.

3.3.6 Transmembrane pressure

Overall, transmembrane pressure was gradually increased during the experimental days in the NP experiment, however; its highest value didn't reach the precautious level (0.3 bars).

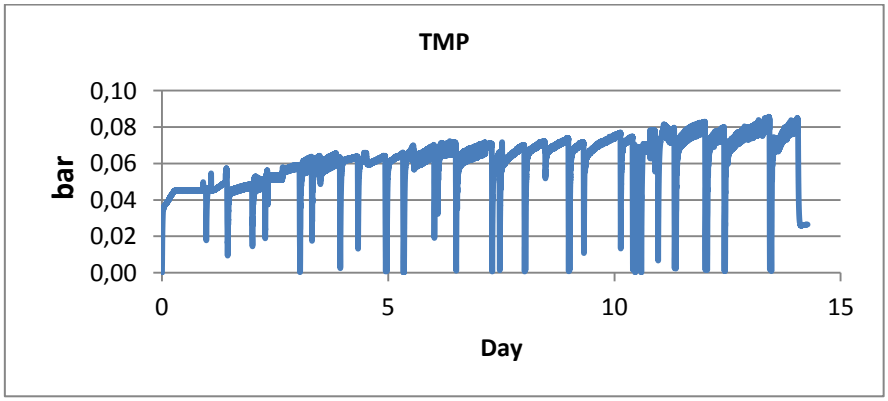

Figure 3.12 Transmembrane pressure (TMP) during the NP experiment.

3.3.7 Egg Production and survival rate of adults in the EP experiment

Egg Production (EP):

The data for egg production is shown in **Figure 3.13** and **Appendix 10**. The EP of cRAS and mRAS slightly fluctuated between 7 and 13 eggs female^{-1} day^{-1} while the EP of FTAS gradually increased from 21 to 32 eggs female^{-1} day^{-1} during the EP experiment. This resulted in significantly higher egg production in the FTAS, compared to the two recirculating systems during experimental days, except at day 10.

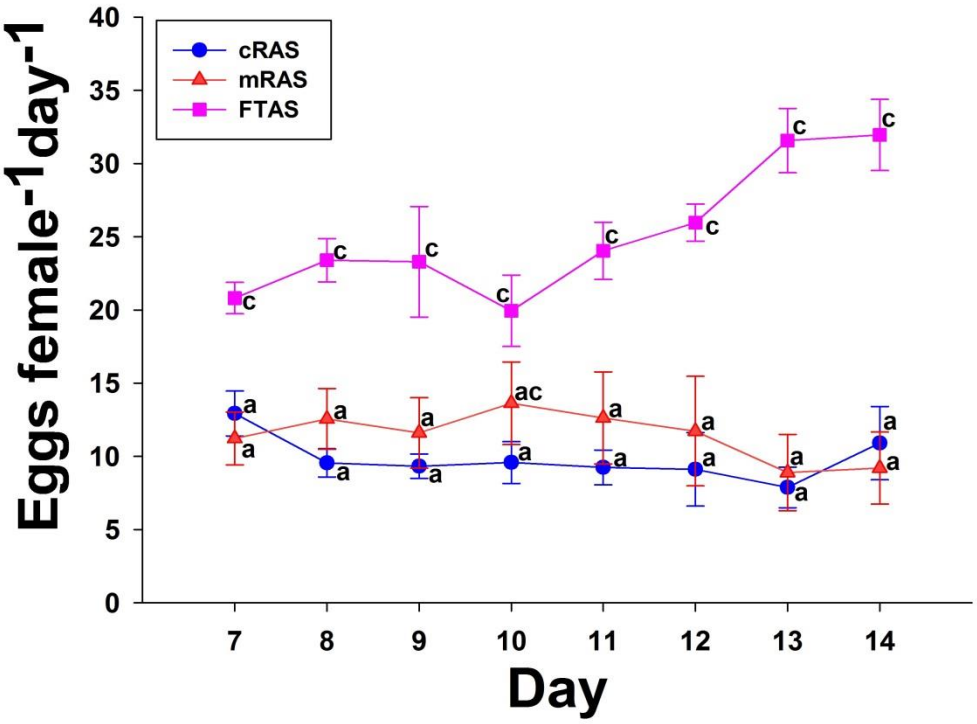

Figure 3.13 Egg Production (Mean ± SE) of the three systems during the second experiment. Different letters in the graph at sampling days denote significant differences between systems.

Survival rate of adults:

The survival rate (%) of adults in the EP experiment is shown in **Figure 3.14** and **Appendix 11**. The adult survival rate decreased very slightly in FTAS but considerably in cRAS and mRAS during the whole EP experiment, which resulted in significant differences between the FTAS and the two recirculating systems during the three last days.

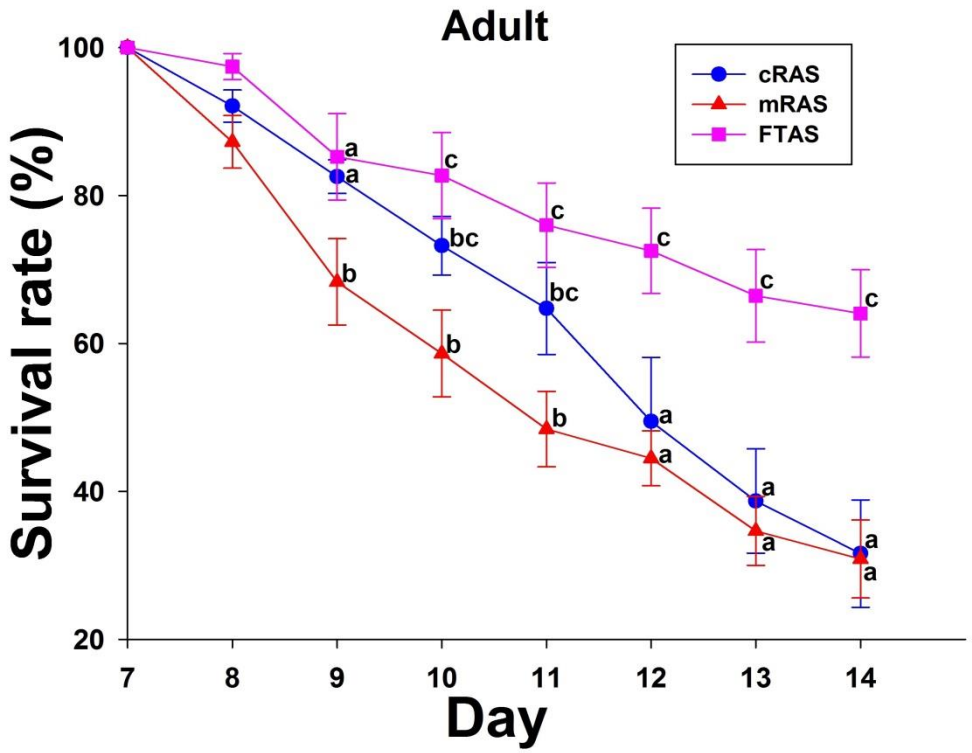

Figure 3.14 Survival rate (%) of *A. tonsa* in three systems during the EP experiment (Mean±SE). Different letters in the graph at sampling days denote significant differences between systems.

3.3.8 Possible relationship between measured parameters

Opportunists (%) and Survival rate (%) in the NP experiment:

The relationships between opportunists (%) and survival rate (%) in the three systems is illustrated in **Figure 3.15**.

The cRAS showed a fluctuation of opportunists (%) within the range above 50% and a gradually considerable decrease in survival; meanwhile, the mRAS showed a considerable up & down of opportunists (%) with the top at day 6, and a gradually sharp decrease in survival during the whole NP experiment. The FTAS showed a fluctuation of opportunists (%) within the range approximately below 50% and a considerable decrease to a plateau of survival (42-41%) during the whole NP experiment.

In the first half of the NP experiment: there was a considerable up & down of opportunists (%) in cRAS (above 50%) and FTAS (approximately below 50%) and a considerable reduction of survival rate by 59.3% in cRAS and by 52.0% in FTAS (**Appendix 8**). However, the mRAS showed a considerable increase in opportunists (%) (from 29.6 to 85.8%) (**Appendix 6**), which resulted in significant differences with the cRAS and FTAS at day 6, and a considerable decrease by 55.6% in survival (**Appendix 8**).

In the second half of the NP experiment: Despite of either up or down, opportunists (%) of cRAS and mRAS was still approximately above 50% and the two systems had the same continuously considerable reduction of survival (by 23.3% in cRAS and by 33.0% in mRAS (**Appendix 8**)). In contrast, the opportunists (%) of FTAS, despite of slightly increasing, was still approximately below 50% and survival of FTAS was relatively stable at around 41% (**Appendix 8**), which led to significant differences with the two recirculating systems at day 14.

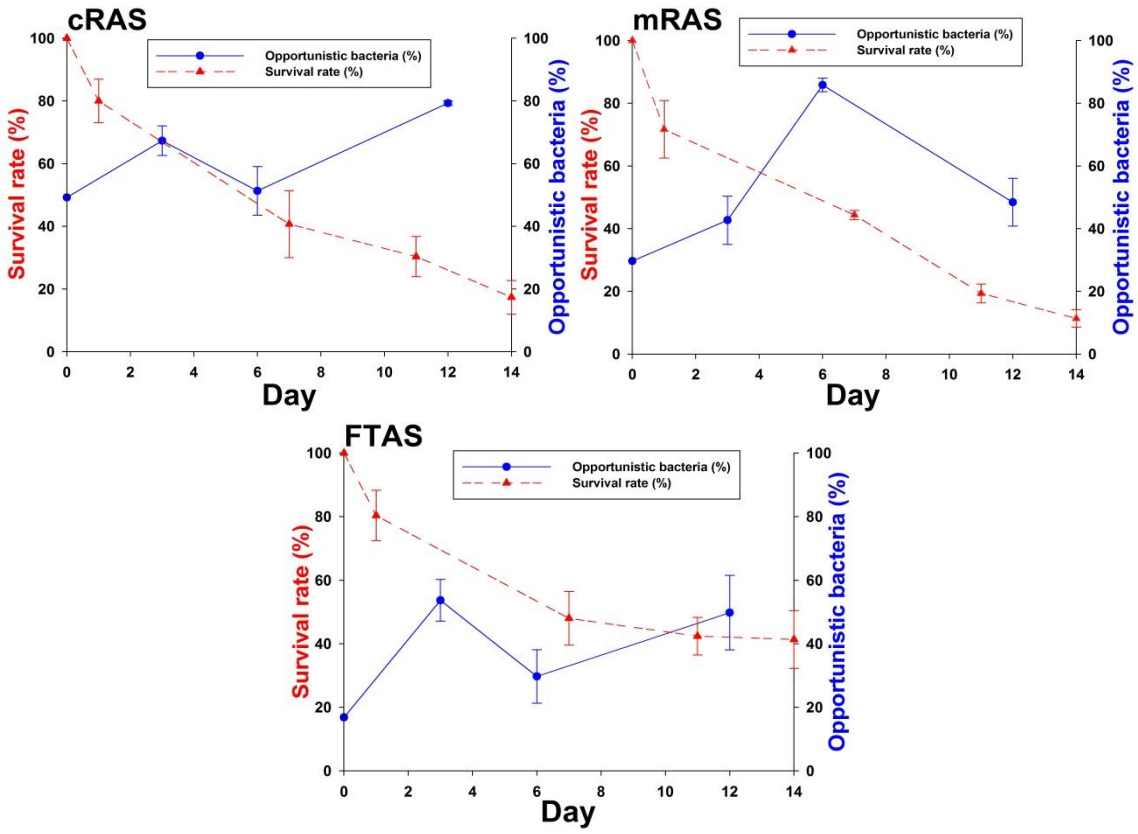

Figure 3.15 Relationships between opportunists (%) and survival rate (%) in the three systems during the NP experiment (Mean±SE).

Besides the relationship of the two parameters in the three systems (**Figure 3.15**), the **Table 3.4** showed the mean survival rate (±SE) of the two groups (≥50% opportunists and <50% opportunists), calculated from the corresponding survival rate of the three systems in all sampling days (**Appendix 1**2). The grouped tanks (**A**), which had above 50% opportunists, had a significantly lower survival rate than the grouped tanks (**B**), which had below 50% opportunists.

Table 3.4 Survival rate (Mean ±SE) of two grouped tanks in three systems, which had ≥ 50% opportunists and <50% opportunists. Different superscripts at a row denote significant differences between the two groups.

Three systems	Group A (n=17) (≥ 50% Opportunists)	Group B (n=19) (<50% Opportunists)
Survival rate (%)	28.0±4.6[a]	55.9±6.2[b]

4. Discussion

The study described an experiment including two parts - Nauplii Production (NP) and Egg Production (EP). In NP experiment, the microbial influences on NP (survival, growth and development) of *A. tonsa* in cRAS, mRAS and FTAS were accessed. In EP experiment, the later effects of microbial community from the NP experiment on EP (number of eggs female^{-1} day^{-1} and survival of adults) were accessed. The results indicated that *A. tonsa* in FTAS showed a better performance in NP and EP experiment, compared to the two recirculating systems.

4.1 Water quality in nauplii production (NP) experiment

4.1.1 Physiochemical water quality parameter

The three systems had a relatively stable water quality condition (temperature, salinity, dissolved oxygen and pH) throughout the experimental period (**Table 3.1**). The temperature, dissolved oxygen and pH of the three systems in the present study was close to the optimal range for cultivation of *A. tonsa* (Blaxter et al., 1998). However, the mean salinity of mRAS (38.3 ppt) and cRAS (37.1 ppt) was slightly higher than the optimal range for the strain of *A. tonsa* (30-32 ppt) (Drillet et al., 2011, Jepsen et al., 2013), and also higher than the rearing salinity of *A. tonsa* at SINTEF Fisheries and Aquaculture (32-35 ppt). Cervetto et al. (1999) tested resistance of *A. tonsa*, that was sampled from the Berre Lagoon, towards different kinds of osmotic shocks in laboratory experiments. The results from experiments of Cervetto et al. (1999) showed that *A. tonsa* had the optimal salinity within the range of 15-22 ppt and expressed a high mortality if the instantaneous change in salinity was greater than 10‑15 ppt. Therefore, the small differences in salinity of mRAS and cRAS, compared to the optimal range at SINTEF (32-35 ppt) could slightly affect the performance of *A. tonsa* in the current experiment. However, the current study didn't test the resistance of the strain of *A. tonsa* toward differences in salinity, so it is unknown to what extent the differences in salinity affected survival, growth and development of the strain of *A. tonsa* in the NP experiment.

4.1.2 Nitrogenous wastes

The highest concentration of un-ionized ammonia nitrogen (NH_3-N) in the rearing tanks and in the inlet water (**Figure 3.1**) were lower than the No Observed Effect Concentrations (NOEC) (0.03 mg NH_3 L^{-1}) for *A. tonsa* nauplii in a batch culture with 48 hours and 72 hours of exposure in a study of Jepsen et al. (2013). Jepsen et al. (2013) also showed that 48-h and 72-h 50% Lethal Concentrations (LC50) values for naupliar stages were 1.257 mg and 0.22

mg NH_3 L^{-1}. Thus, the un-ionized ammonia concentrations in the present study could be in the safe range for the cultivated copepods.

The relatively stable trend at low level of NO_2-N concentration in the rearing tanks and in the inlet water of FTAS throughout the experimental period was probably due to a continuous dilution of new seawater. In contrast, the NO_2-N concentrations of mRAS fluctuated within the range of 0.1 - 0.4 mg L^{-1} throughout the experimental period, while the NO_2-N concentrations of cRAS showed a same trend as the mRAS during the first 11 days, but considerably increased to the highest values at day 14 (1.04 mg L^{-1} and 1.07 mg L^{-1} in **Figure 3.2A** and **Figure 3.2B** respectively). The considerable increase in cRAS from day 11 to day 14 was probably because the system could not remove the fine suspended solids, which could lead to a decrease in biological conversion of ammonia to nitrate through the intermediate nitrite (Andersson et al., 1994, Holan et al., 2013b). Jepsen et al. (2013) also reported that nitrite and nitrate, converted from ammonia during nitrification and denitrification processes in the biofilters, can potentially reach toxic levels for *A.tonsa* but no available studies have been reported about the exact toxic levels for *A.tonsa*. However, Lucas and Southgate (2012) stated that the safe value of nitrite nitrogen in the culture for most marine fish should be less than 1.0 mg L^{-1}. Therefore, the high NO_2-N concentrations in cRAS at day 14 in the current study might adversely affect to the final survival rate of the copepods in this system.

The NO_3-N concentrations in the rearing tanks and in the inlet water for the three systems (**Figure 3.3A** and **Figure 3.3B** respectively) were low at the beginning and high at the end of the experiment, but still lower than 10 mg L^{-1}. Nitrate nitrogen range for aquaculture was 0-400 mg L^{-1} or higher (Meade, 1985, Lawson, 1995, Piper et al., Timmons and Ebeling, 2007); for example, four first-feeding marine fish larvae species (*Heteromycteris capensis, Gaidropsarus capensis, Dipiodus sargus, Lithognathus mormyrus*) showed a very high tolerance to nitrate level (24-h LC_{50} ranging from 4200 to 8700 mg L^{-1}) (Brownell, 1980). NO_3-N is the end-product of nitrification and controlled by daily water exchange rate (Timmons and Ebeling, 2007), hence the NO_3-N concentrations in cRAS and mRAS probably reflected the nitrification process that occurred in the biofilters. Moreover, the algae volume (3.68% replaced water daily in cRAS or mRAS because of 95.54% recirculation rate), which fed to the copepods, contains a small amount of unassimilated nitrate from the growth medium (Conwy medium), which also contributed to the measured NO_3-N concentrations. In short, the NO_3-N concentrations of the three systems in our study could be in the safe range for the cultivated copepods.

4.2 Microbial characterization in the NP experiment

The fluctuations of the microbial community (opportunists (%) and total number of bacteria) in rearing tanks of the three systems during the whole NP experiment probably resulted from the fluctuations of organic particles[10] in the rearing tanks and fluctuations of microbial community and organic particles in the inlet water. The organic particles inside rearing tanks of the three systems originated from uneaten algal sediment, unhatched copepod eggs and egg shells, chitinous exoskeletons of copepods, fecal pellets from copepods, dead copepods. The inlet water of cRAS and mRAS was from the biofilters, while the inlet water of FTAS was from the reservoir (6 m^3) filled with sandfiltered seawater (>40μm).

According to Attramadal et al. (2012b), when microbial maturation was reached at a relatively low microbial CC of the inlet water (water in biofilters), any unexpected changes to higher substrates levels in the rearing tanks still opened a potential for opportunistic proliferation. This trend happened in cRAS where the total number of bacteria in inlet water was stable at low level (**Figure 3.7B**) but the colloidal particles inside rearing tanks (Day, *2014*) was high and gradually increasing throughout the experimental period. This probably opened a potential for opportunistic proliferation in cRAS and probably explained why the opportunists (%) of cRAS fluctuated within the range above 50% (**Figure 3.6A**) throughout the NP experiment. In contrast with cRAS, the opportunists (%) of FTAS fluctuated within the range below approximately 50% (**Figure 3.6A**). This was probably because the colloidal particles inside FTAS rearing tanks was significantly lower than in cRAS, and relatively stable throughout the experimental period (Day, *2014*). Although there was a big gap in the total number of bacteria between rearing tanks and inlet water of FTAS (**Figure 3.7A** and **Figure 3.7B**), the low and stable organic particles (colloidal particles) inside FTAS rearing tanks during the whole NP experiment possibly led to a low substrate supply per bacterium; consequently, this created a K-selective pressure that could inhibit proliferation of opportunists (%) (MacArthur, 1967, Vadstein et al., 1993, Skjermo et al., 1997, Salvesen et al., 1999).

On the other hand, the trend of opportunists (%) in mRAS was opposite to cRAS and FTAS from day 3 to day 6 and from day 6 to day 12 (**Figure 3.6 A**). From day 3 to day 6, the increased opportunists (%) of mRAS was probably because the mean algal density in rearing tanks from day 5 to day 6 increased approximately twofold in mRAS (**Figure 3.11**), but was

[10] Analyses of particles (turbidity, fine solids (2μm-60μm), and colloidal particles (30nm - 1μm)) were conducted simultaneously with microbial analyses. The particle analyses were done by Day (*2014*).

stable in the cRAS & FTAS. Consequently, the increase in algal density possibly resulted in an increased amount of uneaten food in the mRAS rearing tanks because the saturated algal density for the copepods was only 10^4 cells mL^{-1} (Skogstad, 2010). The high amount of uneaten algae can sediment to the bottom[11] of rearing tanks and dissociate into organic substrates. In addition, the fine solid concentration in inlet water in mRAS was significantly higher than in cRAS and FTAS at day 5 (Day, *2014*) that could possibly contribute to the organic substrates in mRAS rearing tanks. The organic substrates in mRAS rearing tanks that resulted from the uneaten algal sediment and the fine solids could then trigger opportunistic bacteria (Martins et al., 2010, Chiam and Sarbatly, 2011, Holan et al., 2013a). From day 6 to day 12, the reduction in opportunists (%) of mRAS (**Figure 3.6A)** was probably because the WER of the three systems increased to 8 times per day and the outlet filter mesh size was replaced from 40 μm to 64 μm during this interval. The increased WER and outlet filter mesh size probably resulted in the increase in the transport of particles from the rearing tanks to the biofilters where the membrane filtration modules were installed between the heterotrophic and nitrification biofilters. The membrane filtration modules effectively removed fine solids (2μm-60μm) in the biofilters of mRAS (a sharp decrease in inlet water of mRAS) and colloidal particles (30nm-1μm) in rearing tanks of mRAS from day 6 to day 12 (Day, *2014*). The effective removal of particles could then lead to a significant reduction in opportunists (%) in the permeates and in the inlet water of mRAS from day 6 to day 12 (**Figure 3.6B**). Lekang (2007) proposed that organic particles contain some nutrients, hence the sufficient water flow (WER) is very important to prevent leakage of nutrients. This probably resulted in a low substrate supplying per capita (i.e. per bacterium) that favored slow growing non-opportunistic bacteria (Odum et al., 1971, Atlas and Bartha, 1981, Andrews and Harris, 1986, Skjermo et al., 1997). The dominance of non-opportunistic bacteria (K-selection) is supposed to inhibit proliferation of opportunistic bacteria in the rearing water of marine fish larvae (Skjermo et al., 1997). This trend happened in mRAS from day 6 to day 12 (**Figure 3.6A**).

The significantly lower total number of bacteria in cRAS, compared to the mRAS and FTAS at day 6 (**Figure 3.7A**), were probably because at day 5 the fraction of copepodites in cRAS (2%) was much lower than that of the mRAS (28%) and the FTAS (26%) (**Figure 3.9**). The more the copepods developed and moulted from nauplii stages into copepodites stages at day 5 in mRAS (**Figure 3.9B**) and FTAS (**Figure 3.9C**), the more their exoskeletons were released into the rearing tanks. The exoskeleton of copepods (**Figure 4.1**) contains the protein

[11] The bottom of the 12 rearing tanks was not siphoned daily due to time-consuming.

chitin which can be considered as an available substrate for bacterial growth (Heidelberg et al., 2002). In fact, Heidelberg et al. (2002) proposed that when nutrient availability become limited for bacterial growth and the number of bacteria is low in ecosystems, selection for specific bacterial species happen in the zooplankton exoskeleton micro-ecosystem, since bacteria attached to copepods have chitin available as a substrate. The study of Heidelberg et al. (2002) also estimated that 2.5 to 4,000 *V. cholerae-V. mimicus* organisms were associated with the exoskeleton of a calanoid copepod. In addition, the bacteria associated with the copepods was not only reported in natural ecosystems (Heidelberg et al., 2002), but also found in intensive systems (Vu Thi Thuy, 2011). In fact, the study of Vu Thi Thuy (2011) with the calanoid copepod (*A.tonsa*) with the same species in this present study, showed that when cultivating the *A.tonsa* in intensive systems (RAS and FTAS), the highest CFU_{0-14} associated with copepods in RAS & FTAS was 0.4-1.1 x 10^4 CFU ml^{-1}. The study of Vu Thi Thuy (2011) also found that fast growing bacteria dominated bacterial community associated with the copepods (35-100%) in the RAS while this fraction was only 4-38% in the FTAS. A study of Hansen and Bech (1996) also showed that a high portion of opportunistic bacteria, like *Vibrio spp.*, *Aeromonas spp.*, and *Pseudomonas spp.*, preferred associating with the copepods to living in the culture water. On the other hand, the chitinous exoskeleton of the naupliar stage 6 (**Figure 4.1**) can sink down to the bottom of rearing tanks, or suspend in the culture water and then dissociate into organic substrates. These substrates can increase organic matter concentration and the microbial carrying capacity (CC) in the rearing tanks, compared to the inlet water (Vadstein et al., 1993, Vadstein et al., 2004). This probably favoured a rapid bacterial growth to the higher CC during some first days of cultivation (Skjermo et al., 1997, Salvesen et al., 1999). This probably explained why the mRAS and the FTAS had the significantly higher microbial carrying capacity (total number of bacteria) than the cRAS.

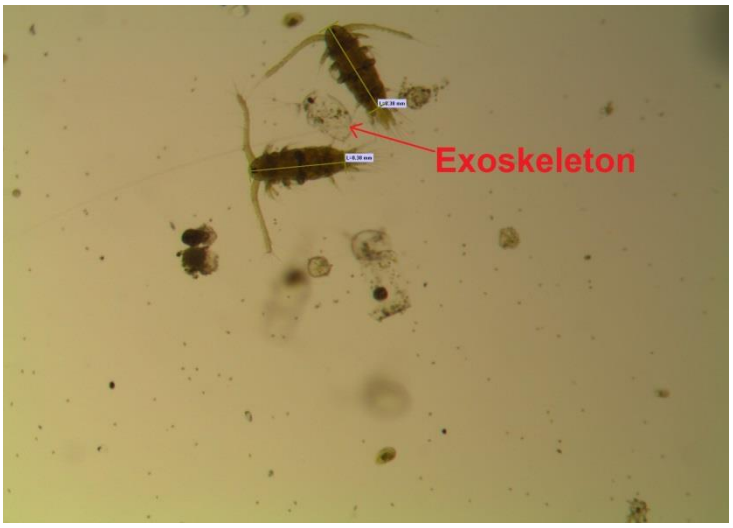

Figure 4.1 Exoskeleton of naupliar stage 6 in the rearing tanks of the cRAS at day 6 of the NP experiment. Photo: Anh Phan Hung, 2014.

4.3 Copepod performance in the NP experiment

4.3.1 Density and survival rate of *Acartia tonsa*

The tendency of density and survival rate of *A.tonsa* in cRAS & mRAS was different to the tendency in FTAS (**Figure 3.8**). This was probably due to the differences in microbial characterization and their influences on the three systems which are discussed in the section **4.4.1**.

The initial density of copepods in the rearing tanks of the three systems in the current study was 75000 ind L^{-1} at day 0. It was much higher than 20000 nauplii L^{-1} in study of (Vu Thi Thuy, 2011) and 1000 ind L^{-1} in study of (Skogstad, 2010) with the same species. The initial density in current study was probably the highest density for *A.tonsa* cultivated in intensive systems (RAS and FTAS) until now since the density in recent studies ranged from 100-2000 ind L^{-1} in (Støttrup et al., 1986, Støttrup, 2003) to 7000 ind L^{-1} in Drillet et al. (2006). Although the current density was 3.75-fold higher than in the study of Vu Thi Thuy (2011), the observed daily average percentage mortality in current study (6.6%) was only 1.6-fold higher than that (3.9%) in the study of Vu Thi Thuy (2011) in the same FTAS system. Moreover, the daily average percentage mortality in the cRAS and the mRAS (13.57% and 17.52% respectively) was just 1.3 – 1.7-fold higher than that (9.9%) in the RAS in the study of Vu Thi Thuy (2011).

4.3.2 Growth and Development of *Acartia tonsa*

The availability of food concentration strongly affects the growth of *A.tonsa*; for example, Skogstad (2010) found that minimum live algal density for maintaining optimum growth rate

was 10^4 cells mL^{-1} (equivalent to 370 µg C L^{-1}) or equal to 500 µg C L^{-1} as found by Berggreen et al. (1988). When culturing *A.tonsa* at high density (20000 ind L^{-1}) in the RAS and FTAS, Vu Thi Thuy (2011) found that the food limitation observed in the RAS was due to a daily limitation of 20L of live algae day^{-1} (5% of total water volume of the RAS replaced per day), which negatively affected the growth, length and weight of the copepods in the RAS, compared to the FTAS. However, in the present study, the live algae concentration in the rearing tanks of the three systems was higher than the saturated level (10^4 cells mL^{-1}) (**Figure 3.11**), so the growth and development of *A.tonsa* was probably not affected by food limitation.

The higher SGR in naupliar stages compared to copepodite stages in the three systems in the present study was also found in the previous studies (Leandro et al., 2006, Skogstad, 2010, Vu Thi Thuy, 2011). The total SGR (from day 0 to day 14) in present study (0.26-0.29 day^{-1}) was lower than 0.45 day^{-1} in the study of Berggreen et al. (1988), but was consistent with the total SGR in study of Vu Thi Thuy (2011).

4.4 Influence of microbial community on nauplii production

4.4.1 Influence of microbes on the survival rate of *A. tonsa*:

The total number of bacteria cannot be used as an absolute sign to evaluate whether the microbial community is beneficial or not (Wold et al., 2014). The low number of bacteria and the low carrying capacity (CC) in itself were not automatically beneficial (Attramadal et al., 2012b). The compositions of the bacterial community probably affect the performance of the rearing species (e.g. fish larvae) more than the absolute abundance of bacteria (Vadstein et al., 1993, Munro et al., 1995, Vadstein et al., 2004, Verner-Jeffreys et al., 2004). Although the microbial compositions (bacterial species) of the three systems in the NP experiment were not analyzed, the opportunists (%) analyses can alternatively be used to evaluate the influences of microbes on the copepod survival in the three systems.

Opportunistic bacteria can become pathogen when the resistance mechanism of the host is lowered by environmental stress factors (Wold et al., 2014). A low fraction of opportunists (%) can suggest a high level of specialists (slow-growing bacteria) and a more mature microbial community (Skjermo et al., 1997, Attramadal et al., 2012a). Water maturation selecting for non-opportunistic or slow-growing bacteria (K-selection) can inhibit proliferation of opportunistic bacteria, then improved performance of marine fish larvae during the early stages of production (Vadstein et al., 1993, Skjermo et al., 1997, Salvesen et al., 1999). In present study, the low fraction of opportunists (%) in FTAS (approximately

below 50%) during the whole NP experiment probably had a positive effect to the survival rate of *A. tonsa* (the relatively stable trend from day 7 to day 14) (**Figure 3.15**).

Furthermore, the statistically significant higher survival rate of the copepods in the grouped tank B (< 50% opportunists) (**Table 3.4**) compared to the grouped tank A (≥ 50% opportunists) showed that the opportunists (%) probably had effects to the survival rate of the copepods. This effect was also found in previous studies when the high fraction of fast growing opportunists adversely affected performance of young marine fish larvae (Nicolas et al., 1989, Vadstein et al., 1993, Munro et al., 1994, Skjermo et al., 1997, Skjermo and Vadstein, 1999, Hansen and Olafsen, 1999, Sandlund and Bergh, 2008, Attramadal et al., 2012b). In short, the high % opportunists (≥50%) in the rearing tanks most probably adversely affected to survival rate of *A.tonsa*.

4.4.2 Influence of microbes on the development of *A. tonsa*

The high ratio of opportunists (%) in cRAS and mRAS (above 50%) in the current NP experiment (**Figure 3.15)** could result in the delay of the first copepodite stage and adult stage in cRAS and mRAS, compared to the FTAS (**Figure 3.9**). Moreover, the significantly higher ratio of opportunists (%) of mRAS at day 6, compared to the cRAS and FTAS (**Figure 3.6A**), could possibly be seen as the reason for the significantly lower fraction of copepodite stages and significantly higher fraction of nauplii stages in mRAS at day 7, compared to the cRAS and FTAS (**Table 3.3**). Shortly, the high fraction of opportunists (>50%) could adversely affect to the development of *A.tonsa* in current study.

Although the bacterial compositions in the current study were not investigated, many previous studies report that in natural ecosystems, when nutrient availability became limited, the bacterial pathogen *V. cholerae* was preferentially attached to the chitinous exoskeleton of calanoid copepods (Heidelberg et al., 2002). The bacterial species digested the chitin as nutrient substrates (Huq et al., 1983, Huq et al., 1984, Nalin et al., 1979) and probably caused damage to the crustacean exoskeleton (Nagasawa, 1988, Sanders and Fryer, 1988), which is the main part of copepod prosome. In addition, when fed the copepods with oversaturated algal concentration, the detritus from the algal sediment and the fecal pellets from copepods could dissolve into organic matter which can proliferate fast growing bacteria species (*Vibrio spp., Pseudomonas spp., Acinetobacter spp.* and *Aeromonas spp.*) (Hansen and Bech, 1996). These bacterial species probably caused harmful or adverse effects to the development of *A. tonsa* in the present study but the detailed mechanisms of these effects on the copepods have not been published or documented.

4.5 Transmembrane pressure

Transmembrane pressure is a parameter to measure the fouling and clogging level of the membrane. During the membrane operation, the solids and particulates remained on the outside of the membrane, while the permeates were drawn through the membrane to the inside of the fibers (http://www.kochmembrane.com/Membrane-Products/Hollow-Fiber/Ultrafiltration/PURON-Series.aspx, 2014) due to a different partial pressure between the outside and inside of the membrane (Judd, 2010). The more WER applied, the more the accumulation of the solids and particulates remained on the outside of the membrane, the higher transmembrane pressure was reached in the mRAS. During experimental days, the transmembrane pressure gradually increased according to the increase in the WER but was still below 0.1 bar, which was much lower than the precautious level (0.3 bar). The low transmembrane pressure level during the last 7 days of the NP experiment could probably be potential for applying a high WER (8 times per day) in the mRAS for cultivation of *A.tonsa* during copepodite stages in the mRAS.

4.6 Influence of microbial community on egg production

Although the copepods, after finishing the NP experiment, were transferred to beakers (stagnant water) and had the same conditions during the EP experiment, the results of EP experiment still showed a different trend between FTAS and the two recirculating systems. It means that the history of cultivating in three different systems during the nauplii and copepodite stages led to the corresponding different EP during the adult stage. In the current study, the significant higher EP of the FTAS, compared to the cRAS and mRAS, during experimental days, except day 10, was probably due to the later adverse effects of opportunistic bacteria from the NP experiment. In fact, the negative influences of microbial community on the copepods in the cRAS and mRAS during the NP experiment (**see section 4.4**) could possibly impact the reproductive performance of the female adults (**Figure 4.2**) in the two corresponding systems in the EP experiment. This can lead to the significantly lower EP in the cRAS and mRAS (**Figure 3.13** and **Appendix 10**) and was probably linked to the significantly lower survival rate of adults in the cRAS and mRAS (**Figure 3.14**), compared to the FTAS, at day 12, 13, 14 of the EP experiment.

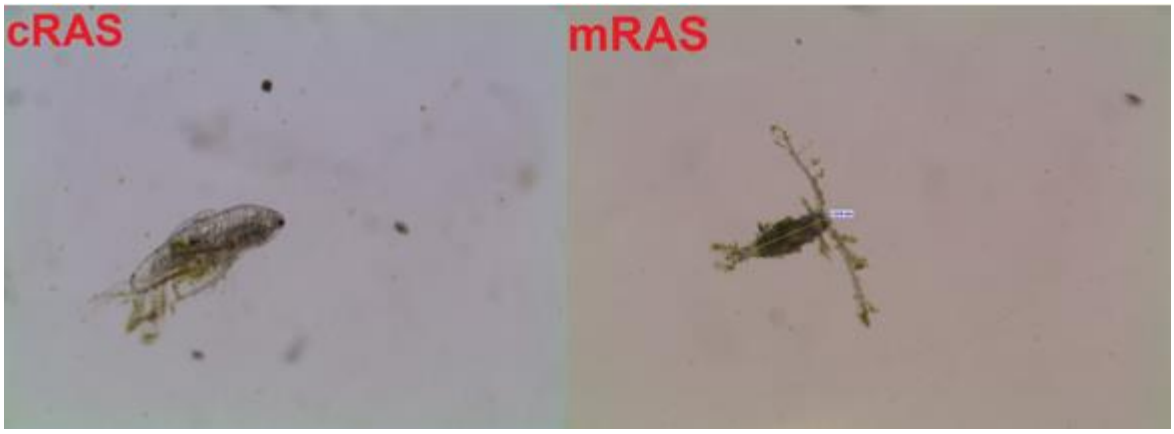

Figure 4.2 Some of *A. tonsa* in the white tanks (Figure 2.1) corresponding to the cRAS and the mRAS at day 15 of the nauplii production (NP) experiment. Photos: Anh Phan Hung, 2014

The experimental parameters (**Table 2.1**) in the EP experiment was similar to several other studies regarding food concentration (Skogstad, 2010), temperature, photo-period, adult stocking density (Holste and Peck, 2006, Peck and Holste, 2006), salinity (Drillet et al., 2008) and oxygen level (Marcus et al., 2004, Sedlacek and Marcus, 2005). Therefore, their influences on the differences between the reproduction capacities of the cultivated copepods can be disregarded in the current study. Moreover, the negative effects of un-ionized ammonia concentration on the copepod adults in the EP experiment should also be ignored because the seawater in the beakers was replaced by the fresh seawater after 24 hours incubation. The study of Jepsen et al. (2013) also indicated that the No Observed Effect Concentrations (NOEC) and the Lowest Observed Effect Concentrations (LOEC) of NH_3, which provides safe levels before cultures are affected, for the adults (*A.tonsa*) were 0.477 mg NH_3 L^{-1} and 1.789 mg NH_3 L^{-1} respectively.

The data for the EP of *Acartia tonsa* was recorded from day 7 to day 14 of the EP experiment which could be seen as the most suitable for measuring the EP. *A. tonsa* were then in the adult stage and this interval was also considered as a period for the high and stable EP according to study of Jónasdóttir and Kiørboe (1996) and Parrish and Wilson (1978). The daily EP in the cRAS and mRAS (7.86 and 12.91 eggs $female^{-1}$ day^{-1}) was lower than the mean EP (25 eggs $female^{-1}$ day^{-1}) in the RAS of the study of Vu Thi Thuy (2011) with the same species. However, the mean EP in the FTAS at day 13 and day 14 (31 eggs $female^{-1}$ day^{-1}) of the current study was higher than the mean EP (25 eggs $female^{-1}$ day^{-1}) in the FTAS of the study of Vu Thi Thuy (2011) but lower than the EP (40-50 eggs $female^{-1}$ day^{-1}) in a small scale experiment of Drillet et al. (2008), Kiørboe et al. (1985) and Holste and Peck (2006).

5. Conclusions and future perspectives

Based on results from the current study, the following conclusions can be drawn:

The fraction of opportunistic bacteria in the rearing tanks was probably more important and influential on the copepod (*A. tonsa*) performance than the fluctuation of total number of bacteria. The low fraction of opportunistic bacteria in FTAS rearing tanks (below approximately 50%) during the whole NP experiment probably had positive effects on the survival rate and development of *A. tonsa* in NP experiment, and probably led to the later positive effects on the adult performance (number of eggs female^{-1} day^{-1}, survival of adults) in the EP experiment. In contrast, the high fraction of opportunistic bacteria in the rearing tanks of cRAS & mRAS (above 50%) in NP experiment probably had adverse effects on the copepod performance in both NP and EP experiment.

When the high water exchange rate (WER) (8 times day^{-1}) was used from day 6 to day 12 in NP experiment, the effective removal rate of particles by the membrane filtration units in mRAS was increased, which led to the considerable reduction in opportunists (%) in the rearing tanks, in the permeate and inlet water (biofilters) of mRAS.

The initial rearing density (75000 nauplii L^{-1}) in the current study, in comparison with previous studies, was probably the highest density for *A.tonsa* cultivated in intensive systems (RAS and FTAS) until now. However, the observed daily average percentage mortality in the current study was relatively similar to previous studies.

Future perspectives:

Since the positive effects of the low fraction of opportunistic bacteria probably led to the better performance of *A. tonsa* in FTAS in the current study, the higher initial rearing density (>75000 nauplii L^{-1}) in FTAS should be further investigated to increase copepod (*Acartia tonsa*) nauplii production to supply as live feed for marine larviculture industry. With a simple design, low investment & operation cost, unlimited algal volume fed to copepods, and continuous removal of organic particles from the rearing tanks, the FTAS should be one of the most suitable systems for intensive production of the copepods (*A. tonsa*).

Using the high WER (8 times day^{-1}) in mRAS at the beginning of NP experiment to take advantage of the effective removal of particles in the membrane filtration units should be further investigated.

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APPENDIXES

Appendix 1: Sampling, analyzing and routine maintenance performed during the NP experiment.

Abbreviations:

Exp. Day: Experimental day

Temp.: Temperature

Sal.: Salinity

CFU: colonies forming units of bacteria on marine agar plates

all tanks: taking 12 samples of 12 tanks of three systems (cRAS, mRAS, FTAS)

1 tank: taking sample in 1 random tank in each system

Exp. day	Temp.	Sal.	O_2	pH	Nitrogenous waste		CFU		Flow cytometry		Taking picture of copepods		Density of Copepod		Cleaning outlet filter
	all tanks	all tanks	all tanks	all tanks	1 tank	all tanks	1 tank	all tanks	1 tank	all tanks	1 tank	all tanks	1 tank	all tanks	all tanks
0	x	x	x	x	x		x		x			x		x	x
1	x	x	x	x								x		x	x
2	x	x	x	x							x		x		x
3	x	x	x	x		x		x		x	x		x		x
4	x	x	x	x							x		x		x
5	x	x	x	x							x		x		x
6	x	x	x	x				x		x	x		x		x
7	x	x	x	x		x						x		x	x
8	x	x	x	x							x		x		x
9	x	x	x	x							x		x		x
10	x	x	x	x							x		x		x
11	x	x	x	x		x						x		x	x
12	x	x	x	x				x		x	x		x		x
13	x	x	x	x							x		x		x
14	x	x	x	x		x				x		x		x	x

On a daily basis, the nitrification biofilters in cRAS and mRAS were each fed 3g of ammonium chloride (NH_4Cl) for a period of 17 days prior to the experiment, while the heterotrophic biofilters in cRAS and mRAS were each fed 3 g of formulated fish feed for a period of 9 days. This amount was increased to 30 gram per day at 4 days before the experiment. The tank water samples for CFU, Flow cytometry, Nitrogenous waste products were taken by using a sucking device connecting with 25ml sterilized sterilin and 40 µm filter tube to make sure that no copepod nauplii were taken together with water samples.

Appendix 2: The expected calculation of *Rhodomonas baltica* for the NP experiment

	Tank	Initial density of copepods	Initial algae concentration after adding to rearing tanks	Reduced algae concentration after 24h	Remained algae in the tank after 24 h	Loss of algae due to water exchange rate in a flow-through system
Thesis of Skogstad (2010)	50 L	1000 ind L^{-1}	518 ± 20.15 µg C L^{-1} Corresponding to : 1,5 x 10^4 cells mL^{-1}	206.25±54.45 µg C L^{-1} Corresponding to: 0.5 x 10^4 cells mL^{-1}	368.85 ± 34.5 µg C L^{-1} Corresponding to: 10^4 cells mL^{-1}	20% of the reduced algae concentration after 24h

Basing on thesis of Skogstad (2010), we can calculate the expected algae volume needed for the experiment.

	Tank	Initial density of copepods (Expected)	Initial algae concentration after adding to rearing tanks (Expected)	Reduced algae concentration after 24h (Expected)	Remained algae in the tank after 24 h (Expected)	Loss of algae due to water exchange rate (Expected)	Live algae needed for 1 rearing tank (Expected) (The present daily harvested algal density at SINTEF was 1.5 x10^6 cells mL^{-1})
Our study	100 L	150000 ind L^{-1}	= 75 x 10^4 cells mL^{-1} + 10^4 cells mL^{-1} = 76 x 10^4 cells mL^{-1}	150 x 0.5 x 10^4 cells mL^{-1} = 75 x 10^4 cells mL^{-1}	10^4 cells mL^{-1}	20% of the reduced algae concentration after 24h	$= \frac{76x10^4x100x10^3}{1.5x10^6}$ $= \mathbf{50.66}\boldsymbol{L}$

With the present daily harvested algal density at SINTEF (about 1.5 x10^6 cells mL^{-1}), the expected volume of live algae needed to supply for 4 cultivation tanks in the present study was **202.64 L (4 x 50.66 L)**. The total incoming water supplying cRAS or mRAS was **212.64 L** (10L for stabilizing salinity and 202.64L of algae), corresponding to **16.67 % of 1275L** (total volume of cRAS or mRAS). However, Timmons and Ebeling (2007) suggested that this amount should be under 10% of the total water volume per day (corresponding to less than **127.5 L** for mRAS or cRAS**)**. Therefore, if the algal density was not increased and the volume of algae supplying cRAS or mRAS was **212.64 L**, it would make the recirculation rate of the cRAS or mRAS decrease significantly (based on the equation **2.2**). A trial test to increase density of present live algae was applied by using sedimentation or centrifugation to concentrate *R. baltica.* However, this test was not successful, so a new procedure (**Appendix 3)** to increase the density of live algae was applied to supply enough food for copepods to maintain maximum growth rate.

Appendix 3: Procedure for cultivating and increasing *Rhodomonas baltica* density for the experiment.

Seawater was sterilized with sodium hypochlorite (25 mL, 10-15% NaOCl/100 L seawater) for 8-12 hours and then neutralized with sodium thiosulphate pentahydrate (3.2 g $Na_2S_2O_3*5H_2O$/25 mL NaOCl) for at least 1 hour under heavy aeration before using for algae cultivation (Hagemann, 2011)

Nine transparent polycarbonate cylinders (40 cm in diameter) with volume of 160 L or 200 L and two transparent plastic bags (300 L each) were used to cultivate *Rhodomonas baltica* at 20^0C. Each container received aeration and was illuminated continuously by six fluorescent tubes (GE Polylux XL 830 F58W) from three sides. The aeration system was added CO_2 to ensure maximum algae growth and stabilize pH for the culture within the range 7.5 – 8.7 (Vu Thi Thuy, 2011).

The production algae was begun by adding 20 L of algae at density of 2.5 – 3 x 10^6 cells mL^{-1} from an intermediate culture keeping in 10 L round lab glass flask with sterilized seawater added Conwy medium (1mL/L seawater) (Walne, 1979). The new sterilized seawater from 2 reservoirs was pumped into the cylinder contained 20 L of algae with 50% dilution every day together with adding Conwy medium (1mL/L new seawater) until reaching density 1.4 – 1.5 x 10^6 cells mL^{-1} in the cylinder 160 L or 200L . 50% algae volume of this cylinder was then transferred to next cylinder and then filling 50% remaining volume by new sterilized seawater plus with Conwy medium (1mL/L new seawater) and continuing this procedure to other cylinders and plastic bag until all of the cylinders and plastic bags were full with algae at density of 1.4 – 1.5 x 10^6 cells mL^{-1}. At this time, the new procedure to increase the algae density for feeding copepods was started. The 9 cylinders 160 L or 200 L, marked as A, B, C, D, E, F, G, I, H, were selected for the trial of increasing algae density in 5 days before starting experiment while the algae production in two plastic bags used for another copepod egg production at SINTEF with 50% dilution every day. Instead of using 50% dilution every day, the new different dilutions were used to increase lived algae density without using centrifugation or sediment method.

According to **Table** below, the dilution was 0% on 5 days before starting experiment, and then 5% total volume of algae at the bottom of cylinder was removed and replace by 5% of new sterilized seawater on the next day since the dead algae was sank down to the bottom and disintegrated into toxic gas or into organic particles which can be the feed source for opportunistic bacteria growth. The two cylinders (160 L and 200L), which was then selected

to harvest 100% algae volume, washed with a brush submerging in a sodium hypochlorite (NaOCl) and cleaned with high pressure hot fresh water before being received 50% splitting volume from another two cylinders (160 L and 200L) respectively. The two cylinders (160 L and 200L) which were selected to split 50% of their algae volume to another two 100% - harvested cylinders (160 L and 200L), was marked as S and **S** in the **Table** respectively. The highlighted row in the **Table** was the days that only 1 cylinder (200L) was selected to harvest 100% algae volume while in other days there were usually 2 cylinders (160 L and 200L) was selected to harvest 100% algae volume. With the procedure below **(Table),** the algae density in each cylinder at 5 days before starting experiment was 1.4 – 1.5 x 10^6 cells mL^{-1} and after 1 day without harvesting, its density increased to 1.9 – 2.2 x 10^6 cells mL^{-1} and then increased to 2.7 – 3.1 x 10^6 cells mL^{-1} after 2 day without harvesting. The key of this culture was 5% of total algae volume at the bottom that was removed and replaced with the same volume of new sterilized seawater in the cylinders without harvesting every day. The usual dose of Conwy medium (1mL/L new seawater) for 50% dilution was changed by using the new dose 100 mL per 160 L algae and 140 mL per 200 L algae for the cylinders with 5% dilution. The culture was kept during 20 days before renewal from intermediate culture.

Table: Procedure for increasing *Rhodomonas baltica* density from 1.4 – 1.5 x 10^6 cells mL^{-1} to 2.24 -3.1x 10^6 cells mL^{-1} with different dilutions (5%, 25%, 100% (harvest all))

	A(200L)	B(200L)	C(200L)	D(160L)	E(160L)	F(200L)	G(200L)	I(160)	H(160L)
5 days before	0%	0%	0%	0%	0%	0%	0%	0%	0%
4 days before	5%	5%	5%	5%	5%	5%	5%	5%	5%
3 days before	**100%**	**S**	5%	**100%**	**S**	5%	5%	5%	5%
2 days before	5%	5%	**100%**	5%	5%	**S**	5%	**S**	**100%**
1 day before	**S**	5%	5%	5%	5%	5%	**100%**	5%	5%
Day 0	5%	**100%**	**S**	**S**	**100%**	5%	5%	5%	5%
Day 1	5%	**S**	5%	5%	5%	**100%**	5%	**100%**	**S**
Day 2	**S**	5%	5%	5%	5%	5%	**100%**	5%	5%
Day 3	5%	5%	**25%**	**100%**	**S**	5%	5%	5%	5%
Day 4	5%	**S**	**100%**	5%	5%	5%	5%	**S**	**100%**
Day 5	**100%**	5%	5%	5%	5%	5%	**S**	5%	5%

Day 6	5%	5%	S	S	100%	100%	5%	5%	5%
Day 7	S	100%	5%	5%	5%	5%	5%	100%	S
Day 8	5%	5%	5%	5%	5%	S	100%	5%	5%
Day 9	5%	S	100%	100%	S	5%	5%	5%	5%
Day 10	100%	5%	5%	5%	5%	5%	S	S	100%
Day 11	5%	5%	S	5%	5%	100%	5%	5%	5%
Day 12	S	100%	5%	S	100%	5%	5%	5%	5%
Day 13	5%	5%	5%	5%	5%	S	100%	100%	S
Day 14	5%	S	100%	5%	5%	5%	5%	5%	5%

Appendix 4: The details of the method for determination of egg production in the second part of the experiment.

After 24 hour incubation, live copepods in each beaker were gently transferred with a plastic spoon into a plastic cup before the beaker (including seawater and sediment) was emptied into a sieve (50 µm) (**Picture**). This beaker was washed with freshwater, and then filled with 1L new seawater and a known volume of algae before supplied with light aeration. The live copepods in the plastic cup were counted and then transferred into the beaker. Dead copepods, eggs and newly hatched nauplii were transferred to a Petri dish (**Picture**) and counted under stereomicroscope.

Picture: Equipment for transferring copepod adults and collecting eggs and newly hatched nauplii. Photo: Anh Phan Hung, 2014.

Appendix 5: Total ammonia nitrogen (TAN) in rearing tanks and inlet water of the three systems. Different superscripts at a row denote significant differences between treatments.

TAN in rearing tanks (mg/L)	Mean ±SE		
	cRAS	mRAS	FTAS
Day 0	0	0.0400	0
Day 3	0.0367±0.0267[a]	0.0767±0.0167[a]	0.0667±0.0260[a]
Day 7	0.3133±0.0338[a]	0.0533±0.0203[b]	0.0767±0.0470[a]
Day 11	0.1633±0.0371[a]	0.1567±0.0367[a]	0.1933±0.0167[a]
Day 14	0.3167±0.0570[a]	0.1800±0.0208[a]	0.1833±0.0240[a]

TAN in inlet water (mg/L)	Mean ±SE			
	cRAS	mRAS	FTAS	Permeate
Day 0	0	0.0400	0	0
Day 3	0.0133±0.0133[a]	0.0067±0.0067[a]	0.0067±0.0033[a]	0.0150±0.0050
Day 7	0.3767±0.0233[a]	0.0900±0.0666[b]	0.0433±0.0219[a]	0.0450±0.0150
Day 11	0.0700±0.0100[a]	0.0100±0.0100[a]	0[a]	0.0050±0.0050
Day 14	0.0567±0.0145[a]	0.0267±0.0145[a]	0.0367±0.0367[a]	0.0300±0.0100

Appendix 6: Percentage (%) of opportunistic bacteria in the rearing tanks and in the inlet water of the three systems at sampling days during the NP experiment. Different superscripts at a row denote significant differences between treatments

Percentage (%) of opportunistic bacteria in the rearing tanks	**Mean ±SE**		
	cRAS	**mRAS**	**FTAS**
Day 0	**49.22**	**29.66**	**16.82**
Day 3	**67.31±4.72** [a]	**42.68±7.73** [a]	**53.67±6.55** [a]
Day 6	**51.30±7.76** [a]	**85.83±2.15** [b]	**29.71±8.42** [a]
Day 12	**79.31±0.78** [a]	**48.42±7.63** [a]	**49.78±11.75** [a]

Percentage (%) of opportunistic bacteria in the inlet water	**Mean ±SE**			
	cRAS	**mRAS**	**FTAS**	**Permeate**
Day 0	**53.82**	**61.15**	**0.00**	**42.62**
Day 3	**51.18±2.60** [a]	**31.38±2.95** [b]	**2.63±1.73** [c]	**47.73±1.86**
Day 6	**21.76±1.61** [a]	**72.60±2.95** [b]	**3.79±2.44** [c]	**53.64±0.49**
Day 12	**70.33±1.62** [a]	**52.91±0.45** [b]	**0.00±0.00** [c]	**17.01±6.25**

Appendix 7: The total number of bacteria in the rearing tanks and in the inlet water of the three systems at sampling days during the NP experiment. Different superscripts at a row denote significant differences between treatments.

The total number of bacteria in the rearing tanks (million mL^{-1})	**Mean ±SE**		
	cRAS	**mRAS**	**FTAS**
Day 0	**1.43**	**0.43**	**0.17**
Day 3	**1.73±0.08^{a}**	**1.80±0.23^{a}**	**2.59±0.56^{a}**
Day 6	**0.82±0.11^{a}**	**1.45±0.06^{b}**	**1.72±0.19^{b}**
Day 12	**1.68±0.18^{a}**	**1.14±0.13^{a}**	**1.08±0.14^{a}**
Day 14	**2.53±0.49^{a}**	**1.93±0.70^{a}**	**2.64±0.51^{a}**

The total number of bacteria in the inlet water (million mL^{-1})	**Mean ±SE**			
	cRAS	**mRAS**	**FTAS**	**Permeate**
Day 0	**0.44**	**0.36**	**0.12**	**0.18±0.01**
Day 3	**0.39±0.05^{a}**	**0.78±0.03^{b}**	**0.29±0.16^{a}**	**0.23±0.05**
Day 6	**0.33±0.01^{a}**	**0.70±0.05^{b}**	**0.14±0.01^{c}**	**0.10±0.04**
Day 12	**0.41±0.07^{a}**	**0.29±0.02^{a}**	**0.05±0.01^{c}**	**0.10±0.04**
Day 14	**0.38±0.01^{a}**	**0.77±0.29^{a}**	**0.27±0.06^{a}**	**0.13±0.06**

Appendix 8: Density and survival of *Acartia tonsa* of the three systems during the NP experiment. Different superscripts at a row denote significant differences between treatments.

Survival rate of *Acartia tonsa* (%)	**Mean ±SE**		
	cRAS	**mRAS**	**FTAS**
Day 0	**100**	**100**	**100**
Day 1	**80.01±6.95^{a}**	**71.67±9.18^{a}**	**80.34±7.95^{a}**
Day 7	**40.68±10.70^{a}**	**44.35±1.48^{a}**	**48.01±8.45^{a}**
Day 11	**30.34±6.41^{bc}**	**19.34±3.01^{b}**	**42.34±5.92^{c}**
Day 14	**17.34±5.37^{a}**	**11.34±2.80^{a}**	**41,34±9.09^{c}**

Density of *Acartia tonsa* (individuals L^{-1})	**Mean ±SE**		
	cRAS	**mRAS**	**FTAS**
Day 0	**75000**	**75000**	**75000**
Day 1	**60006±5212^{a}**	**53754±6884^{a}**	**60255±5964^{a}**
Day 7	**30510±8027^{a}**	**33259±1109^{a}**	**36009±6340^{a}**
Day 11	**22755±4803^{bc}**	**14504±2256^{b}**	**31757±4443^{c}**
Day 14	**13004±4030^{a}**	**8505±2097^{a}**	**31005±6821^{c}**

Appendix 9: Specific growth rate (SGR day^{-1}) and of *Acartia tonsa* of the three systems during the NP experiment. Different superscripts at a row denote significant differences between treatments.

	Mean ±SE		
Specific Growth Rate (SGR day^{-1})	**cRAS**	**mRAS**	**FTAS**
Nauplii (Day 0 – Day 7)	**0.321±0.008** [a]	**0.307±0.009** [a]	**0.298±0.011** [a]
Copepodites (Day 7 – Day 14)	**0.199±0.006** [b]	**0.177±0.006** [b]	**0.188±0.013** [b]
Total (Day 0 – Day 14)	**0.283±0.007** [c]	**0.268±0.006** [c]	**0.294±0.006** [c]

Appendix 10: Egg Production in three systems during the EP experiment (Mean ± SE). Different superscripts at a row denote significant differences between treatments.

	Mean ± SE		
Sampling days	**cRAS**	**mRAS**	**FTAS**
7	12.92±1.54 [a]	11.22±1.81 [a]	20.81±1.07 [c]
8	9.55±0.96 [a]	12.55±2.08 [a]	23.40±1.48 [c]
9	9.32±0.83 [a]	11.60±2.41 [a]	23.29±3.78 [c]
10	9.58±1.43 [a]	13.62±2.82 [ac]	19.95±2.44 [c]
11	9.24±1.19 [a]	12.62±3.15 [a]	24.04±1.95 [c]
12	9.10±2.50 [a]	11.72±3.74 [a]	25.97±1.27 [c]
13	7.87±1.39 [a]	8.89±2.61 [a]	31.57±2.20 [c]
14	10.90±2.50 [a]	9.19±2.46 [a]	31.96±2.43 [c]

Appendix 11: Survival rate (%) of *A. tonsa* in three systems during the EP experiment (Mean±SE) and fraction (%) males or females of survival rate in each system. Different superscripts at a row in highlighted columns denote significant differences between treatments.

Sampling day	cRAS			mRAS			FTAS		
	%survival	%male of survival	%female of survival	%survival	%male of survival	%female of survival	%survival	%male of survival	%female of survival
7	100	1.8	98.2	100	2.4	97.6	100	1.4	98.6
8	92.1±2.2^{a}	1.9	98.1	87.3±3.6^{a}	1.4	98.6	97.4±1.8^{a}	0.7	99.3
9	82.6±2.3^{a}	2.1	97.9	68.3±5.8^{b}	1.7	98.3	85.2±5.8^{a}	0.8	99.2
10	73.2±4.0^{ab}	2.4	97.6	58.6±5.9^{b}	2.0	98.0	82.7±5.8^{a}	0.8	99.2
11	64.7±6.2^{ab}	2.7	97.3	48.4±5.1^{b}	2.5	97.5	76.0±5.7^{a}	0.0	100
12	49.4±8.7^{b}	3.4	96.6	44.5±3.7^{b}	2.8	97.2	72.5±5.8^{a}	0.0	100
13	38.7±7.1^{b}	4.3	95.7	34.7±4.7^{b}	3.7	96.3	66.5±6.3^{a}	0.0	100
14	31.6±7.3^{b}	0.0	100	30.9±5.3^{b}	0	100	64.1±5.9^{a}	0.0	100

Appendix 12: Data of %Opportunistic bacteria and Survival rate (%) in the NP experiment. The tanks (A) represent for the tanks that had above 50% opportunistic bacteria; the tanks (B) represent for the tanks that had lower 50% opportunistic bacteria.

Abbreviation:

%Op: percentage (%) of opportunistic bacteria

%Sur: Survival rate

CFU S. : CFU sampling days

Sur S. : Survival sampling days

T: grouped tank (A or B)

cRAS					mRAS					FTAS				
T	**%Op**	**CFU S.**	**%Sur**	**Sur S.**	**T**	**%Op**	**CFU S.**	**%Sur**	**Sur S.**	**T**	**%Op**	**CFU S.**	**%Sur**	**Sur S.**
B	**48.19**	0	**65.34**	1	B	**33.99**	0	**69.34**	1	B	**21.05**	0	**89.34**	1
B	**47.77**	0	**98.68**	1	B	**26.96**	0	**77.34**	1	B	**0.00**	0	**94.67**	1
A	**51.70**	0	**76.01**	1	B	**28.03**	0	**92.00**	1	B	**29.41**	0	**58.67**	1
A	**73.52**	3	**17.35**	7	B	**29.78**	3	**41.35**	7	B	**47.58**	3	**28.01**	7
A	**58.04**	3	**48.01**	7	A	**56.51**	3	**48.01**	7	B	**46.67**	3	**69.35**	7
A	**70.37**	3	**30.68**	7	B	**41.74**	3	**45.35**	7	A	**66.75**	3	**48.01**	7
B	**38.37**	6	**14.67**	11	A	**90.10**	6	**12.00**	11	B	**33.97**	6	**28.00**	11
A	**50.35**	6	**38.67**	11	A	**83.24**	6	**18.67**	11	B	**41.69**	6	**50.67**	11
A	**65.19**	6	**25.34**	11	A	**84.14**	6	**26.68**	11	B	**13.48**	6	**37.35**	11
A	**78.01**	12	**10.67**	14	A	**60.58**	12	**2.67**	14	A	**73.27**	12	**25.34**	14
A	**80.70**	12	**29.34**	14	B	**34.34**	12	**10.67**	14	B	**37.62**	12	**62.68**	14
A	**79.21**	12	**6.67**	14	A	**50.35**	12	**12.00**	14	B	**38.44**	12	**29.34**	14

The survival rate (%) according to the two grouped tanks (**A**) and **(B**) of the three systems at all sampling days was combined and analyzed for significant differences ($p < 0.05$) (t-test). Different superscripts at the last row denote significant differences between the two groups.

	A (n=17)	**B (n=19)**
	76.01	65.34
	17.35	98.68
	48.01	**14.67**
	30.68	69.34
	38.67	77.34
	25.34	92.00
	10.67	41.35
	29.34	45.35
	6.67	10.67
	48.01	89.34
	12.00	94.67
	18.67	58.67
	26.68	28.01
	2.67	69.35
	12.00	28.00
	48.01	50.67
	25.34	37.35
		62.68
		29.34
Mean±SE	**28.007±4.599^{a}**	**55.939±6.265^{b}**