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Trondheim, August 2021

Xingyu Li

ABSTRACT

Elevation in water temperature due to the climate change is considered one of the major factors to influence the microalgae and zooplankton community. The copepod genus *Calanus* is ecologically important as an energy bridge connecting lower to higher trophic level. However, physiological activities of *Calanus* may be affected due to the climate change. Increased water temperature also causes eutrophication which stimulates excessive growth of algae with variable nutritional quality. This, in turn, may influence *Calanus* as it grazes upon microalgae. The aim of this study was therefore to investigate the effect of increased temperature along with decreased food quality on *Calanus* with respect to phosphorus. The North Atlantic species *Calanus finmarchicus* was reared at two elevated temperature regimes, 12°C and 14°C and fed phosphorous-limited microalgal phytoplankton *Rhodmonas baltica*. Phosphorus limitation on microalgae was induced by reducing NaH_2PO_4 of the original Conwy medium (100%) to 50% and 12.5 %. Cellular carbon, nitrogen, phosphorus analysis of microalgae showed that the P level in algal cells decreased with decreasing medium P level, while carbon and nitrogen content were lowest with moderate P limitation. Fatty acid composition in the algae showed significant differences where strong P limitation reduced the eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) content. Adult *C. finmarchicus* reared at 14°C were generally smaller in prosomal volume than those at 12°C. Neither temperature nor food quality significantly affected lipid storage of copepods. Fatty acid composition of *C. finmarchicus* was analyzed. Major differences were found in the long chain fatty acids 22:1n-11, which were generally more abundant at 14°C, while the level of EPA and DHA were higher at 12°C. Food quality did not seem to have a clear effect on the fatty acid composition. Egg production rate of copepods was reduced at 14°C which is likely due to the lower content of the essential fatty acids EPA and DHA which are needed for egg production. Hatching success of copepod eggs in the present study was affected by temperature but not food quality. Egg size did however increase with increasing temperature. Surprisingly, egg size was negatively correlated to the egg production rate in the present study. Overall, temperature seems to have a strong effect on *C. finmarchicus* rather than food quality, and no additive effect was observed in this study.

KEY WORDS: *Calanus finamrchicus*; *Rhodomonas baltica*; Temperature; Phosphorus limitation; Reproduction; Lipid; Fatty acids

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1. Introduction

The Arctic is characterized by its cold temperature, seasonality in production, low biodiversity, and therefore, simple food chain structures are common. It is not surprising that species residing in the Arctic area rely on lipid depots to survive the cold winter with its minimal sunlight and food production. Marine zooplankton species residing in the Arctic also relies on the lipid reserve for many activities such as overwintering, development, and egg production (Lee et al., 2006). However, it will become more challenging due to the global climate change. Rising ocean temperatures may increase metabolic rate which could lead to lower capacity to deposit sufficient lipid to survive winter or that lipid stores are exhausted before spring bloom (Schulte P, 2011; Schulte P, 2015; Seebacher et al., 2015; Somero G, 2011). In addition, the frequent and intense elevation of the temperature may threaten the organisms at an even faster rate if the organisms are already in stressful conditions such as lack of food (Somero, 2010; Tewksbury *et al*., 2008). As algal populations are also temperature sensitive, such scenarios would also lead to alteration in algal communities with lower suitability as feed organisms (Yong et al., 2016). Elevated water temperatures may therefore change community structure by disturbing fitness of certain species, threatening individual survival, causing population bottlenecks, and thus loss of gene variety (Chew et al., 2015). The law of conduction suggests that heat sensitivity and body size are negatively correlated hence the smaller the body size, the faster the changes in body temperature and therefore more sensitive to environmental temperature (Beaugrand, 2015). This suggests smaller organisms such as zooplankton are more exposed to temperature change, elevated temperature may affect the physiological activity of small organisms to a significant extent. Therefore, zooplankton are in the interest as they can be at serious risk due to their small body size (Pörtner and Knust, 2007).

1.1 Copepod *Calanus finmarchicus*

Copepods, the most abundant zooplankton on Earth (Grieve et al., 2017), are common in a variety of habitats ranging from moist soils to lakes and open oceans (Craig et al., 2001). Planktonic copepods play a major role in aquatic trophic chains as a major source of food for both larger invertebrates and vertebrates (Craig et al., 2001). The copepod genus *Calanus* is an abundant genus in the North Atlantic Ocean, and it significantly contributes to the total zooplankton biomass. *Calanus* graze upon phytoplankton and are preyed upon by larger species such as fish larvae, seabirds and marine mammals (Wold et al., 2011). Therefore, *Calanus* is regarded as key species since they represent an energy bridge connecting lower trophic levels to higher trophic levels (Runge, 1988; Falk-Petersen, 2009).

The North Atlantic species *Calanus finmarchicus* is the most abundant species in the North Sea, Norwegian Sea and adjacent fjords (Planque and Batten, 2000). The life cycle of *C. finmarchicus* (Figure 1.1) includes six naupliar stages (N1-N6) followed by six copepodite stages (C1-C6) after egg hatching. The final copepodite stage is the adult stage (Miller and Tande, 1993). The late copepodite stages (C3-C5) synthesize and accumulate large amounts of wax esters from carbohydrate, protein and fatty acid constituents from phytoplankton diet, and this accumulation is found to be higher in the copepods in deep or cold water (Sargent et al., 1978). The C5 stage of *C. finmarchicus* undergo a vertical migration from sea surface to deep water where they enter a dormancy state called diapause (Hirche, 1996). During mid-winter they exit diapause and molt to the adult stage. Females will then ascend to surface waters to spawn (Health, 1999; Hirche, 1996). The reproduction of *C. finmarchicus* is highly dependent on food supply to maintain spawning, and thus peak egg production happens during or right after phytoplankton blooms (Kjellerup et al., 2012).

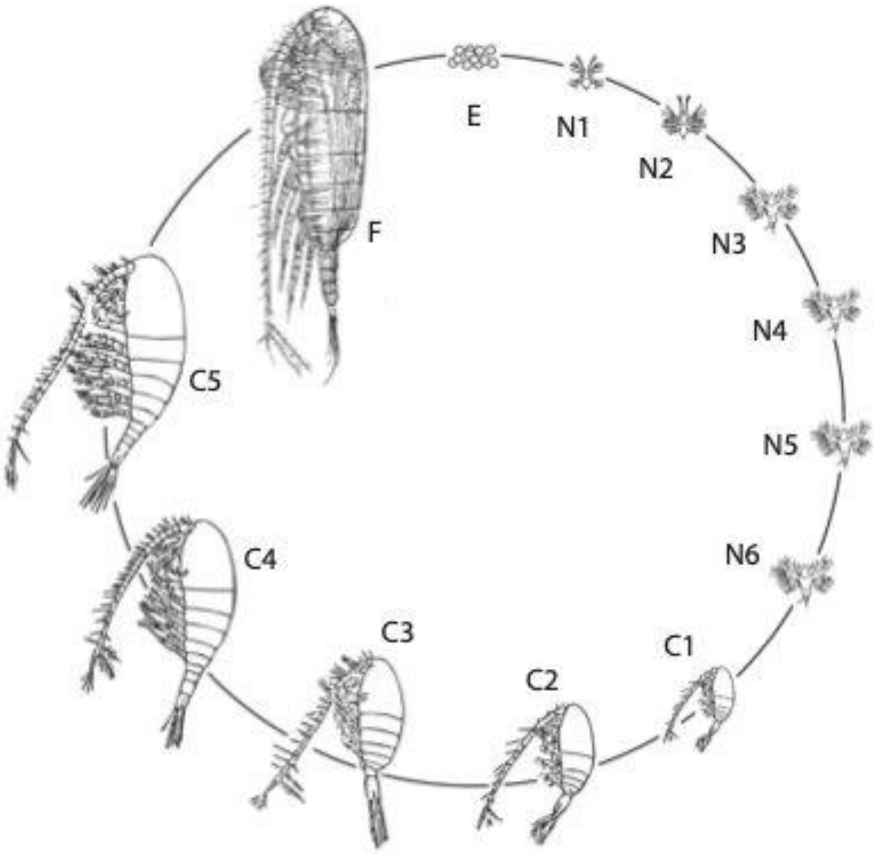
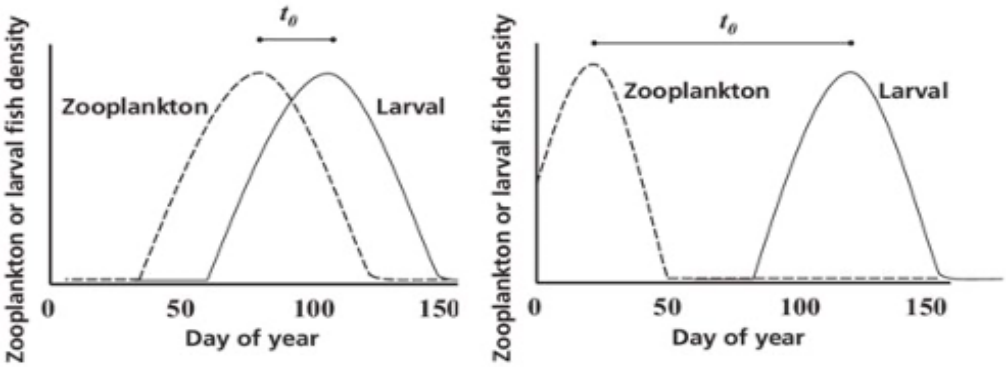


Figure 1.1 Life cycle of *Calanus finmarchicus*. Developing from egg stage (E), six naupliar stages (N1-N6), and six copepodite stages (C1-C6), while C6 stage is the adult stage distinguished as male and female (Baumgartner, 2009).

1.2 Temperature effects on *C. finmarchicus*

As mentioned before, temperature is one major factor affecting organisms especially ectothermic marine organisms that have a relatively narrow tolerance range to temperature change (Beaugrand, 2015). Zooplankton is poikilothermic and varies its body temperature within the range of variations in the environmental temperature, and therefore variations in the ambient temperature can significantly alter physiological processes such as growth and reproduction. Increased temperature due to the natural and anthropogenic influences, may deplete energy stores at a much higher rate as a consequence of the increased metabolic rates. This may lead to failure to survive diapause. Additionally, the findings from a recent paper by Kvile et al (2014) shows that elevated temperature might cause peak *C. finmarchicus* copepodites occurs earlier due to the faster individual growth rates in combination with faster development rates, and thus affecting abundance and seasonal timing of young copepodites

of *C. finmarchicus*. These changes in turn may influence predators (e.g., Northeast Arctic cod larvae) feeding on these life stages due to the mismatch in abundance timing (Figure 1.2), and therefore it is highly likely to impose an impact on higher trophic levels due to the mismatch of predators with their prey (Huey and Berrigan, 2001; Kvile et al., 2014). Besides the possible mismatch between *C. finmarchicus* and higher trophic species, mismatch with microalgae may also occur because of the earlier abundance than algal blooms. Rising temperature in the ocean is therefore believed to have a real potential to interfere and restructure marine ecosystems (Kvile et al., 2014). It is critical to study the effect of global climate change though this is still to be elucidated.



Sources: Barange & Perry, 2009

Figure 1.2 Match/mismatch hypothesis from Cushing's theory. On the left is the match relationship between zooplankton prey and fish larval abundance timing, and the right is the mismatch between prey and predator. t_0 is the separation time between two peaks.

1.3 Nutrient limitation

Microalgae are mostly unicellular organisms that reside in the worldwide aquatic system (Francisca et al., 2019). They are primary producers via photosynthetic carbon fixation, and using organic and inorganic carbon, inorganic nitrogen and phosphorus for their growth while excreting oxygen (Latsos et al., 2020). Therefore, microalgae can be regarded as the true lung of the earth. Nevertheless, the current shifts of climate change by anthropogenic activities have the potential to pose various impacts on microalgal communities (Yong et al., 2016). Increased temperature on land may change the rainfall patterns, and nutrients from land (mainly nitrogen and phosphorus) could be washed out and flow into the waterbodies due to the increased precipitation (Yong et al., 2016). This, eutrophication, may stimulate algal growth. However, excessively dense microalgae in the waterbody may deplete the

surrounding nutrients at a faster rate, and thus causing microalgae to have poor quality for their upper trophic level species. Many studies have already studied nutrient deficiency effect such as nitrogen limitation in growth medium (Reitan et al., 1994; Yaakob et al., 2021). Nevertheless, more research is required to fully understand the effect of nutrients limitation on the natural microalgae community.

The microalgae of the genus *Rhodomonas* sp. is often regarded as an excellent food source for calanoid copepods due to its abundant content of the essential fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Latsos et al., 2020). However, in a previous study it was found that phosphorus limitation reduced the content of EPA and DHA (Reitan et al., 1994). This could affect the reproduction of *C. finmarchicus* fed P limited *R. baltica*, as they require EPA and DHA for their egg production (Bergvik et al., 2012; Evjemo et al., 2008).

1.4 Aim of the study

Increased water temperature due to climate change may affect physiological processes of *C. finmarchicus*. Whilst more often precipitation on land because of the increased temperature could cause eutrophication, and this may affect the nutritional status of algal community. It is highly likely that wild *C. finmarchicus* may experience more stressful conditions than expected, as algal food with poor quality may not fulfill the demand of *C. finmarchicus* with more energy budget due to the elevation in temperature.

How will elevated temperature in combination with decreased food quality can influence the fitness of *C. finmarchicus*? To answer this question, the objective of the present study is therefore to firstly assess chemical contents and fatty acids composition of *R. baltica* cultivated under different extent of phosphorus limitation to evaluate the effect of nutrients deficiency on microalgae. Development and reproduction of *C. finmarchicus* were then assessed when fed on p-limited *R. baltica* while reared at elevated temperature regimes. In the present study, the cultivation of *C. finmarchicus* was carried out at two temperatures,

12°C and 14°C. Results are then intended to be used for elucidating possible effects of climate change and changes in nutrient composition for the feed algae.

The following research questions were defined in the present study:

1) Does phosphorus limitation affect the cellular contents, as well as fatty acids composition of microalgae *R. baltica*?
2) Does temperature or food quality affect development and reproduction of *C. finmarchicus*?
3) Can food quality be an additive effect to temperature? If so, to what extent does temperature along with food quality affect *C. finmarchicus?*

2. MATERIAL AND METHOD

2.1 Experimental design and set-up

The experiment was conducted at NTNU Centre of Fisheries and Aquaculture (SeaLab) in January 2021. Copepods that had been used for the experiments were from the continuous in-house culture. For the feeding of copepods in the experiment, unicellular algae *Rhodomonas baltica* were cultivated at Sealab.

The experiment was designed to have two factors; one is temperature and another one is the food quality. The later one was determined by microalgal species *R. baltica* that had been cultivated under three different phosphorus levels by changing the concentrations of NaH_2PO_4 whilst increasing the amount of $NaNO_3$ with 25% manipulating the N:P ratio in the Conwy medium (Walne, 1996). These algae were then used as food sources to feed the copepod *C. finmarchicus* reared at two different temperatures, 12°C and 14°C (Figure 2.1).

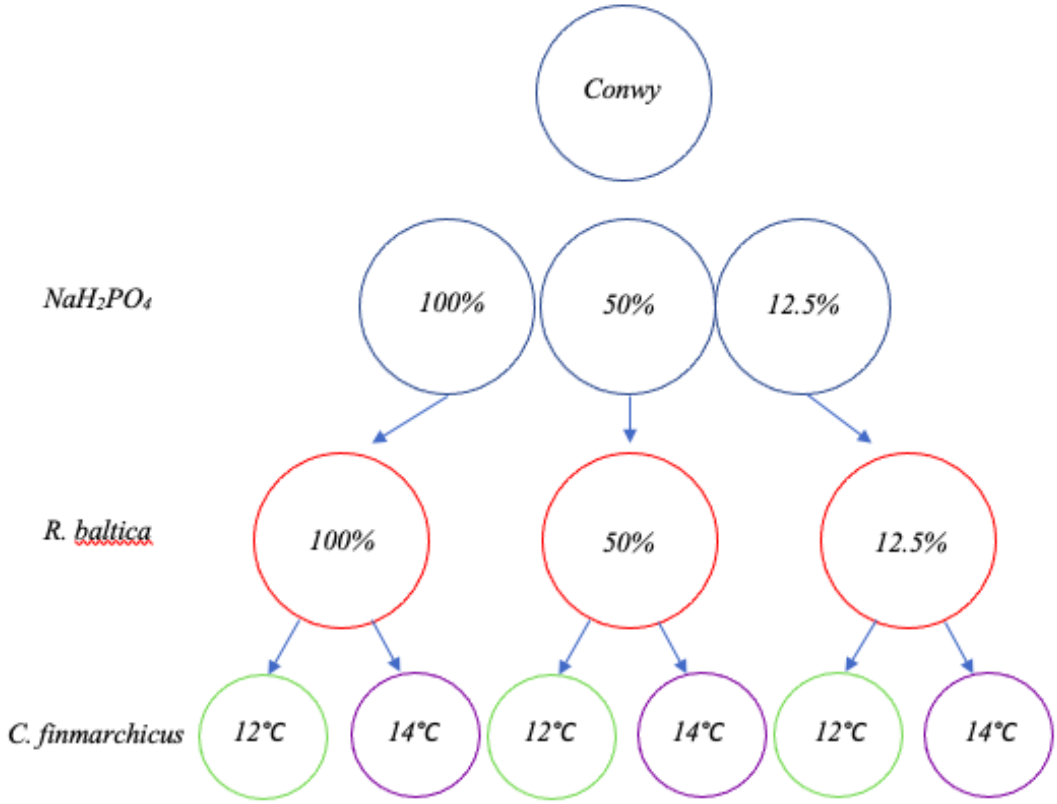


Figure 2.1 Explanatory depiction of the experimental variables. Different levels of NaH_2PO_4 indicating the modification in phosphorus level of Conwy medium.

2.2 Cultivation of the microalgae *R. baltica*

The unicellular algae species *Rhodomonas baltica* were cultivated and used as diet for the copepods. *R. baltica* was grown in round 10L flasks with flat bottom at 20 ± 2°C and horizontally illuminated with 3 fluorescence light tubes (Philips, TLD 36W/965). The algae cultures were aerated with compressed air added CO_2 to a concentration of 2300 ± 100 ppm to prevent the aggregation of cells at the bottom, and meantime to sustain growth for their cultures. By use of modified Conwy medium (Walne 1966) in order to obtain a gradient in phosphorous limitation (Figure 2.1). *R. baltica* were cultivated in Conwy medium that contains 100%, 50% or 12.5% phosphorus level of its original recipe (See appendix) to assess the effect of non, moderate, and strong phosphorus limitation on *R. baltica*. Algae were grown for one week to test the carrying capacities of the cultures, and the growth rates were obtained (See appendix). The microalgae cultures showed good growth during the cultivation period and use of medium with the least P level still produced cells in excess for feeding the cultures of *C. finmarchicus* (See appendix). Therefore, all experimental microalgae groups (100%, 50%, and 12.5% of P) were used in the experiment. These modified Conwy mediums were made by co-supervisor Dag Altin at NTNU Sealab.

Throughout the rearing of *C. finmarchicus*, the diet microalga groups of *R. baltica* were harvested every week for lipid analyses. Approximately 180ml of the culture were harvested from each flask and centrifuged, algae were then stored in the -80°C bio-freezer waiting for the further steps.

Algae were also harvested for cellular carbon, nitrogen, and phosphorus analysis. 50 ml of the cultures was sampled and filtered by using vacuum filtration flask (Figure 2.2). Filters with concentrated algae were then stored in the -20°C freezer for further analyses.

Figure 2.2 Vacuum filtration flask from TBS (Trondheim Biological station); Beaker with a magnet at the bottom for containing algae sample (A). Plug with magnet at the top, filter was placed and set at green part by two magnets (B). Flask for the water (C). Vacuum tube connected to water tap and flask (D). Photos by student Ane Torgersen Selnes at Sealab.

2.3 Cultivation of *C. finmarchicus*

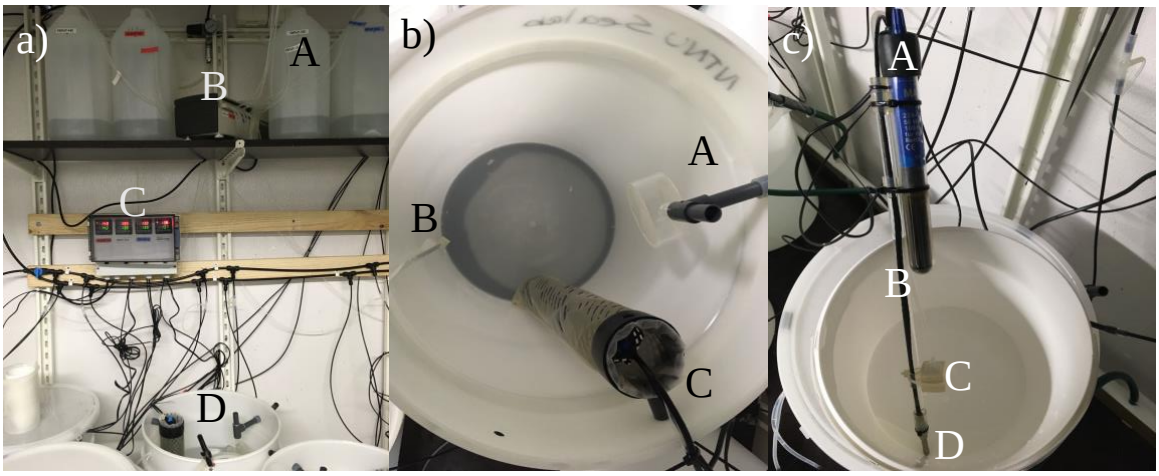


Figure 2.3 Illustration of experimental set-up. a) represents an overview of the set-up, while A is the microalgal feeding stock culture, B is the pumping unit, C is the heating unit regulator and D is the experimental tank that contains copepods. b) represents an overview in the tank with heating system, A is the outlet tubes with bongo mesh, B is the feeding tubes with diffuser at the tip, C is the heating unit that is immersed in the water and surrounded by a sieve. c) is a shot for detailed information of heating unit, A is the heating unit itself connect to the regulator, B is the glass that contains heating sensor in it to prevent sensor from being floating, C is the tubes with diffuser at the tip to produce smooth air bubbles for the aeration, D is the sensor to measure the water temperature in the experimental tanks.

C. finmarchicus were grown at two different temperatures 12°C and 14°C, fed the unicellular alga *R. baltica* that was grown in Conwy medium with three different levels of phosphorus (see 2.1 & 2.2). The main rearing of *C. finmarchicus* was carried out in a climate room where the temperature was manually regulated to generate 12°C in tanks. Each tank contained 45L of filtered seawater, tubes for feeding, and aeration to prevent uneven distribution of algae. Whereas in the tanks at 14°C, besides the aeration and feeding system mentioned above, a heating system (Aquarium Heaters, 50W) connected to a control unit (Auber, SYL-2372) was applied to regulate the temperature in the tanks. The heating unit was immersed in the water and surrounded by a fine sieve to prevent copepods from entering. Along with the heating unit, a sensor (PT100) was also applied to control the water temperature. Moreover, another aeration tube was applied to gently mix the water inside to avoid excessively hot water being concentrated near the heating unit which may cause temperature gradients in the tanks and potentially increase the mortality. Whereas the feeding stock was connected to the two different pumps, one for pumping out the algae into the experimental tank to feed copepods, another one for aeration to mix the algae in the feeding stock to avoid algae being concentrated at the bottom (Figure 2.3-2.4).

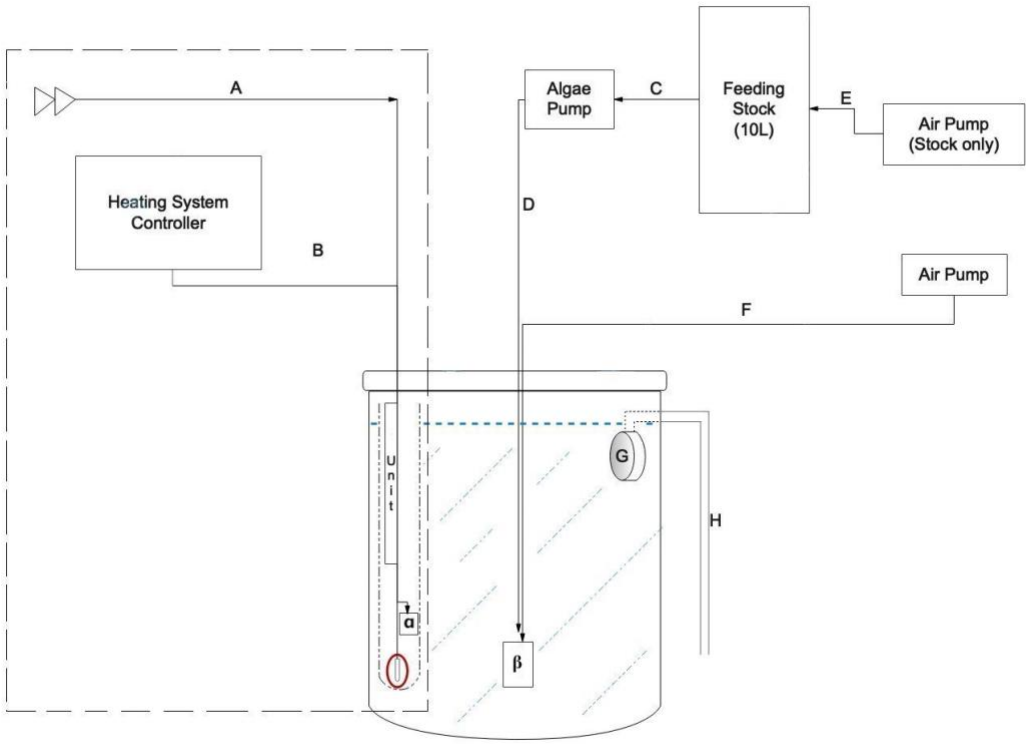


Figure 2.4 A schematic drawing of the experimental design in the tanks at 14°C. α: Diffuser to generate smooth and gentle bubbling air. β: Diffuser to generate smoother air bubbles to distribute algae evenly. A: Aeration tube that connects air valve and α diffuser. B: Electric wires that connect the heating regulator to the heating unit and sensor. C: Tube that connects stock culture to pump. D: Tube that delivers algae into the tank, note that tube is not connected to diffuser. E: Aeration tube that connects to the air pump. F: Tube that connects Air pump and β diffuser. G: 100µm Bongo mesh attached to outlet tube to prevent copepods from entering H: Outlet tube for discarding excessive water. Blue dashed lines indicate water, small tip surrounded by red circle stands for the temperature sensor of heating system and 'Unit' stands for the heating unit that partly immersed in water. Unit, α diffuser and sensor are surrounded by a fine sieve indicated by the denser dashed line. Double arrowheads on the left-top corner indicate an air valve that regulates the airflow, whereas small black arrows show the directions of air and water flow, while the area depicted by dashed line rectangle on the left side indicates the whole heating system.

2.3.1 Feeding of *C. finmarchicus*

The different treatments of algae were cultivated semi-continuously to feed *C. finmarchicus* throughout the experiment. Each treatment had one additional flask as a replicate to allow the feeding being conducted between two flasks, this was especially for providing algae enough time to regrow to the density before the feeding. Nevertheless, culture may still be crashed during the experiment, new cultures were prepared when the ongoing culture started showing pale colour.

For the procedure of daily feeding routine, 3L of stock culture was poured out and refilled again with filtered (5 µm), and dechlorinated seawater. 1.5ml of Conwy media per liter of seawater was also added based on the trial of algae cultivation in which the amount of growth medium added were found to support the growth of algae. The harvested algae were counted on a Coulter Counter (Multisizer^{TM} 3 Coulter Counter, Beckman Coulter Inc., Miami, FL, USA) at 400 dilution rates for measuring the density. Additionally, cell densities in the experimental tanks were also measured daily before preparing the feeding stocks, and the measured numbers from the counter were put on the Excel sheet to calculate the volume of algae needed to reach a set biomass of algae of 200µg C/L for that day (Equation in appendix), Excel sheet was saved daily for tracking and further analysis. Once the volume was obtained, feeding stocks were disconnected temporary from the system and remaining algae from the day before was discarded. Algae from stock culture were then added to the feeding stock according to the volume from Excel sheet and refilled back to 10L by adding filtered seawater, then feeding stocks were connected to the system again and left running for another 24 hours. However, 10L of feeding stock needed more than 25 hours to be completely drained, this was to provide the flexibility to the system and to avoid the feeding stocks to run dry.

2.3.2 Handling of copepods and eggs

When starting the experiment, 300 individuals at stage C3 were counted manually for each tank and transferred into the tanks. Algae were added before copepods to reach 200ug C/L in the tanks at the very beginning so that copepods can immediately graze upon the algae rather than waiting for the food from pumps.

When copepods entered stage C5, 20 individuals were taken from each tank, stage was confirmed by using a dissecting microscope (Leica, M80 + Leica Base TL) and an image of each individual was taken by Dag Altin, by using a computer with software (NIS Elements F v/4.60) connected to a dissecting microscope (Leica, MZ APO) with a camera (DS-FI1/DSU2). Anesthesia (FINQUEL, Argent Chemicals) was applied to stop the movement of copepods during processing. 10 individuals were transferred into the same kimax tube, flushed under N_2 and stored in -80°C bio-freezer immediately for the lipid analysis.

After two weeks from copepods molting into adult, egg production was started and the eggs were collected. The water of the experimental tank (hereby referred to original tank) was transferred to another empty tank by applying a special siphon with a bongo sieve that has fine 100 µm mesh glued at the sides to prevent copepods from being sucked into the tubes and be transferred along with water. Siphon was removed when the level of water in the original tank is approximately 2-3 cm above the bottom. Copepods were concentrated due to the limited space so that copepods can be easily scooped out and be placed in a 300 µm sieve in a small bucket, while the original tank was cleaned and all visible particles on the bottom were removed thoroughly, then both copepods in the sieve and siphoned water free of eggs were carefully transferred back into the original tank. This was to evaluate the egg production only for 24 hours period. One day after, the eggs laid for 24 hours were collected, after lowering the water level, both copepods and water passed through 300µm fine mesh to let eggs go through but capturing the copepods, and eggs were further separated from water by using 125 µm mesh, and finally transferred to the petri dish, images were taken by Dag Altin. Meantime, the number of eggs was also recorded for the further calculation. Eggs were then transferred to well plates (VWR Tissue Culture Plates, 24 wells), each well accommodates up to 25 eggs, each treatment used up to 6 wells, and be placed into hatching chamber for 72 hours at 12°C and 14°C.

After the collection of eggs, all copepods were scooped out with the same procedure mentioned above in which by lowering the water level in the tanks. Copepods were transferred into a tube and filled up with ethanol, kept in the fridge for the counting of survived individuals and sex distribution among them.

2.3.3 Temperature adjustment.

The temperature of water in the experimental tanks was measured every day during the growing session and recorded on an Excel sheet. The temperature, however, was not stable and adjustments were needed occasionally. Climate room temperature was set at 12°C first week, and the temperature was consistently lower in the 12°C treatment tanks. Therefore, the room temperature was set at 13°C due to the heating capacity and heat conductivity of air requires more energy to heat water, and temperature increased and stabilized at 12°C after increasing room temperature to 13°C. While temperature in the tanks at 14°C treatment fluctuated some days, excessively high temperature was recorded. The adjustments were done by adjusting aeration and opening the lid slightly when temperature was excessively high.

2.4. Biometric analysis

Copepods and eggs were observed under microscopic for biometric analysis, and software ImageJ version 1.5.3 with the aid of tablet (Wacom, Intous3 Co., Ltd, Saitama, Japan) was used for measuring the copepods length of prosome, area of lipid sac, and egg diameter based on scaled images. While images for copepods from this study were taken horizontally, additional images were taken vertically by Dag Altin at Sealab, though not the same animal from this study. However, even though copepods vary in the body size and thus volume, they have similar ratio of width to length. Therefore, this is an important measurement as it gives the width of animals, and it is required to calculate the prosomal volume as the volume calculation of copepods can be regarded as the calculation of ellipsoid. Additional copepods of 50 were measured for its width by student Simen Sæther at Sealab, and width to length ratio was obtained. Then the width, and therefore the prosomal volume of copepods from this study can be calculated.

The length measurement was carried out by simply drawing the line across the prosome (Figure 2.5), and the length in mm was obtained by calculating the numbers of pixel that had been calibrated by using the standard to correlate the numbers of pixel to the known length (733pixels/mm and 583 pixels/mm respectively for both C5 and adult stage). Same for the measurement of egg diameter and lipid sac area.

Figure 2.5 Biometric measurement of *C. finmarchicus* and its egg. a) is the measurement of length and height of prosome, white line is the length and yellow line is the height. b) lipid sac area measurement, yellow line indicates the area of lipid sac. c) measurement of egg diameter

2.5 Chemical analysis

2.5.1 Carbon, Nitrogen and Phosphate analysis of the microalgae

The filters with concentrated algae were delivered to Trondheim Biological Station (TBS) and be prepared for C, N and P analysis. Small pieces from the total filters (factor 16 of the total filter) were cut out and be enveloped in the tin capsules. The tin capsules were then transferred into the well plates and be heated overnight in the heating cabinet. Each filter had two replicates and analyzed for cellular nitrogen and carbon on an elemental analyzer (vario EL cube, Elementar Analysensyteme GmbH, Germany).

Small filter pieces with same factor 16 were put in scintillation vials, 10 ml of aquadest, 2 ml oxidizing reagent and 0.1 ml of sulfuric acid were added, giving a total of 0.0121L liquid added. Samples were then autoclaved, cooled, and be analyzed. The method follows the Norwegian standard for Phosphate analysis. NS-EN ISO 6878, 2004. Each filter had two replicates and analyzed for cellular phosphorus content. 1 part samples were diluted by 3 parts aquadest to fit the standard curve for the reading.

2.5.2 Fatty acids composition analysis

Total lipid was extracted, and fatty acid composition of copepods and algae were analyzed by Gas-Chromatography (GC). The tubes that contained copepods samples were added Chloromethane (2:1, v/v) and homogenized (Whereas algal samples were harvested by centrifuge at 4000 rpm for 3 min). 0.88% potassium chloride was then added into the tubes and centrifuged at 4000 rpm for 3 min. The lower phase was collected and went through the filter, evaporated under N_2 gas. Once it has been dried out, samples were then transferred into desiccator for 30 to 60 min to remove the water to a higher extent, dried samples were then weighed, and 10mg/ml of Chloroform was added and stored in the freezer after flushing with N_2. Fatty acid methyl esters (FAME) were obtained from the extracted lipids. 20µL of the extracted lipids was transferred to another tube, chloroform and 1% sulphuric acid: methanol were added, the tube was placed onto the heating block and being methylated for 16-18 h at 50C. Isooctane and Sodium chloride (NaCl) were added after methylation, and upper phase was collected and evaporated under N2. Fatty acids and alcohol were separated on HPTLC silica gel plates. 20µL of methylated lipid was transferred onto the plate by using 10ml Hamilton microliter syringes. The plate was then placed in Hexane: Diethyl ether: Acetic acid (90:10:1, v/v) and visualized by Iodine. Separated fatty acids and fatty alcohol were collected by scraping the silica gel and Hexane: Diethyl ether (1:1, v/v) was added and evaporated under N_2. 50µL of Isooctane was used to prepare the tubes for gas chromatograph. However, fatty alcohol was not able to detect in the present study.

2.6 Data analysis

2.6.1 Egg production rate and hatching success

Egg production was evaluated to individual level as the number of total eggs varied significantly among the treatment group. Egg production rate per female per day was calculated based on the following equation:

$$Egg\ production\ rate = \frac{total\ eggs}{total\ females} \cdot Day^{-1}$$

Whereas the hatching success was calculated by the following equation:

$$Hatching\ success = \frac{nauplii\ presented}{total\ eggs} \cdot 100\%$$

2.6.2 Statistics

All statistical analyses were conducted using R version 3.3.1; SPSS version 27.0.1.0 and alpha value was set at 0.05 for all tests. Data with no normal distribution or heterogeneity was transformed by log or square root, and the non-parametric alternatives were carried out when ANOVA assumption was violated. For the analysis of algal C, N and P content, and fatty acids composition, the non-parametric alternative of one-way ANOVA, Kruskal Wallis H test was used to test the significances, followed by Mann Whitney U post hoc test. Two-way ANOVA, followed by Tukey's multiple comparison post hoc test was carried out for all copepod analysis. R version 3.3.1 and SigmaPlot were used for figure making and editing.

3. RESULTS

3.1 Composition of the diet microalgae *R. baltica*

3.1.1 Carbon, Nitrogen and Phosphorus contents.

Carbon, nitrogen, phosphorus contents and nitrogen:phosphorus (N:P) ratio of *R. baltica* grown at different phosphorus limitation of the growth media is shown in Table 1.

Significant differences in carbon, phosphorus, nitrogen content of *R. baltica* cells and ratio of nitrogen to phosphorus (weight) were found ($p < 0.05$, Kruskal Wallis H). The C content per cell in treatment with 50% NaH_2PO_4 (P50) in growth medium was significantly lower than other treatments ($p < 0.05$, Mann Whitney U), No differences were found between cells from medium with 100% NaH_2PO_4 (P100) and 12.5% NaH_2PO_4 (P12.5) ($p > 0.05$). The N content per cell in treatment P100 was significantly higher than other treatments ($p < 0.05$), while the contents between P50 and P12.5 were also significantly different ($p < 0.05$), P50 had the lowest N contents. The P content per cell in the three treatments were all significantly different. While P100 showed the highest, P12.5 showed the lowest P content per cell ($p < 0.05$). The highest N:P ratio was found in treatment P12.5 ($p<0.05$), whereas P100 and P50 had similar ratio ($p > 0.05$).

Table 1. Carbon, nitrogen, phosphorus content, and N:P ratio. Mean ± SD. Kruskal Wallis non-parametric test were carried out for data analysis. Significant differences were indicated by letters, where values that sharing the same letter meaning no differences.

	P level		
	P100	P50	P12.5
pg C $cell^{-1}$	55.74 ± 8.69^a	37.40 ± 1.62^b	50.00 ± 1.81^a
pg N cell−1	11.38 ± 1.70^a	7.80 ± 0.36^b	8.41 ± 0.43^c
pg P $cell^{-1}$	1.45 ± 0.14^a	1.01 ± 0.04^b	0.32 ± 0.04^c
N:P pg N pg P^{-1}	7.81 ± 0.59^a	7.72 ± 0.41^a	26.91 ± 3.55^b

3.1.2 Fatty acid composition

The fatty acid composition of *R. baltica* cultivated with different P limitation is summarized in Table 2. Significant difference was found in C18:1n-9 ($p < 0.05$, Kruskal Wallis H, followed by Mann Whitney U post hoc test), percentage of C18:1n-9 in treatment P12.5 was significantly higher than P100 and P50 ($p <0.05$). No major differences were found in other SFA and MUFA.

Fatty acids belong to n-3 in PUFA, however, showed significant differences. C18:4n3 in treatment P100 was significantly higher than P12.5 ($p < 0.05$). Treatment P12.5 had significantly higher ∑MUFA, lower C20:5n-3, C22:6n-3, ∑n-3 and ∑PUFA than P100 and P50 ($p < 0.05$), P100 and P50 had no significant difference ($p > 0.05$).

Table 2. Fatty acid compositions of *R. baltica* cultured under phosphorus limitation, by using phosphorus-modified Conwy media with. Fatty acid composition is expressed as percentage values of total fatty acids. Mean ± SD, n = 5. Kruskal Wallis non-parametric test was carried out for data analysis. Letters indicate significant differences, while values that sharing the same letter meaning no differences. ∑SFA, sum of saturated fatty acid; ∑MUFA, sum of monounsaturated fatty acid; ∑PUFA, sum of polyunsaturated fatty acid.

		P level	
FA %	**P100**	**P50**	**P12.5**
C14:0	11.37 ± 2.84	10.86 ± 3.57	10.29 ± 1.73
C16:0	13.84 ± 7.25	13.67 ± 4.11	18.80 ± 6.67
C18:0	1.98 ± 1.30	2.79 ± 2.10	3.36 ± 2.21
C20:0	0.04 ± 0.06	0.25 ± 0.54	0.07 ± 0.08
C22:0	0.25 ± 0.29	0.43 ± 0.72	0.38 ± 0.48
∑SFA	27.47 ± 6.93	28.01 ± 4.37	32.91 ± 7.62
C16:1n-7	0.82 ± 0.14	0.73 ± 0.33	0.80 ± 0.27
C18:1n-9	4.26 ± 4.56[a]	3.47 ± 4.08[a]	10.22 ± 5.96[b]
C18:1n-7	2.41 ± 0.44	2.20 ± 0.64	2.76 ± 0.49
C22:1n-11	0.23 ± 0.54	0.10 ± 0.15	0.14 ± 0.13
∑MUFA	7.72 ± 4.60[a]	6.50 ± 4.21[a]	13.93 ± 6.46[b]
C18:2n-6	21.07 ± 6.46	22.35 ± 6.44	19.85 ± 5.74
C20:3n-6	0.30 ± 0.22	0.31 ± 0.21	0.37 ± 0.24
C20:4n-6	1.94 ± 1.00	2.05 ± 0.62	1.40 ±0.81
∑n-6	23.31 ± 7.55	24.71 ± 7.00	21.62 ± 6.44
C18:3n-3	13.49 ± 1.08	13.69 ± 3.57	11.88 ± 3.19
C18:4n-3	8.04 ± 3.31[b]	5.22 ± 2.35[a]	5.12 ± 2.05[a]
C20:5n-3	3.10 ± 0.65[a]	2.86 ± 0.70[a]	1.80 ± 0.79[b]
C22:6n-3	3.93 ± 1.07[a]	3.92 ± 1.33[a]	2.50 ± 1.37[b]
∑n-3	28.56 ± 3.58[a]	26.69 ± 7.44[a]	21.30 ± 7.06[b]
∑PUFA	51.87 ± 7.80[a]	51.40 ± 6.00[a]	42.92 ± 12.05[b]

3.2 Effect on *C. finmarchicus*

3.2.1 Temperature monitoring

Water temperature in the experimental tanks was recorded every day, and fluctuations of the water temperature were observed (Figure 3.1). The average of measured temperature of the tanks at 12°C was 11.9 ± 0.2°C, and 14.0 ± 0.3°C in the tanks at 14°C. Additionally, the highest temperatures (HT) observed from each treatment were also recorded. HT at 12°C were 0.2°C greater, whereas HT at 14°C were generally 0.5°C greater.

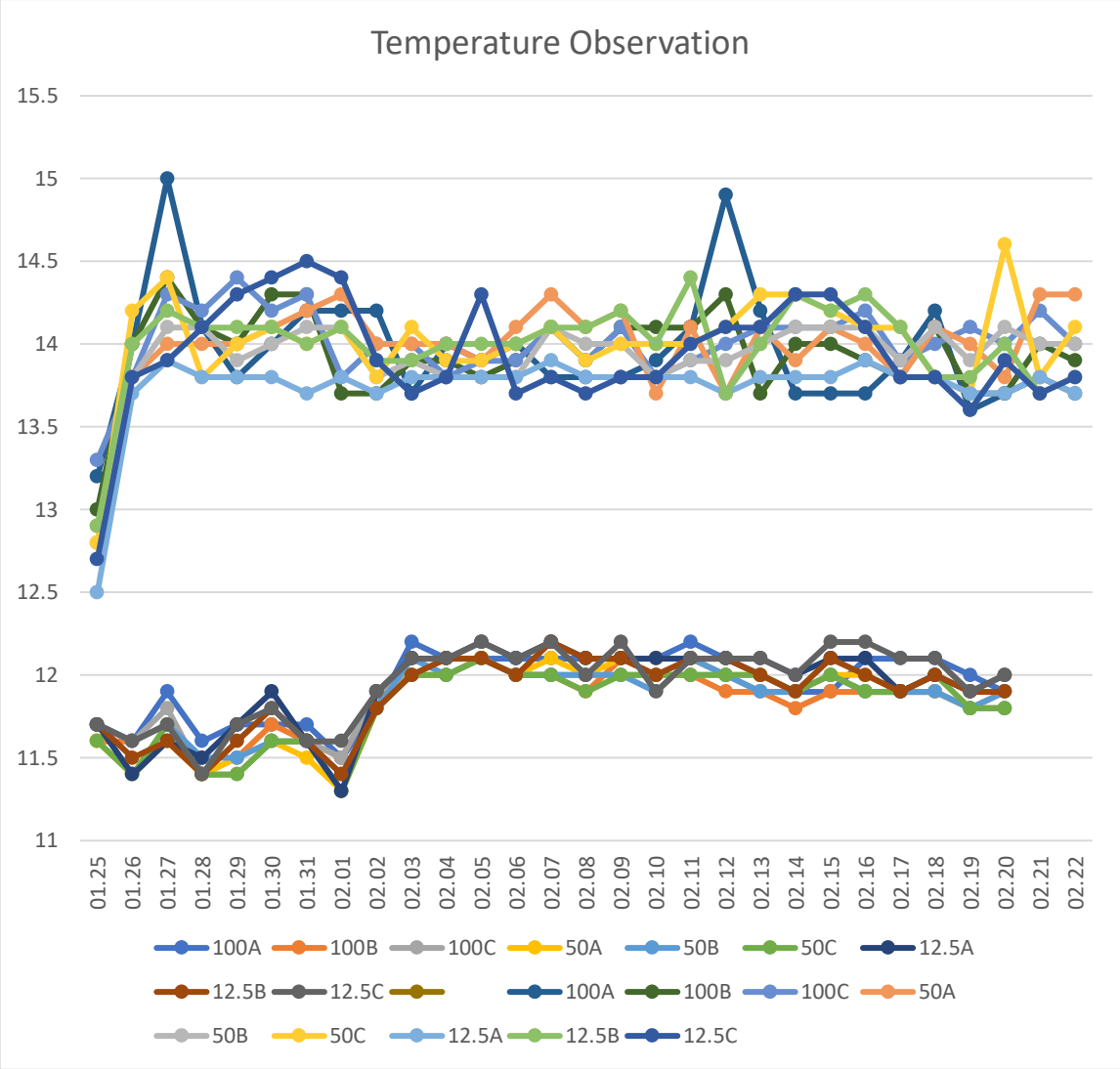


Figure 3.1 Temperature was observed and recorded daily throughout the experiment. The upper trace is the record from the treatment group at 14°C, and the lower trace is the treatment group at 12°C

3.2.2 The volume of prosome and ratio of lipid sac

The assessments of prosome volume for *C. finmarchicus* at C5 and adult were conducted (Figure 3.2). Neither temperature or phosphorus limitation nor interaction effect was found on prosome volume for copepods at C5 ($p > 0.05$, two-way ANOVA).

Significant differences were found for *C. finmarchicus* at adult stage. Temperature had a significant effect on prosome volume: adult *C. finmarchicus* were significantly smaller when reared at 14°C than when reared at 12°C ($p < 0.05$). Neither phosphorus level nor interaction effect with temperature was found ($p > 0.05$).

The ratio of lipid sac to prosome volume was calculated for both *C. finmarchicus* at C5 and adult stage to assess the lipid storage throughout the experiment (Figure 3.2). No significant differences were found at C5 stage ($p > 0.05$, two-way ANOVA). Similarly, temperature had no effect for *C. finmarchicus* at adult stage ($p > 0.05$). However, phosphorus limitation in *R. baltica* had significant effect on the lipid storage of *C. finmarchicus* in which treatment P50 had the least lipid storage compared to other two treatments ($p < 0.05$). No interaction effect between temperature and phosphorus limitation was found ($p > 0.05$).

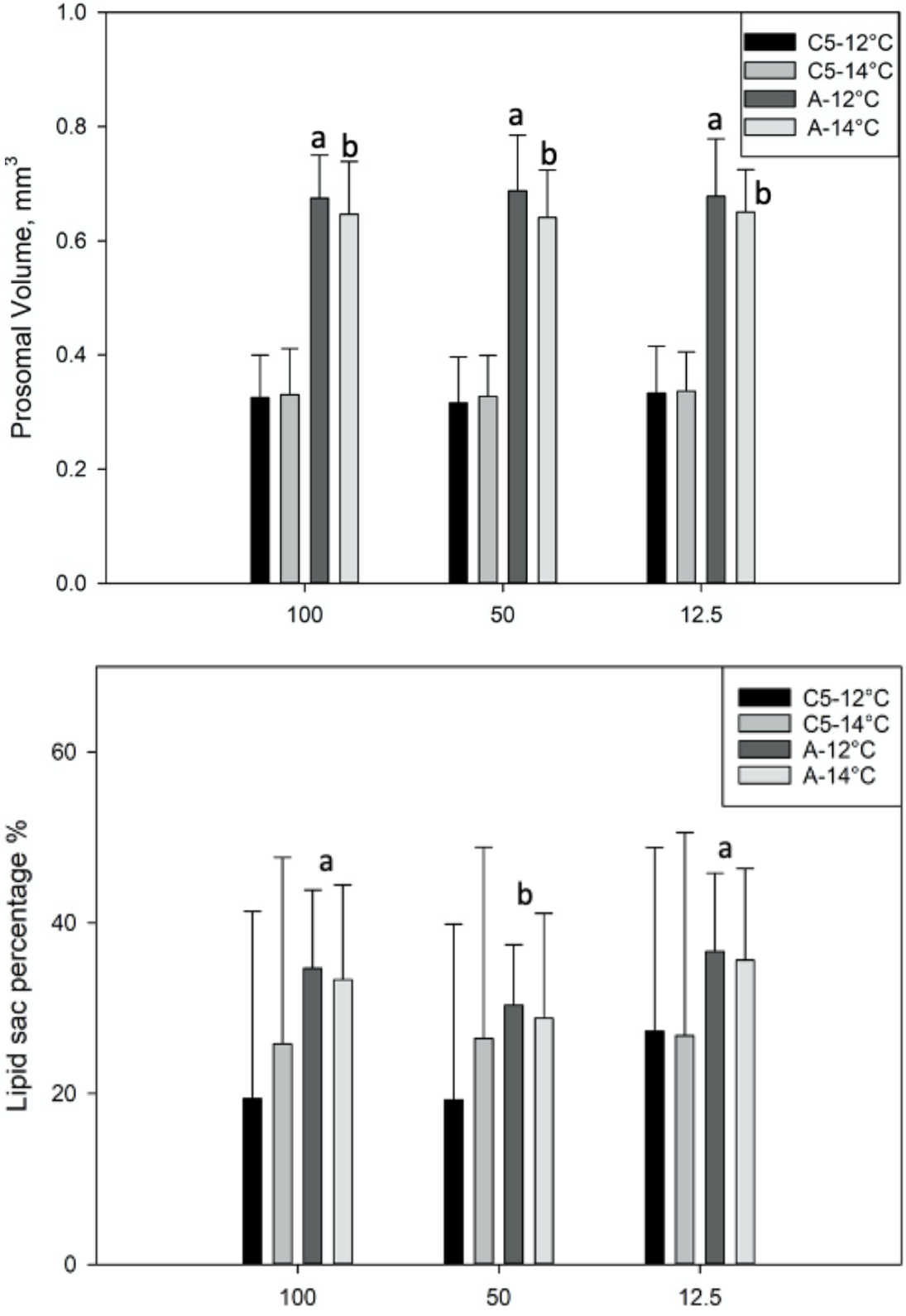


Figure 3.2 Barplot of prosome volume (mm^3), and lipid sac percentage in body of copepods (%) at stage C5 and Adult. Mean ± SD, n ≤ 3. Horizontal axis shows phosphorus level of the growth media. Different letters represent a significant difference among groups.

3.2.3 Egg size, production, and hatching success

Egg diameter was measured for evaluating its size (Figure 3.3). Temperature has a significant effect on egg size, eggs from *C. finmarchicus* reared at 14°C were significantly larger in diameter than eggs from copepods reared at 12°C ($p < 0.05$, two-way ANOVA), whereas eggs from P100 had significantly larger diameter compared to treatment groups P50 and P12.5 ($p < 0.05$), indicating significant effect at phosphorus limitation. No interaction effect between temperature and phosphorus limitation was found ($p > 0.05$).

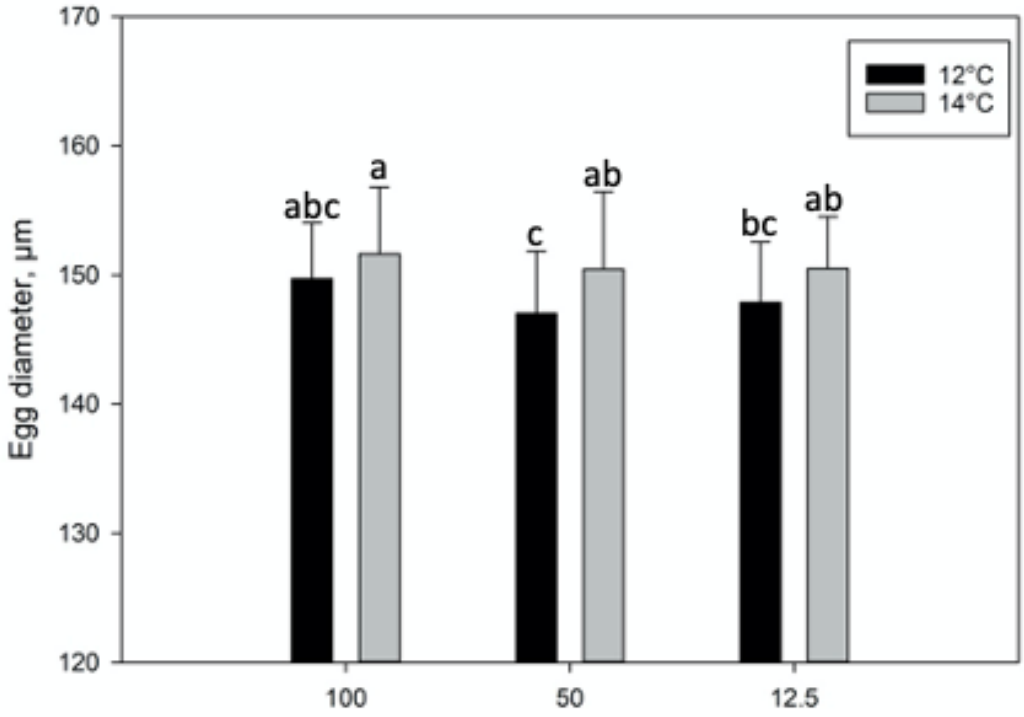


Figure 3.3 Barplot of egg diameter (µm). Mean ± SD, n ≤ 3. Horizontal axis shows phosphorus level of the growth media. Different letters represent a significant difference among groups.

Egg production per female during 24 hours and hatching success were evaluated (Figure 3.4). Temperature had significant effect on egg production, where copepods at 14°C produced fewer eggs than copepods at 12°C ($p < 0.05$, two-way ANOVA). In contrast, phosphorus had no effect on egg production ($p > 0.05$), and no interaction effect between temperature and phosphorus limitation was found ($p > 0.05$).

Hatching success (Figure 3.4) after 72 hours were significantly different in temperature ($p < 0.05$, two-way ANOVA, followed by tukey's post hoc test). Eggs from treatment P100 and

P50 at 14°C had significantly lower hatching success compared to other treatments ($p < 0.05$). Eggs hatching success at 12°C was significantly higher than eggs at 14°C ($p < 0.05$), no interaction effect between temperature and phosphorus limitation was found ($p > 0.05$).

Egg production against egg diameter was evaluated (Figure 3.5), and the clear relationship was found in P50 and P12.5 at different temperatures where copepod at 12°C tended to produce more eggs but smaller in diameter, and vice versa at 14°C.

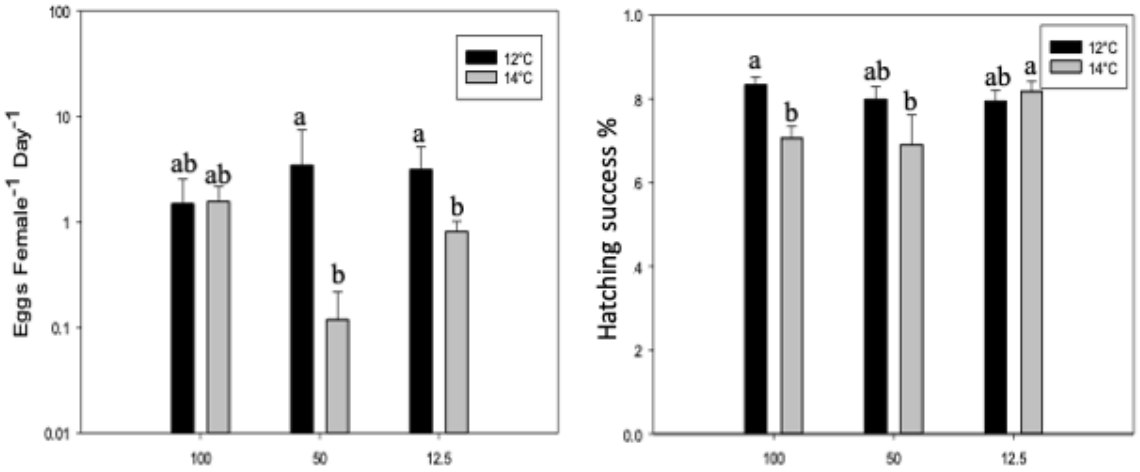


Figure 3.4 Egg production per female and hatching success in different treatments. Horizontal axis shows phosphorus level of the growth media, vertical axis is transformed to logarithmic value. Mean ± SD, n ≤ 3. Different letters represent a significant difference among groups.

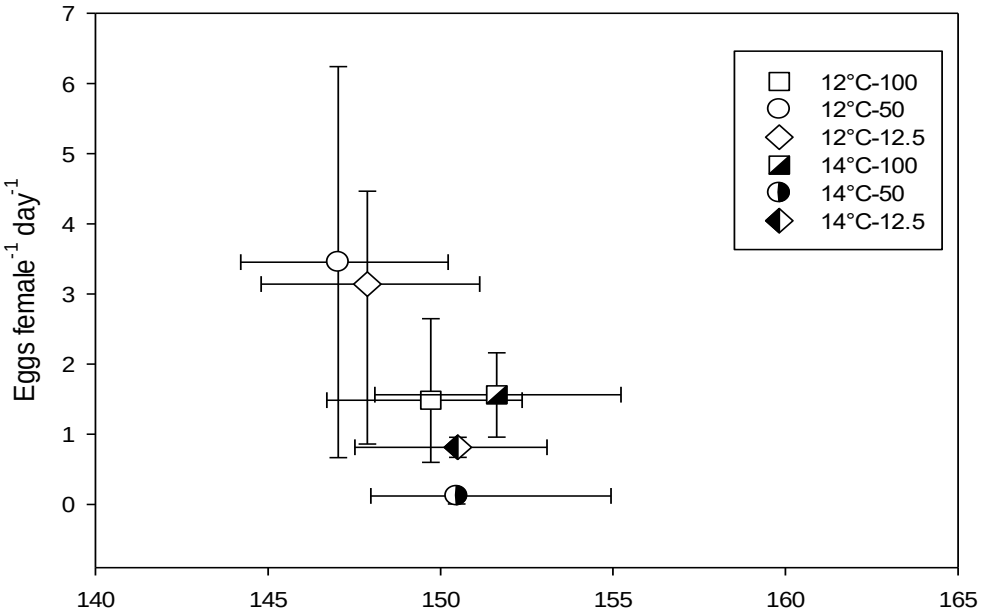


Figure 3.5 Egg production and Egg size correlation, x lab shows the diameter of egg in µm, y lab shows the egg production per female per day. Mean ± SD.

3.2.4 Fatty acid composition

The fatty acid composition of *C. finmarchicus* in different treatments were summarized in Table 4. Significant difference was detected in ∑SFA, treatment P100 at 12°C had significantly higher percentage than the other treatments ($p < 0.05$, two-way ANOVA, followed by Tukey's post hoc test). Moreover, among SFA, percentage of C16:0 and C18:0 in treatment P100 at 12°C was significantly higher than treatment P100 and P12.5 at 14°C ($p <0.05$), C22:0 in P100 at 12°C was significantly higher than the other treatments ($p < 0.05$).

Significant difference was found in ∑MUFA, P12.5 was significantly higher than the other treatments ($p < 0.05$). Whereas fatty acid C22:1n-11 was significantly lower in treatment P100 compared to the other treatments. Additionally, percentage at 14°C was significantly higher than percentage at 12°C ($p<0.05$).

∑PUFA of treatment P100 at 12°C was significantly lower than the other treatments ($p < 0.05$), among PUFA, ∑n-6 of treatment P100 at 12°C was significantly lower than the other treatments ($p < 0.05$), and ∑n-3 of treatment P100 was significantly lower than the other treatments ($p < 0.05$). Moreover, P100 at 12°C had significantly lower C20:4n-6, C20:5n-3 and C22:6n-3 compared to the other treatments ($p < 0.05$).

Table 4. Fatty acid compositions of C. finmarchicus fed by *R. baltica* that had grown under phosphorus limitation. Fatty acid composition is expressed as percentage values of total fatty acids. Mean ± SD, n = 3. Two-way ANOVA test was carried out for data analysis. Letters indicate significant differences while values that sharing the same letter meaning no differences. ∑SFA, sum of saturated fatty acid; ∑MUFA, sum of monounsaturated fatty acid; ∑PUFA, sum of polyunsaturated fatty acid.

	P level					
	P100		P50		P12.5	
FA%	12°C	14°C	12°C	14°C	12°C	14°C
C14:0	10.63 ± 6.56	10.84 ± 3.98	9.29 ± 4.18	10.46 ± 1.34	10.08 ± 2.90	16.58 ± 3.41
C16:0	34.21 ± 7.77^{a}	19.45 ± 0.69^{b}	22.75 ± 6.39^{ab}	24.49 ± 4.70^{ab}	22.11 ± 3.05^{ab}	18.43 ± 1.85^{b}
C18:0	12.12 ± 4.46^{a}	4.29 ± 1.52^{ab}	5.25 ± 3.46^{ab}	8.52 ± 3.44^{ab}	6.14 ± 2.13^{ab}	2.79 ± 0.62^{b}
C20:0	0.22 ± 0.06	0.17 ± 0.07	0.21 ± 0.12	0.26 ± 0.06	0.17 ± 0.07	0.16 ± 0.07
C22:0	0.28 ± 0.41	0.48 ± 0.30	0.95 ± 0.66	0.68 ± 0.23	0.51 ± 0.23	0.58 ± 0.26
∑SFA	57.47 ± 7.50^{a}	35.23 ± 1.43^{b}	38.44 ± 6.44^{b}	44.40 ± 7.44^{b}	39.01 ± 2.62^{b}	38.54 ± 4.99^{b}
C16:1n7	1.48 ± 0.88	1.60 ± 0.22	1.78 ± 0.37	1.78 ± 0.05	1.84 ± 0.38	2.31 ± 0.22
C18:1n9	2.60 ± 1.53	1.82 ± 1.51	2.68 ± 2.56	0.93 ± 0.07	2.50 ± 1.18	3.81 ± 0.41
C18:1n7	0.63 ± 0.32	0.52 ± 0.34	0.65 ± 0.44	0.31 ± 0.01	0.82 ± 0.41	1.02 ± 0.05
C22:1n11	0.14 ± 0.24$^{\mathbf{a}}$	3.91 ± 0.21$^{\mathbf{ab}}$	2.86 ± 2.12^{ab}	5.68 ± 0.71^{a}	3.30 ± 1.03^{ab}	7.04 ± 3.08^{b}
∑MUFA	4.85 ± 2.01^{a}	7.84 ± 1.92^{ab}	7.97 ± 5.49^{ab}	8.70 ± 0.66^{ab}	8.46 ± 1.50^{ab}	14.18 ± 3.56$^{\mathbf{b}}$
C18:2n6	5.34 ± 2.55	8.44 ± 0.07	7.63 ± 0.86	7.85 ± 1.44	8.43 ± 0.65	7.90 ± 0.96
C20:3n6	0.76 ± 0.23	0.54 ± 0.10	0.62 ± 0.27	0.70 ± 0.14	0.80 ± 0.02	0.69 ± 0.28
C20:4n6	1.30 ± 0.57$^{\mathbf{b}}$	3.47 ± 0.19^{a}	2.90 ± 0.11^{a}	3.01 ± 0.46^{a}	2.96 ± 0.24^{a}	2.63 ± 0.49^{a}
∑n-6	7.40 ± 1.93$^{\mathbf{a}}$	12.44 ± 0.33$^{\mathbf{b}}$	11.15 ± 0.70^{ab}	11.57 ± 1.88^{ab}	11.69 ± 0.90^{ab}	10.61 ± 2.44^{ab}
C18:3n3	5.39 ± 2.97	9.38 ± 0.42	8.67 ± 2.03	8.69 ± 1.67	10.11 ± 1.16	7.87 ± 3.85
C18:4n3	2.28 ± 2.36	5.73 ± 0.25	3.64 ± 1.58	2.28 ± 3.00	5.62 ± 0.79	5.19 ± 1.66
C20:5n3	2.65 ± 2.13$^{\mathbf{b}}$	8.51 ± 0.80^{a}	8.55 ± 0.12^{a}	5.14 ± 2.64^{ab}	7.24 ± 0.51^{a}	5.03 ± 0.66^{ab}
C22:6n3	4.95 ± 2.44$^{\mathbf{b}}$	10.10 ± 1.38^{a}	10.49 ± 2.69^{a}	6.44 ± 3.12^{ab}	8.94 ± 1.23^{a}	6.03 ± 1.42^{ab}
∑n-3	15.27 ± 5.40$^{\mathbf{a}}$	33.73 ± 2.61$^{\mathbf{b}}$	31.35 ± 0.79^{ab}	22.55 ± 8.55^{ab}	31.92 ± 1.13$^{\mathbf{b}}$	24.12 ± 6.21^{ab}
∑PUFA	22.67 ± 7.01$^{\mathbf{a}}$	46.17 ± 2.88$^{\mathbf{b}}$	42.51 ± 1.49$^{\mathbf{b}}$	34.12 ± 8.00^{ab}	43.61 ± 1.92$^{\mathbf{b}}$	35.74 ± 8.08^{ab}

4. Discussion

This study was designed to gain better insight into the fitness of *C. finmarchicus* fed a diet of *R. baltica* cultured at three different phosphorus concentrations 100, 50 and 12.5 % NaH_2PO_4 in the growth medium and reared at two different temperatures. Discussion section is mainly divided up into three parts; microalgae, copepods and experimental conditions and evaluations.

4.1 Microalgae *Rhodomonas baltica*

The objective of this algal experiment was to evaluate the effect of phosphorus deficiency on the *R. baltica* stoichiometric content, N:P ratio and the total lipid fatty acid profile. The most significant differences were found in algae maintained at the lowest, 12.5%, phosphorus for both fatty acid composition, and the N:P molar ratio.

4.1.1 Cellular carbon, nitrogen, phosphorus, and N:P ratio

R. baltica cultivated in semi-continuous Conwy media with different phosphorus level had significantly different C, N and P content. As expected, the P content of *R. baltica* decreased with decreasing NaH_2PO_4 content in the medium. The reduction was not a clear dose-response relationship where reductions were notably lower in P50 and P12,5 showing a substantial ability to extract P from the media. In the natural environment, P concentrations are generally low (Brown & Shilton, 2014; Lomas et al., 2010; Paytan et al., 2007). As an adaptation, microalgae can increase P uptake and store P whenever needed. While C content in treatment P100 and P12.5 was all above 50pg/cell, P50 had the lowest content which was lower than 40pg/cell. In previous studies, *R. baltica* generally had more than 53pg/cell C content (Dutz, 1998), and the reason for this abnormally lower C content in P50 was unclear.

The N content has previously been reported to be around 11.5pg/cell (Dutz, 1998). The N content also varied between the groups followed the same manner, cellular nitrogen of *R. baltica* in treatment P50 was 7.8pg/cell while in P12.5 was 8.4pg/cell and 11.4pg/cell in P100. Despite similar N content in treatment P100, the other two treatments had lower value than other studies (Dutz, 1998). However, cultivated *R. baltica* during the experiment did not turn to green/yellow from its original red colour (Thoison, 2017), indicating that *R. baltica* had

sufficient nitrogen content in the growth medium for normal growth. It has well known that nitrogen is one of the essential macronutrients for algal growth, while 25% increased concentration of $NaNO_4$ (See appendix) used in this study was to maintain the algal cell growth and proliferation for the feeding of *C. finmarchicus.* Nevertheless, nitrogen content in P50 and P12.5 were lower than P100.

Redfield ratio hypothesis (1958) states that N:P ratio of 16:1 in which 16 mol of N and 1 mol of P in the growth medium may favor the algal cultivation, and this is very widely acknowledged ratio in the study of nutrients in aquatic system (Arbib, 2013). Therefore, Redfield ratio is described as the ideal ratio for the growth of majority microalgae since the chemical formula of a typical microalgae possess 16 of N and 1 of P. However, many studies have found that Redfield ratio should be used as a guidance rather than a rule since this ratio in microalgae is strain specific (Whitton et al., 2016). In addition, N:P ratio was found to be dynamic in microalgae that ranges from 8:1 up to 45:1 (Hecky et al., 1993). In the present study, N:P ratio was found similar between treatment P100 and P50 in the algae. The main reason of identical ratio between P100 and P50 was due to the lower N content in P50. Whereas ratio in P12.5 was significantly higher due to the lower level of P content. N:P molar ratio in Conwy medium that has been used in this study was 10.14 in P100, 20.28 in P50 and 40.57 in P12.5 respectively. However, the growth curve among groups P100 and P50 were identical, and this might be due to the similar cellular N:P ratio in *R. baltica.* In P12.5, exponential growth phase was longer than P100 and P50 (See appendix), indicating that strong phosphorus limitation might have caused changes in microalgal cellular metabolism and its content. Surprisingly, cultivation of *R. baltica* in P12.5 crashed only twice throughout the experiment while other groups crashed more often. However, this might also be due to the unexpected different laboratory conditions. Future study is required to understand the effect of strong phosphorus limitation on growth rates, length of different growth phases, and more detailed information such as genetic expression as well as metabolic pathway.

4.1.2 Fatty acid composition of *R. baltica*

One significant effect of P limitation was the effect it had on the total lipid fatty acid composition. In the P12.5 treatment in particular, there was a notable decrease in the long chain PUFA content including EPA and DHA. This is in line with previous studies showing that

PUFA synthesis is impaired with P starvation (Reitan et al., 1994). However, this can also be explained by the different harvesting time, as microalgae at different growth phase has different lipid content (Lovio-Fragoso et al., 2021), and harvesting of algae that had been done during the experiment did not happen at the same algal growth phases.

The P concentration used in the present study had strong effect on the fatty acid composition of *R. baltica,* but not decreasing in production, as *R.baltica* in P12.5 still performed well regarding cell proliferation. Nevertheless, the main consequence of reduced PUFA content is that the feed quality of *R. balcica* will be lower. Plankton feeding on such algae will have reduced supply of essential fatty acids which may affect growth, recruitment, and welfare.

4.2 *Calanus finmarchicus*

This study was designed to investigate the effect of feeding the different P limited microalgae to *C. finmarchicus* at two temperature regimes where one temperature is regarded as reaching upper tolerance limit (Heath et al., 2000). The results clearly show the temperature effects on development, egg size, egg production per female per day and its hatching success. Prosome volume, egg production and hatching success were reduced with increasing temperature while egg diameter increased. There were no clear impacts of phosphorus level on prosome volume at C5 and adult stage, whereas major reductions of phosphorus also affect lipid storage and the median egg diameter, especially at 12°C. Overall, however, P starvation did not show any consistent effect on copepod development or its egg production. Nevertheless, the hatching success seemed to be decreased with decreasing phosphorus level despite P12.5 at 14°C was high.

4.2.1 Developmental conditions

Stormer (1929) noted that *Calanus* in colder environments had slower developmental rates than those in warmer temperatures. This gave a progressive reduced size with increased temperature. This assumption has also been confirmed in later studies (Atkinson, 1994; Campbell et al., 2001). The smaller size of *C. finmarchicus* kept at 14°C compared to 12°C agrees with these earlier studies.

The mean value of lipid sac percentage in *C. finmarchicus* was lower at 14°C compared to 12°C though this was not statistically significant. However, this finding is in line with previous study, which found that the higher the temperature, the lower the lipid deposit in copepods (Zhou & Sun, 2017). It is likely that copepods at higher temperature may possess smaller lipid storage, and with faster growth rates, there is a real possibility of which adults may have to deal with less energy storage and more energy consumption due to the elevated metabolic rates. Besides temperature, lipid deposition is also sensitive to the quality of food. Previous studies found that copepods fed low quality of food had lower lipid accumulation (Chen et al., 2018; Mayor et al., 2006). In this study, however, only *C. finmarchicus* in treatment group P50 had lowered lipid sac percentage compared to the other treatments. This might be related to the lower C content of *R.baltica* in P50 since daily food concentration of 200µg C/L may not be achieved. Nevertheless, lipid reserve measurement at adult stage is not fully reliable as the sampling was intended to select the individuals with visible lipid sac only for the further lipid analysis, though it was not possible to perform due to the lack of time. Therefore, it is not clear to state that lower lipid storage was due to the lower C content in food, or it was because of the bias in sampling. Nevertheless, then constantly lower percentage found at 14°C seems to be caused by the effect of high temperature.

4.2.2 Fatty acids composition of *C. finmarchicus*

C. finmarchicus relies on the lipid reserves to be able to survive diapause, migrate from deep to surface water, and is essential for molting and gonad development (Jonasdottir, 1999). Therefore, it is critical to understand the effect of temperature on lipid accumulation and lipid content of *C. finmarchicus.* Additionally, the fatty acids in *C. finmarchicus* were found to be related to the phytoplankton that they feed on (Graeve et al., 1994), at this point, food quality is also a critical factor need to be studied. In the present study, P100 had significantly higher content of SFA compared to the other treatment groups, contributed by C16:0, C18:0 and C22:0, this might be due to the lower content in EPA and DHA. Fatty acids EPA and DHA need to be increased via ingestion and then copepod can utilize some of the SFA for energy or synthesis of the fatty alcohols as a precursor (Bergvik et al., 2012). Nevertheless, it is unclear that noticeably high content of SFA in *C. finmarchicus* in P100 at 12°C was only due to the low content of EPA and DHA, this can also because of the laboratory conditions, mistake of fatty acid separation for GC analysis, or other factors that we do not know.

MUFA in treatment P12.5 was significantly higher than other treatments due to the excessively higher C22:1n-11. While C22:1n-11 increased with decreasing P level, it also increased with elevated temperature. Previous studies have found that C22:1n-11 is a prominent component of wax esters, and it can be synthesized by *C. finmarchicus* (Kattner and Krause, 1989; Sargent, 1977). However, this fatty acid is found to be scarce during the phase of lipid accumulation since all the synthesized C22:1n-11 is converted to the fatty alcohols C22:1n-11, but starts accumulating fatty acids C22:1n-11 once they have accumulated enough wax esters (Bergvik et al., 2012). Higher content of this fatty acid at 14°C might be the result of faster development that *C. finmarchicus* at 14°C had accumulated wax esters in a faster rate than those at 12°C, as warmer temperature is known to accelerate the development of *C. finmarchicus* (Marshall and Orr, 1972; Stormer, 1929). Whereas Increasing content of C22:1n-11 with decreasing P level might be due to the same relationship found in *R. baltica* regarding fatty acids C16:0 and C18:0. It is well studied that the fatty acid C22:1n-11 are thought to be synthesized from carbohydrate and protein precursors with the intermediates C14:0, C16:0 and C18:0 (Bergvik et al., 2012). Therefore, higher content of C22:1n-11 might be related to the lipid content in food source, *R. baltica* in P12.5 had the highest contents of fatty acids C16:0 and C18:0 and higher content of fatty acid C18:0 in P50, which favored the synthesis of C22:1n-11 in *C. finmarchicus.*

While n-3 and n-6 PUFA in treatment P100 at 12°C was always lower than other treatments, n-6 PUFA in other treatments were identical, and n-3 PUFA decreased with increasing temperature. EPA and DHA seems to be reduced with elevated temperature though it were not statistically significant. Previous study found that DHA proportion of total fatty acids in *C. finmarchicus* was lower at higher temperature (Helenius et al., 2020), whereas PUFA concentrations are thought to be increased at low temperatures for their membrane fluidity (Masclaux et al., 2012). Due to this, lower content of EPA found at 14°C might also because of the effect of elevated temperature. EPA and DHA were also reduced with P limitation though it was not statistically significant. The fatty acid compositions of *Calanus* were found to be sensitive to the fatty acid compositions in food source (Leiknes et al., 2016). Therefore, it is expected to see reduced EPA and DHA in *C. finmarchicus* with decreased food quality, though the effect was not as strong as temperature. EPA and DHA in treatment P100 at 12°C,

however, were the least, the reason for this is unclear. Possible reason is that *C. finmarchicus* in this treatment might just about to start the lipid accumulation in wax ester, and therefore they had higher content in SFA but lower content in EPA, DHA and C22:1n-11. Alternatively, this may also because of the mistake of fatty acids separation for the GC analysis, fatty acid was lost somehow during the preparation which resulted significantly lower content of EPA and DHA.

4.2.3 Egg profile

Egg production decreased with increasing temperature in the treatments P50 and P12.5, whereas the egg production in P100 at the two different temperatures were similar. Previous studies of copepods have shown that egg production rate increases linearly with increasing temperature (Evjemo et al., 2007; Hirche et al., 1997; Kjellerup et al., 2012). This was not the case in the current study. Moreover, total egg production rate in this study was noticeably lower than other studies (Campbell et al., 2001; Hirche et al., 1997). It is likely that temperature regimes in the present study reduced egg production rate, as elevated temperature at 13°C reduced egg productions severely in the previous studies (Heath et al., 2000; Hirche et al., 1998). However, egg production is still possible as high as 15°C (Heath et al., 2000), indicating that *C. finmarchicus* may still produce the eggs at upper tolerance limit. However, reduced egg production may be due to the strong aeration during early developmental stage that had affected the growth of *C. finmarchicus*, which resulted too young females in the tanks during the collection. Alternatively, sex ratio in the tanks was seriously biased that most of the females were not fertilized. Nevertheless, significant differences were observed. The most pronounced effects were impaired egg production by the combination of high temperature and P limitation (P50, P12.5 at 14°C). It is also interesting to note that these groups, in general were the ones with reduced levels of total lipid EPA and DHA which again relates to the fatty acids of *R. baltica*. The results are consistent with previous findings that the reproductive capability of copepods is dependent high supplies of these fatty acids (Bergvik et al., 2012; Evjemo et al., 2008). For reasons yet unexplained, *C. finmarchicus* in treatment P100 at 12°C, had the lowest content of EPA and DHA. However, they still produced more eggs than P50 and P12.5 at 14°C, though it was lower than P50 and P12.5 at 12°C. This finding seems that there was an unexpected analytical mistake involved during the lipid analysis.

In the current trial, hatching success after 72 hours were generally good and higher than 70%. There was also a significant temperature effect where high temperature reduced the hatching. Hatching success of *C. finmarchicus* at different temperature regimes in previous studies have found that higher temperature did not lower the hatching success though temperature ranges of the study from Weydmann was from 0°C to 10°C. Whereas hatching success from Preziosi study was reduced from 19°C (Preziosi et al., 2017; Weydmann et al., 2015). There did not appear to be an interaction between food quality and temperature that was expected from the lowered PUFA content from P limitation and high temperature. It therefore appears that once the *Calanus* matures and produces eggs, then progression into hatching will proceed regardless. It might have affected the survivability of the nauplii, but this was not a goal of study in the present experiment. It was also noticeable that there were great variations within treatments which could have been affected by unknown factors of the experimental setup.

Egg size was generally bigger at 14°C than those at 12°C, and eggs from treatment P50 both at 12°C and 14°C were the smallest (Figure 3.2), this was consistent with the lowest lipid reserve of *C. finmarchicus* in this treatment. However, it is unclear whether smaller egg size was due to the lower lipid storage since lipid reserve evaluation in this study is not fully reliable. Moreover, mean value of eggs from treatment P100 were the biggest, this is likely due to the variation.

Surprisingly, egg size was negatively correlated to egg production rate in this study despite similar relationships in treatment group P100 at two different temperatures (Figure 3.5). To our knowledge, no previous studies have found this phenomenon. It is likely that *C. finmarchicus* at higher temperature along with low lipid content tended to reduce the egg production to guarantee the quality of egg and maintain the eggs at relatively high hatching rate, whereas *C. finmarchicus* in P100 at 14°C had similar egg production rate but slightly bigger eggs compared to P100 at 12°C. This might be because of the high lipid content at 14°C. Nevertheless, further study is required to investigate if this relationship is true or not.

4.3 Experimental conditions

The variations observed throughout the experiment regarding the temperature, aeration intensity in the tank were not part of the experimental design. However, the variations are discussed in this section to better understand the results in this study. Based on the findings reported in the previous studies, these variations may affect development, egg production and hatching success.

4.3.1 Variation in temperature

In this study, temperature itself was intended as a fixed factor. Ideally, temperature should be maintained around 12°C and 14°C, and fluctuation within 0.5°C was considered as acceptable. However, water temperature of the treatment group at 12°C were consistently lower than nominal temperature and the fluctuation was out of range during first week. This was due to the lack of knowledge regarding different heat conductivity between air and water. To achieve certain temperature of water, air temperature needed to be higher since water requires more energy to increase its temperature (Daniel. H, 2003). Therefore, the room temperature was set at 13°C along with slight increase of 0.5°C of the inlet water temperature. Treatment group at 14°C, however, has fluctuated significantly. Water temperature of tanks occasionally exceeded the nominal temperature, and the highest temperature was recorded higher than 14.5°C which was not acceptable. Previous study has pointed out that mortality was increased at 12°C compared to lower temperature (Campbell et al, 2001) and therefore, further elevation of temperature might increase the mortality. The other previous studies also found that *C. finmarchicus* has followed similar patterns that the higher the temperature, the higher the mortality (Grenvald et al., 2013; Clarke and Bonnet, 1939; Hirche, 1987).

Mortality, however, was not one of the parameters included in this study, and therefore it must be handled carefully, as enough individuals were important for lipid analysis, egg production and so on. Therefore, an emergency operation was carried out to prevent such excessively high temperatures in the tank when detected, lid was removed immediately and decreased the preset temperature of heating unit to stop heating temporary, and the aeration intensity was also increased to mix the water to a greater extent. However, this also caused another problem that will be discussed in the next section.

4.3.2 Aeration intensity

Aeration system was applied for preventing temperature gradient and algae concentration in the tank. This, however, has produced a severe problem. Due to the poor performance of the heating unit for the treatment group at 14°C, the aeration valve needed adjustment to enhance the mixing extent by increasing the airflow into the tanks. However, the system involved several tanks and thus, adjusting aeration intensity for only one tank was impossible, increasing one tank to a suitable extent may cause other tanks to possess extraordinarily high aeration. Appreciate the tiny size of *C. finmarchicus*, it was not surprising to see that majority of the individuals were sucked up onto the mesh (see Figure 2.2), and this may happen at a slower pace that was, inconspicuous right after the adjustment but became serious overnight. Therefore, *C. finmarchicus* might have experienced difficulties in feeding for approximately 20 hours since moving area was significantly restricted. Previous studies have found that reduced food may significantly decrease the growth rate with lower metabolic activity (Campbell et al., 2001; Wagner et al., 1998), this may cause *C. finmarchicus* to develop at a slower pace and therefore different time window of massive egg production from other treatment groups since these individuals may still need time to reach their full reproductive potential. Studies from Campbell et al (2001) also suggested that too young female may produce fewer eggs than those fully matured individuals. Besides developmental lagging, starvation may also decrease the egg production rate at adult stage, especially at higher temperature (Pasternak et al., 2013; Preziosi et al., 2014). However, aeration seemed not affected the behaviour of adult since *C. finmarchicus* was fully grown and its body size was big enough to be away from the sieve. Nevertheless, too much aeration during early developmental stage needed to be considered when looking into the findings in this study.

On the other hand, strong water flow caused by aeration may also increase the metabolic rate as *C. finmarchicus* was already at higher temperature and trying to flee from the mesh by jumping repeatedly with relatively short intervals, this might cause the extra cost of energy (Schulte P, 2011; Schulte P, 2015; Seebacher et al., 2015; Somero G, 2011). Possible outcome of strong water flow might be that *C. finmarchicus* has tolerated stressful circumstances with limited food supply because of the hindered feeding behaviour, as well as repeated metabolic activity and therefore utilized significant amount of energy. Interestingly, this was reflected

by the high daily concentration of algae in the tank coincides with its high mortality (See appendix for detailed information).

4.3.3 Mortality

In this study, cultivation of *C. finmarchicus* had triplicates for each treatment group, and placed in a random order, replicates were supposed to have a similar performance during and after the experiment. However, one tank of *C. finmarchicus* population in treatment P50 at 12°C and another in P12.5 at 14°C had crashed, only few individuals survived and reached the adult stage, and therefore produced eggs in total from these tanks were 0 and 1 respectively. This might be due to the laboratory conditions or other factors that we do not understand, but we are confident to state that starting population of *C. finmarchicus* was distributed equally since daily algal density in the tanks did not have a noticeably huge bias, and all tanks were treated similarly except the differences in aeration intensity. Therefore, these two tanks were excluded when evaluating egg production and hatching success as they may significantly affect the statistical analysis.

4.4 Evaluation of experiment and improvement

Throughout the experiment, few problems were observed regarding the set-up and design. As mentioned above, aeration intensity has significantly affected the animals in the tank. Possible solution is that placing the heating unit at the bottom, outside of the tank rather than submerging it at the corner of the tank. Then aeration intensity can be fixed and steady throughout the experiment as heating from the bottom can evenly distribute temperature until the surface because of the heat conductivity.

24 hours of egg collection used in this study caused huge variation among different treatment groups. Therefore, egg collection can be extended to 72 hours since animals might have different time window for massive egg production.

Algae cultivation was mainly for feeding of *C. finmarchicus* in this study, new culture was used only when the original culture was crashed. This, however, may cause algae to possess different lipid content as they may be harvested at different growth phases, it may affect the

analysis and cause *C. finmarchicus* to feed differently when the algae were at different phases but harvested at the same time. Therefore, the recommendation is that replacing the original culture with the new one every week and harvest them on the 5th day. In this way, algae will likely be at the same growth phase.

4.5 Future study

Combined effects of elevated temperature and food quality on *C. finmarchicus* have been studied. However, this study was started with *C. finmarchicus* at stage C3 and stopped right after the hatching of nauplii. It is still unknown how these can affect the early life stages or the next generation of *C. finmarchicus*. Moreover, the effect of phosphorus limitation on microalgae *R. baltica* can be studied deeply, including more detailed factors such as growth rate, length of growth phase, changes in genetic expression. While *R. baltica* was used as a food source in this study, other microalgal species can be used the same under nutrients limitations.

5. Conclusion

The present study shows that the modification of phosphorus level in the growth medium did not limit the cell growth and proliferation of microalgae. However, phosphorus content in algal cell was decreased along with decreased content of EPA and DHA. This apparently indicates the real risk to the aquatic ecosystem as many organisms rely on these fatty acids, further study on this effect is required. Whereas *C. finmarchicus* fed this p-limited food at high temperature seemed had no effect on development and lipid accumulation. Nevertheless, *C. finmarchicus* at higher temperature was smaller in prosomal volume and lipid sac percentage, and this was same with many other studies, indicating the higher temperature makes animal grow faster with less lipid reserves. Faster growth rates may cause peak copepodites happens earlier, and this shift in timing may cause mismatch of predator. Less lipid contents may also affect the species at higher trophic level. Arctic food web is relatively simple, and simple food web structure is prone to the changes.

Fatty acids composition of *C. finmarchicus* were found differently but followed similar pattern, EPA and DHA was reduced with increasing temperature. This finding indicates that there is an effect of temperature on the fatty acid compositions. In addition, *C. finmarchicus* at higher temperature fed algae under strong P limitation still produced many eggs and its hatching success was surprisingly high, though the egg productions in the present study were all lower. Reduced contents of EPA and DHA is likely the reason of decreased egg production. Egg size in the present study was larger at higher temperature, and it seems that lower production rate caused larger egg since *C. finmarchicus* was struggling to maintain the egg quality and its hatching rate at relatively high success as a compensation in harsh circumstances. Nevertheless, this result is firstly found in the present study, and further research is required.

Lastly, the experimental set-up in this study had caused few crucial problems and improvement of this is needed if similar study is considered in the future.

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APPENDIX

Appendix A.

Recipe of modified Conwy medium used in this study, prepared by Dag Altin

Major nutrients	Amount (mg/ml)/(g/L)	Exact amount
$FeCl_3 \times 6H_2O$	1.30 g	1.31 g
$MnCl_2 \times 4H_2O$	0.36 g	0.3595 g
H_3BO_3	11.20 g	11.2405 g
$Na_2EDTA \times 2H_2O$	30.00 g	30.0024 g
$NaNO_3$	125.00 g	125.1095 g
Vitamins		
Thiamine-HCL	0.10 g	0.10 g
Cyanocobalamine Stock (5.0 ml 1mg/ml = 5.0mg)	5.00 mg	5.00 mg
Trace Metal Stock (50% strength)	2.00 ml	2.0 ml
Deionised water	1.00 L	1.00 L

Cyanocobalamine Stock (04.02.2020, frozen)	Amount (g/ml)	Exact amount
Cyanocobalamine (vit. B_{12})	0.25 g	0.2509 g
Deionised water	250 ml	250 ml

Trace Metal Stock (50% strength) (05.07.2018)	Amount (g/ml)	Exact amount
ZnCl2	2.6250 g	2.6293 g
CoCl2	2.5000 g	2.5044 g
(NH4)6Mo7O24 x 4H2O	1.1250 g	1.1215 g
CuSO4 x 5H2O	2.5000 g	2.5015 g
Deionised water	250ml	250ml

NaH_2PO_4	Amount (g/ml)	Exact amount
100%	17.40 g	17.40 g
50%	8.71 g	8.71 g
12.5%	2.17 g	2.17 g

Ref: Andersen, 2005

Appendix B.

Growth condition of *R. baltica*

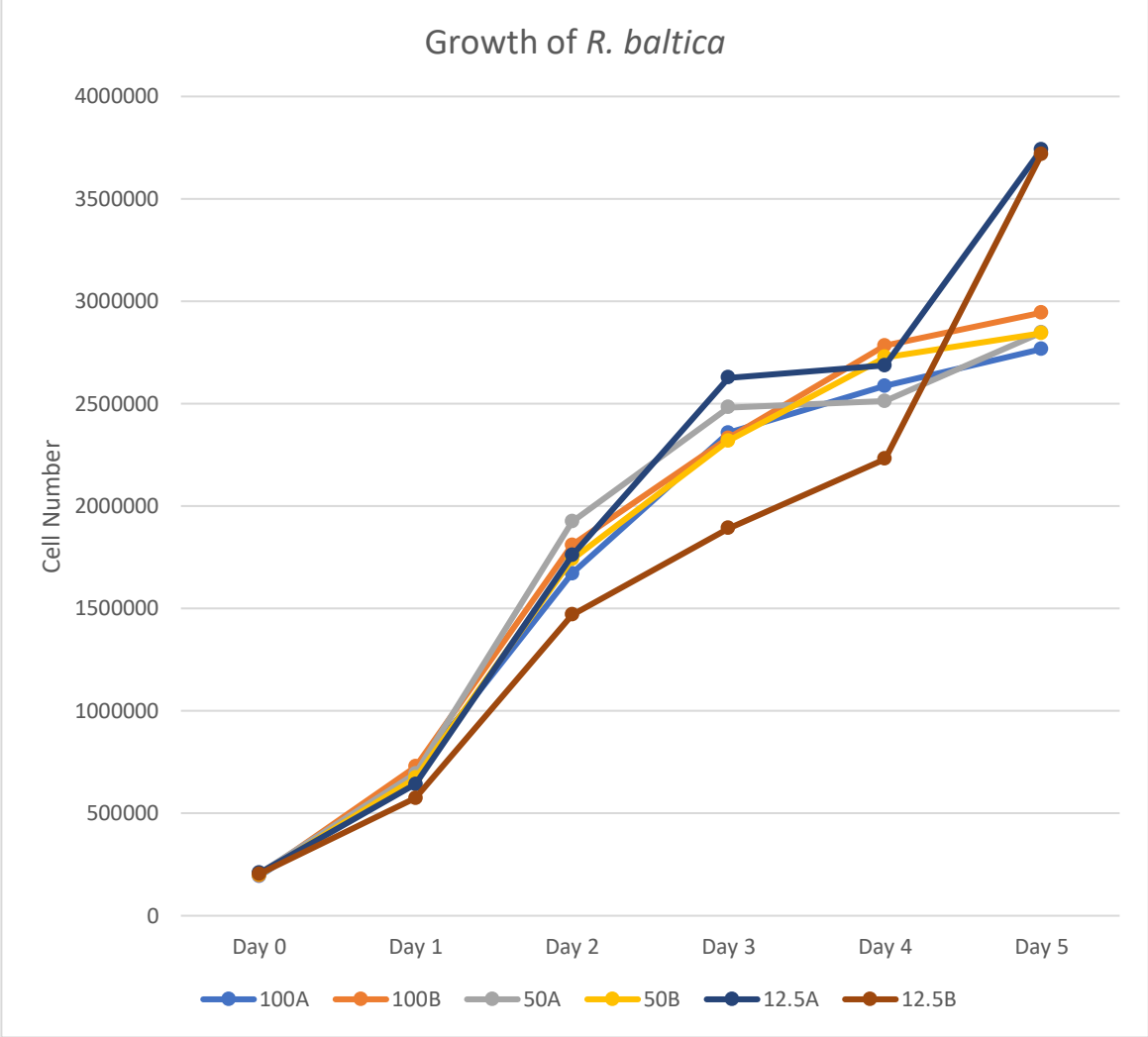

Figure B1. Growth rate was evaluated by the actual cell number in the flask. *R. baltica* was grown in p level modified Conwy medium

Appendix C.

Feeding summary of *C. finmarchicus*

Table C1. Feeding status of *C. finmarchicus* at 12°C. The value is the concentration of carbon in µg per liter in the experimental tanks.

	Treatment								
Day	P100A	P100B	P100C	P50A	P50B	P50C	P12.5A	P12.5B	P12.5C
1	168	163	140	140	138	165	185	176	184
2	146	155	139	166	169	190	162	133	145
3	195	208	169	162	160	202	217	200	195
4	189	201	180	168	168	214	147	136	124
5	151	175	129	162	150	202	176	168	153
6	171	189	142	155	155	220	172	152	144
7	158	194	149	134	142	204	164	150	150
8	133	155	128	141	148	209	153	146	143
9	136	160	91	117	122	161	165	146	152
10	116	121	101	142	146	239	148	137	129
11	241	183	150	200	212	310	160	151	150
12	193	188	146	171	150	213	143	138	131
13	168	172	142	156	145	206	152	148	139
14	162	169	133	139	137	191	144	134	131
15	147	159	130	166	140	193	164	161	146
16	134	139	115	118	113	160	152	141	125
17	149	163	136	132	129	181	174	160	147
18	140	151	123	124	122	178	157	137	132
19	145	154	126	128	126	182	172	151	140
20	174	170	151	135	135	194	173	158	147
21	160	166	141	127	123	181	156	144	131
22	177	156	146	131	136	181	152	139	130
23	158	150	136	116	120	167	154	143	132
24	163	167	141	126	125	170	139	138	127
25	146	158	137	110	109	160	151	142	140
26	152	157	133	115	108	165	151	133	136
27	153	165	133	128	116	174	152	147	148
28	159	166	129	142	146	205	140	128	123

Table C2. Feeding status of *C. finmarchicus* at 14°C. The value is the concentration of carbon in µg per liter in the experimental tanks.

	Treatment								
Day	P100A	P100B	P100C	P50A	P50B	P50C	P12.5A	P12.5B	P12.5C
1	152	164	148	124	128	128	221	174	195
2	136	145	133	180	201	166	188	134	140
3	183	184	169	188	149	168	256	198	208
4	161	175	171	193	164	190	188	127	130
5	142	143	144	180	141	155	227	150	164
6	164	153	149	176	146	167	225	148	152
7	146	147	134	157	130	162	206	139	143
8	141	149	135	174	131	158	214	141	139
9	179	176	167	125	95	124	218	144	163
10	133	145	137	178	97	141	209	129	133
11	181	193	176	231	180	237	241	169	165
12	165	180	163	182	159	207	201	135	138
13	148	163	144	192	162	187	208	143	152
14	152	159	142	170	138	155	199	129	131
15	147	152	135	174	147	164	216	151	155
16	132	138	120	134	114	139	197	129	134
17	159	166	146	151	149	158	217	142	144
18	143	151	132	141	125	143	222	156	150
19	140	155	128	148	131	144	192	135	133
20	170	180	161	151	139	149	189	142	144
21	158	174	156	147	132	142	170	128	131
22	152	170	155	162	143	153	173	131	135
23	147	158	148	141	133	140	166	125	142
24	151	166	147	141	128	147	167	119	130
25	151	167	143	134	128	148	175	130	138
26	131	154	135	134	129	145	170	126	134
27	135	181	141	140	140	157	172	127	139
28	163	188	175	137	132	130	186	149	150

Appendix D.

Equation of the food supply for *C. finmarchicus*

Daily concentration that needed for the feeding of *C. finmarchicus* was calculated based on the equations as following:

$$Water\ Volume = Inlet\ water\ (ml/min) \cdot 1440\ (min) \cdot 3\ (tanks)$$

Daily volume of the water that had been flowed into for three tanks (Replication) in 24 hours was calculated, while inlet water flow was measured and used as a constant number.

$$Actual\ C\ in\ tanks = \frac{Cell\ density\ in\ tanks \cdot 54.6\ (pg\ C/Cell)}{1000}$$

Actual carbon status of the experimental tanks was obtained, while cell density was measured by Coulter Counter, 54.6 is a constant number of the carbon content in *R. baltica*

$$Required\ density = \frac{54.6\ (pg\ C/Cell) \cdot 1000}{(200\ (\mu g\ C/L)\ - Actual\ C\ in\ tanks\ (\mu g\ C/L)) + 200(\mu g\ C/L)}$$

Required microalgal cell number per ml for that day was calculated. While 200µg C/L is the constant concentration that was set for the feeding of *C. finmarchicus*.

$$Required\ volume = \frac{Water\ Volume \cdot Required\ density}{Stock\ culture\ density}$$

Required microalgal volume for the tanks in ml was calculated, while stock culture density was measured by Coulter Counter.

$$Corrected\ volume = \frac{Required\ volume}{10000ml \cdot 9000ml}$$

Corrected volume for the tanks as the final volume was obtained, while 10000ml is the capacity of feeding stock tank, 9000ml is the usable volume per day to prevent *C. finmarchicus* from starvation when feeding routine was not able to be done on time.

Appendix E.

Survived total number of *C. finmarchicus*, and its sex distribution at 12°C

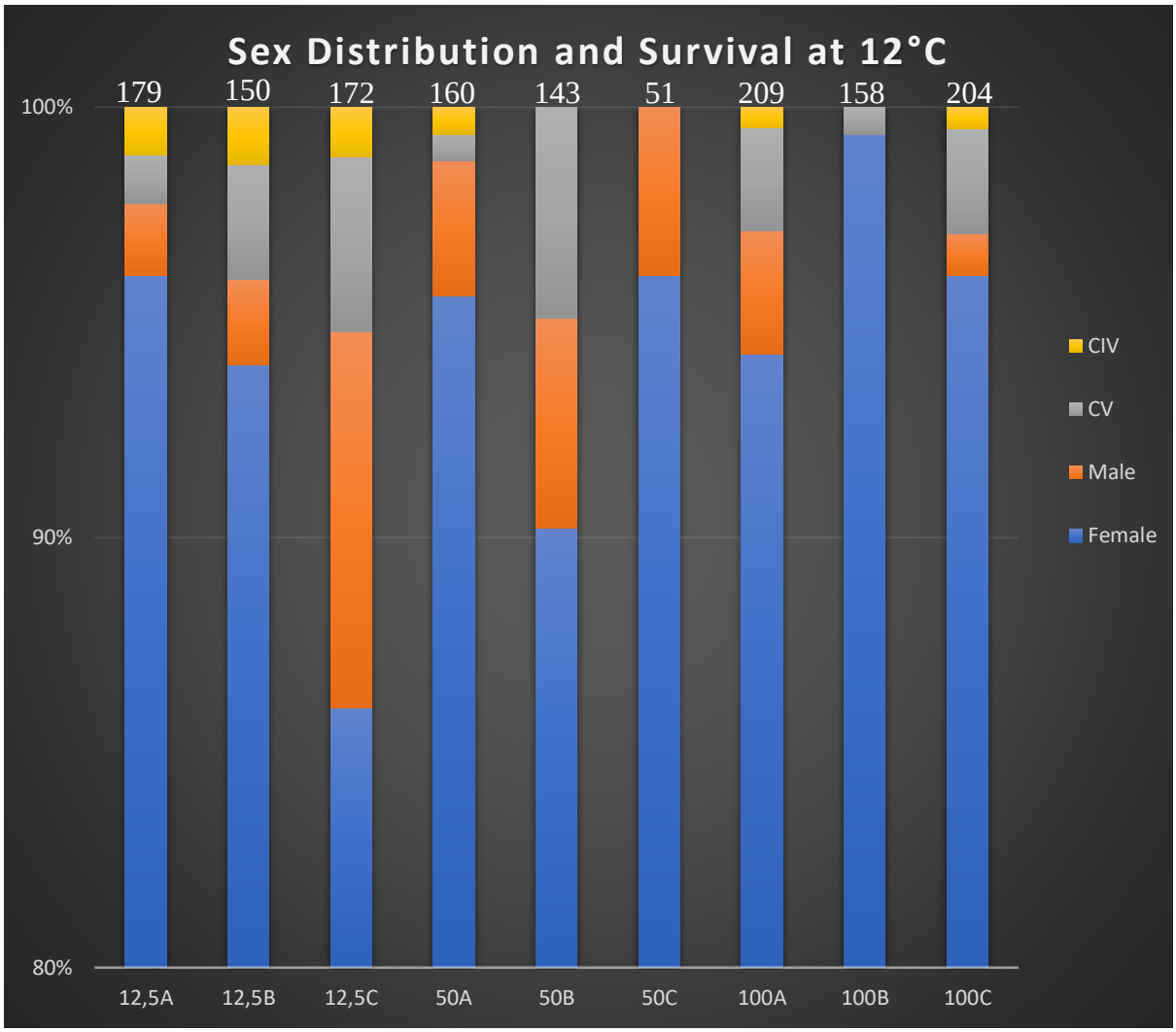

Figure E1. Sex and stage ratio of the population and total number survived throughout the experiment at 12°C. The bars indicate the distribution, and the numbers are the number of individuals that had survived.

Survived total number of *C. finmarchicus*, and its sex distribution at 14°C

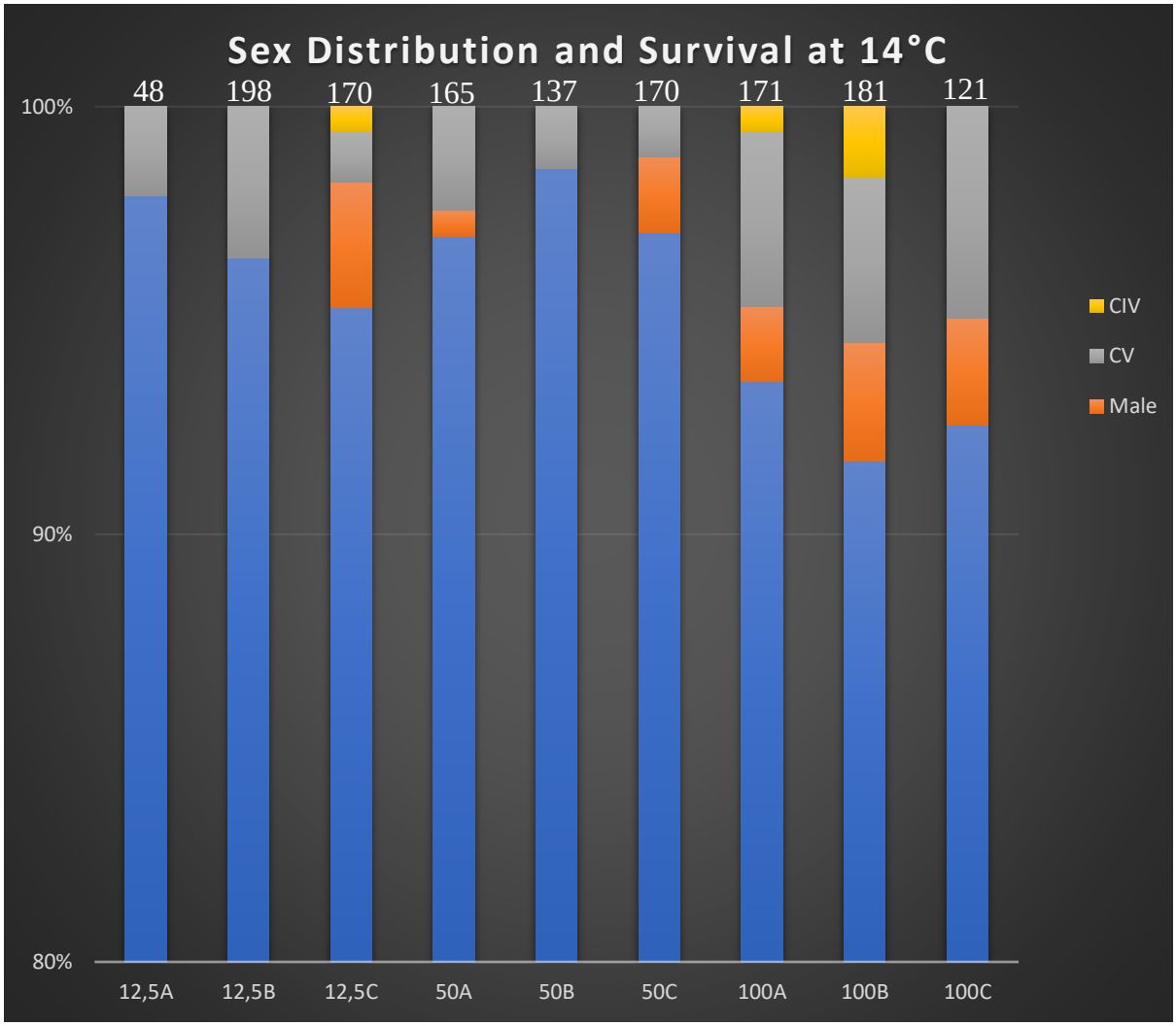

Figure E2. Sex and stage ratio of the population and total number survived throughout the experiment at 14°C. The bars indicate the distribution, and the numbers are the number of individuals that had survived.