Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

Ragnhild Lind Rong

Microbial quality of the copepod *Acartia tonsa* for use as live feed for marine larvae

A process-evaluation

Master's thesis in Chemical Engineering and Biotechnology
Supervisor: Olav Vadstein
June 2020

C-Feed

NTNU
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Science and Technology

CFEED

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I would like to give a huge thanks to my supervisor, Olav Vadstein, for his patience, interesting discussions and for pushing me to create as good a product as possible. Thanks to C-Feed for wanting to work with me on this thesis. Without Yngve Attramadal and Maren Gagnat believing in me and showing an interest in the research, this thesis would not have been a reality. I would also like to thank Amalie Horn Mathisen for teaching me more about working in a lab, than I learned in my previous four years of this education.

Summary

Copepods have been found to be a superior live feed for marine larvae compared to more traditional feed like *Artemia* and rotifers. This makes them an important food source in aquaculture. However, like every other live feed option, the risk of pathogen transfer to the marine larvae is present. Here, the microbial quality in the process of rearing the copepod *Acartia tonsa* was assessed. Using the quantitative measures of total cell concentration and CFU, as well as the qualitative measures of high RNA content, growth potential and fast growing microbes, the microbial quality has been assessed based on r- and K-theory. Haemolytic activity and taxonomy were used to determine the risk of pathogens within the process, and bacterial cell contribution from sub-processes was estimated. Microbial community diversity was assessed, using both phenotypic- and genotypic fingerprinting methods. It was found that the water associated with copepod rearing had an unstable and undetermined selection regime, shifting from r-selection to K-selection. But without reaching a stable microbial water quality through the production cycle. The inn-water had an unfavorable selection regime (r-selection), while the algae reservoir had more favorable K-selection. As the latter contributed to the copepod water with 97-99% of the supplied microbial cells, the selection regime of the inn-water was deemed less important. Due to limiting analysis methods, the selection regime of the copepods could not be safely determined. No heamolytic activity was discovered within the process. The fish pathogen associated genera *Flavobacterium* and *Tenacibaculum* were discovered for *A. tonsa* and associated water. However, the absence of haemolytic activity lead to the conclusion that the copepods were a good quality live feed choice for marine larvae.

Sammendrag

Copepoder har vist seg å være et godt levendefôr-alternativ for marine larver. Overlegent sammenlignet med mer tradisjonelt levendefôr, som *Artemia* og rotatorier. Dette gjør copepoder til en viktig fôrkilde innenfor akvakultur. Den samme utfordringen som ved andre levendefôr-kilder er likevel tilstede, i form av risiko for overføring av patogener. Her har jeg vurdert den mikrobielle kvaliteten i produksjonsprosessen av copepoden *Acartia tonsa*. Ved hjelp av de kvantitative målene total cellekonsentrasjon og CFU, samt de kvalitative målene av celler med høyt RNA-innhold, vekstpotensiale og rasktvoksende mikrober, har den mikrobielle kvaliteten blitt vurdert med grunnlag i r- og K-teorien. Hemolytisk aktivitet og taksonomi ble brukt til å bestemme risikoen for patogener tilstede i prosessen. Mikrobielt cellebidrag fra delprosesser ble også estimert. Mikrobielt samfunnsmangfold ble vurdert, både ved hjelp av fenotypiske og genotypiske fingerprinting-metoder. Vannet assosiert med copepodene ble funnet til å være ustabilt og seleksjonsregimet så ut til å sakte skifte fra r- til K-seleksjon. Det ble imidlertid ikke observert noen stabilisering av mikrobiell vannkvalitet gjennom produksjonssyklusen. Inn-vannet ble vurdert til å ha den lite gunstige seleksjonen for r-strateger, mens algereservoiret hadde mer gunstig K-seleksjon. Siden sistnevnte sto for 97-99% av det mikrobielle cellebidraget, ble innvannet vurdert som mindre viktig. På grunn av begrensende analysemetoder kunne ikke seleksjonsregimet til copepodene bestemmes. Ingen hemolytisk aktivitet ble funnet i produksjonsprosessen. Bakterieslektene *Flavobacterium* og *Tenacibaculum*, assosiert med fiskepatogener, ble funnet for *A. tonsa* og vannet assosiert med copepodene. Fraværet av hemolytisk aktivitet gjorde imidlertid at copepodene ble vurdert til å ha en høy kvalitet som levendefôr for marine larver.

Table of Contents

Summary i

Sammendrag i

Table of Contents iii

List of Tables vii

List of Figures ix

Abbreviations xiii

1 Introduction **1**
- 1.1 Copepods role as live-feed in aquaculture 1
- 1.2 Marine Microbial Ecology and the theory of r- and K-selection 2
- 1.3 Production of the copepod *Acartia tonsa* 4
- 1.4 Aim 5

2 Principals of analytical methods used **7**
- 2.1 Determining percentage of fast-growing microbes 7
- 2.2 Quantification of microbes with haemolytic activity 8
- 2.3 Total cell count from flow cytometry 8
- 2.4 Using flow cytometry for diversity analysis 9
- 2.5 Using RNA content to estimate percentage of fast -growing microbes . . . 10
- 2.6 Estimating community composition and diversity by Illumina sequencing 10

3 Material and methods **13**
- 3.1 Sampling strategy and sample handling 13
- 3.2 Analytical methods 14
  - 3.2.1 Plate counting of bacteria 14
  - 3.2.2 Haemolytic activity 15

3.2.3 Flow cytometry for counting and phenotypic fingerprinting of bacteria . . . 17
3.2.4 Illumina sequencing . . . 19
3.3 Calculations . . . 21

4 Results **23**
4.1 Algae reservoir . . . 23
4.1.1 Microbial density: CFU and total cell count . . . 23
4.1.2 Percentage of fast growing microbes . . . 26
4.1.3 Growth potential . . . 27
4.1.4 Percentage of microbes with high RNA content . . . 27
4.2 In-water . . . 28
4.2.1 Microbial density: CFU and total cell count . . . 28
4.2.2 Percentage of fast growing microbes . . . 31
4.2.3 Growth potential . . . 31
4.2.4 Percentage of microbes with high RNA content . . . 32
4.3 Copepod water . . . 33
4.3.1 Microbial density: CFU and total cell count . . . 33
4.3.2 Percentage of fast growing microbes . . . 35
4.3.3 Growth potential . . . 36
4.3.4 Percentage of microbes with high RNA content . . . 36
4.4 Copepods . . . 37
4.4.1 Microbial density from CFU's . . . 37
4.4.2 Percentage of fast growing microbes . . . 38
4.5 Haemolytic activity . . . 38
4.6 Statistical comparison of cell densities between sample types and cultivation rounds . . . 39
4.7 Microbial cell contribution to copepod tanks . . . 39
4.8 Diversity analysis based on phenotypic fingerprinting . . . 41
4.8.1 Alpha diversity . . . 41
4.8.2 Beta diversity . . . 43
4.9 Illumina sequencing . . . 44
4.9.1 Taxonomy . . . 45
4.9.2 Alpha diversity . . . 47
4.9.3 Beta diversity . . . 48

5 Discussion and conclusion **51**
5.1 Discussion . . . 51
5.1.1 Total cell concentration and RNA content . . . 51
5.1.2 Nutrient availability . . . 52
5.1.3 CFU and fast growing microbes . . . 53
5.1.4 Haemolytic activity . . . 54
5.1.5 Growth rate and cell contribution . . . 54
5.1.6 Phenotypic fingerprinting . . . 55
5.1.7 Genotypic fingerprinting . . . 56
5.2 Conclusion . . . 57

5.3 Further research 59

6 Bibliography 61

Appendix 67
A Raw data parameters for the tanks 67
B Illumina 16S rRNA sequencing 70
B.1 Sample information 70
B.2 Mastermix for first round of PCR 70
B.3 Indexing and mastermix for second round of PCR 71

List of Tables

1.1 Some key attributes for crowded versus uncrowded environments which affect fitness. Table reproduced from Andrews and Harris (1986). 3

4.1 Average values of investigated quantities for all sample types, with standard deviation. 39
4.2 Alpha diversity in terms of Hill order 1, 2 and 3 and evenness (E) for all sample types, separated by production cycles (R). 47

6.1 Raw data for rearing of the copepods during the production cycle in Round 1. 68
6.2 Raw data for rearing of the copepods during the production cycle in Round 2. 68
6.3 Raw data for rearing of the copepods during the production cycle in Round 3. 69
6.4 Samples and corresponding number analyzed by Illumina 16S rRNA sequencing. 70
6.5 Recipe for mastermix of a total volume of 25 μl. 70
6.6 Recipe for mastermix of a total volume of 25 μl. 71

List of Figures

1.1 A) shows an *A. tonsa* nauplius in growth stage 3 (n3). B) shows an *A. tonsa* copepodite in growth stage 5 (c5). *Source: C-Feed*. 4

1.2 In addition to producing copepods, C-Feed also produces the algae *R. baltica* that is used as feed for the copepods. The figure shows the tanks where *R. baltica* is produced and a 400X magnification of *R. baltica*. *Source: C-Feed*. 5

2.1 A schematic overview of a flow cytometer. For every cell the laser hit, emitted light will be detected by specific detectors that correspond to a given wavelength. Here this is illustrated in different colors. (*Illustration by IDEX Health & Science, Semrock*). 9

3.1 Schematic drawing of both algae and copepod production at C-Feed's facillity. The red arrows indicate sampling points in the production. 1) algae reservoir, 3) copepod water and 4) the copepods were sampled on day 0, 1, 2, 3, 5, 7, 9, 12 and 14. 2) in-water was sampled on day 3, 5, 7, 9, 12 and 14. 13

3.2 Example of colony growth on the M-65 medium after ten days. 15

3.3 CFU's on a blood agar plate 20 hours after incubation. 16

3.4 Four different gating plots, with two different gates (total cell count and cells with high RNA content) in each plot are shown as an example, for the FL1-channel (y-axis) and FSC-channel (x-axis). R2 means round 2 or tank 2, Ar is the algae reservoir, Cw is the copepod water, Iw is the in-water and I means initial sampling. The numbers corresponds to dilution (100 is 1:100 dilution with TE buffer and 10 is 1:10 dilution. 18

4.1 Cell concentration [cfu/ml] from CFU's of the algae reservoir (y-axis) as a function of time (x-axis) for the three different replicates. Standard deviation for each sample point is given. 23

4.2 Total cell concentration, obtained from flow cytometry, [cells/ml] (y-axis) as a function of time (x-axis) for the three replicates from the algae reservoir. 24

4.3 Average total cell concentration (from flow cytometry and from CFU's) [cells/ml] (y-axis) as a function of time (x-axis) are shown for the algae reservoir. 25
4.4 Average total cell concentration (from flow cytometry and from CFU's) [cells/ml] (y-axis) for each of the three replicates (x-axis) are shown for the algae reservoir. 25
4.5 Percentage of fast growing microbes calculated from the CFU's, as percentage of visible colonies on the plates after three days of incubation, is shown for the algae reservoir. 26
4.6 Doubling number for growth potential, obtained as shown in Equation 3.4 (y-axis) plotted against time (x-axis). 27
4.7 Percentage of microbes with a high RNA content obtained from the flow cytometry analysis are shown for all three replicates as a function of time (x-axis) for the algae reservoir. 27
4.8 Cell concentration [CFU/ml] obtained from CFU's on the in-water plates (y-axis) as a function of time (x-axis) is shown for the three different replicates. Standard deviation for each sample point is given. 28
4.9 Total cell concentration, obtained from flow cytometry, [cells/ml] (y-axis) as a function of time (x-axis) for the three replicates from the in-water. . . 29
4.10 Average total cell concentration (from flow cytometry and from CFU's) [cells/ml] (y-axis) as a function of time (x-axis) are shown for the in-water. 29
4.11 Average total cell concentration (from flow cytometry and from CFU's) [cells/ml] (y-axis) for each of the three replicates (x-axis) are shown for the in-water. 30
4.12 Percentage of fast growing microbes calculated from the CFU's, as percentage of visible colonies on the plates after three days of incubation, is shown for the in-water. 31
4.13 Doubling number for growth potential, obtained as shown in Equation 3.4 (y-axis) plotted with time (x-axis). 31
4.14 Percentage of microbes with a high RNA content obtained from the flow cytometry analysis are shown for all three replicates as a function of time (x-axis) for the in-water. 32
4.15 Cell concentration [cfus/ml] from CFU's of the copepod water (y-axis) as a function of time (x-axis) for the three different replicates. Standard deviation for each sample point is given. 33
4.16 Total cell concentration, obtained from flow cytometry, [cells/ml] (y-axis) as a function of time (x-axis) for the three replicates from the copepod water. 34
4.17 Average total cell concentration (from flow cytometry and from CFU's) [cells/ml] (y-axis) as a function of time (x-axis) are shown for the copepod water. 34
4.18 Average total cell concentration (from flow cytometry and from CFU's) [cells/ml] (y-axis) for each of the three replicates (x-axis) are shown for the copepod water. 35

4.19 Percentage of fast growing microbes calculated from the CFU's, as percentage visible colonies on the plates after three days of incubation, is shown for the copepod water. 35
4.20 Doubling number for growth potential, obtained as shown in Equation 3.4 (y-axis) plotted with time (x-axis). 36
4.21 Percentage of microbes with a high RNA content obtained from the flow cytometry analysis are shown for all three replicates as a function of time (x-axis) for the copepod water. 37
4.22 Cell concentration [cells/copepod] obtained from CFU's of the copepod plates (y-axis) as a function of time (x-axis) is shown for the three different replicates. Standard deviation for each sample point is given. 37
4.23 Percentage of fast growing microbes calculated from the CFU's, as percentage of visible colonies on the plates after three days of incubation, is shown for the copepods. 38
4.24 The percentage contribution of microbial cells to the copeod rearing tanks from the algae reservoir relative to the in-water. 40
4.25 The cell concentrations in terms of net change, loss and supply are plotted with time. Benchmark, or start concentration was set to $2.0 \cdot 10^{12}$ cells per milliliter to give the same starting point for all three average concentration rates. Because of little consistency in the start up of the copepod tanks, day 2 was chosen as the starting point. 40
4.26 The cell concentrations in terms of net change and loss are plotted with time. Benchmark, or start concentration was set to $2.0 \cdot 10^{12}$ cells per milliliter to give the same starting point for both average concentration rates. Because of little consistency in the start up of the copepod tanks, day 2 was chosen as the starting point. 41
4.27 Development in Phenotypic diversity with time for the different sample types are shown for Hill orders 0, 1 and 2. Abbreviations Ar, Cw and Iw corresponds to algae reservoir, copepod water and in-water, respectively. The line for each sample type represents the mean value at the specific day. 42
4.28 Principal Coordinates Analysis (PCoA) plot, based on Bray-Curtis metrics, showing beta diversity. The different symbols corresponds to sample types (Ar: algae reservoir, Cw: copepod water and Iw: in-water). The colors corresponds to the sampling day. In total 62.2% of the variance are explained by this plot. 43
4.29 The DNA fragment size of the products is according to the ladder approximately 500 bp. The ladder used was GeneRuler 1 kb plus DNA ladder (Thermo Fisher Scientific). Sample 1, 2, 5, 6, 10, 14 and 18 are from the copepods; sample 2, 7, 11, and 15 are from the algae reservoir; sample 4, 8, 12 and 16 are from the in-water; sample 5, 9, 13 and 17 are from the copepod water. PC represents the positive control and NC represents the negative control. Additional information about the individual samples are found in Appendix, Table 6.4 44

4.30 Taxonomy on phylum level, for the samples taken on day 5 and 7 of the cycle for all three production cycles, in percentage. Cop and Naup means *A. tonsa* at copepodite and nauplii stage, respectively. Cw is the copepod water, Iw is the inn-water and Alg is water from the algae reservoir. C and N corresponds to the water samples associated with either the copepodite (C) or the nauplii (N), and R1, R2 and R3 corresponds to production round 1, 2 and 3, respectively. 45
4.31 Taxonomy on class level, for the samples taken on day 5 and 7 of the cycle for all three production cycles, in percentage. Cop and Naup means *A. tonsa* at copepodite and nauplii stage, respectively. Cw is the copepod water, Iw is the inn-water and Alg is water from the algae reservoir. C and N corresponds to the water samples associated with either the copepodite (C) or the nauplii (N), and R1, R2 and R3 corresponds to production round 1, 2 and 3, respectively. 46
4.32 A) shows the Bray-Curtis distance (56.7% of the variance explained) and B) shows the Dice-Sørensen distance (58.7% of the variance explained). For both plots the filled symbols corresponds to samples associated with the copepodites and the other symbols corresponds to samples associated with the nauplii. The triangles are the copepod samples, circles are the copepod water, diamonds are the algae reservoir samples and squares correspond to the inn-water. 48

Abbreviations

bp	=	base pair
CFU	=	Colony forming units
CV	=	coefficient of variation
D	=	Specific dilution rate
d	=	dilution factor
DN	=	Doubling number
DOM	=	Dissolved organic matter
FCR	=	Feed conversion ratio
GPV	=	logarithmic growth potential value
NAD	=	Nicotinamide adenine dinucleotide
NC	=	Negative control
OTU	=	Operational taxonomic unit
p	=	probabillity value
PC	=	Positive control
PCA	=	Principal component analysis
PCoA	=	Principal coordinate analysis
PCR	=	Polymerase chain reaction
R	=	Specific net rate of change
r	=	net change factor
RAS	=	Recirculating Aquatic Systems
rRNA	=	ribosomal RNA
S	=	Specific supply rate
s	=	supply factor
SD	=	standard deviation
UV	=	Ultraviolet

Chapter 1

Introduction

1.1 Copepods role as live-feed in aquaculture

Copepods are believed to be the most abundant multicellular organisms in the world (Hammervold et al., 2015). This makes the copepods an important food source for many different species across the higher trophic levels, both in fresh- and salt water. It is also a grazer and omnivorous species on the lower trophic levels (Leandro et al., 2006). Since the copepods are such an important food source for fish fry in the wild, it makes them a good choice of feed for reared fry. In particular for fry that are considered more challenging to rear (Højgaard et al., 2008). It has been shown that aspects like size, behaviour and nutritional value makes the copepods superior compared to more traditional feed like *Artemia* and rotifers. A lot of research have been done on production of copepods, as well as copepods impact in the wild (Berggreen et al. (1988), Castonguay et al. (2008), Peterson et al. (1991)). However, there is minimal research on the microbial growth associated with copepod production. With live feed there is always a risk of transferring pathogens to the vulnerable fry. Especially since it is challenging, if not impossible, to properly disinfect live copepods. More research into the microbial growth in regards to copepod production is therefore necessary to ensure the safest feed option for fry. Demand for such options will only keep increasing in the future.

In the last decades there have been a rapid increase in the human population and with that an increased demand for food production, and especially high-quality protein. Historically seafood have been a good source for such protein. Seafood have a lower FCR (food conversion efficiency, e.g. how many kilos of feed is needed to produce 1 kg of meat) than conventional meat production, like beef and pork (Martínez-Córdova et al., 2017). A major problem, however, is the over-exploitation of fish-populations. In 2009 it was estimated that only about 12% of the marine populations had not been exploited, 60% had been exploited to a certain degree and 30% had been severely over-exploited (Martínez-Córdova et al., 2017). This shows the importance of aquaculture to take some of the pressure off the wild marine populations. While the amount of fish caught by capture fisheries sta-

bilized around 90 million tonnes in 1994, aquaculture production more than doubled in the 1990s, and have steadily increased with about 6% per year in the period 2000-2014 (Guillen et al., 2019). All of this shows how important aquaculture is for food demand in the future. However, aquaculture still face some challenges.

In aquaculture microbes have been a major focus over the years. In particular the pathogens (De Schryver and Vadstein, 2014). Antibiotic use and disinfection methods have been widely used to battle the microbes (Summerfelt (2003), Kasai et al. (2002), Jorquera et al. (2002)). However, knowledge of the microbial growth in the different aspects of rearing is important to ensure both the best quality and yield of the reared species. It has even been suggested that microbes can be beneficial in fish rearing. Olav Vadstein proposed that the approach to microbes should be be changed from the traditional view of 'beat them' to 'join them' (Vadstein et al., 2018).

1.2 Marine Microbial Ecology and the theory of r- and K-selection

To get a better idea of how microorganisms behave in aquaculture, a brief understanding of marine microbial ecology is necessary. According to Kirchman (2010) a simple definition for marine microbial ecology is 'The study of the ecology of microbes in marine systems' where 'Microbes' are defined as all organisms smaller than 100 μm (Kirchman, 2010). The microogranisms are responsible for a lot of important chemical processes in marine systems. For example: Primary producers fixate CO_2 to produce organic material using light as an energy source. The photoheterotrophs also use light as an energy source, but need organic material as a carbon source. Heterotrophic procaryotes mineralize and oxidize dissolved organic matter (DOM) to produce biomass and inorganic byproducts. Nitrifiers oxidate ammonium to nitrate and denitrifiers release N_2 during either oxidation of ammonium or reduction of nitrate (Kirchman, 2010). The nitrifiers and denitrifiers are also important in waste water treatment and a key component in Recirculating Aquatic Systems (RAS) (Klas et al., 2006). The mentioned processes are just a small fraction of everything the microorganisms are responsible for in marine ecology and simply a means to illustrate their importance for a stable water environment.

For microbial ecology there have been, and still are, limitations in methods and knowledge necessary to explain the behaviour, composition and symbiosis in microbial communities. A proposed theory that have been investigated in relation to microbial ecology is Robert Mac Arthur's 'The theory of island biogeography' (1967), where he introduced the terms r- and K-strategists (Mac Arthur and Wilson, 1967). Here he proposed that so-called r-strategists grow fast and have a general adaptation to an environment. On the other hand, the K-strategist has a slower increase in population, but over a longer time period they are able to utilize more specific parts of the environment. The K refers to carrying capacity and the r stands for the maximum intrinsic rate of natural increase (r_{max}) (Pianka, 1970). This means that in a stable environment the K-strategists are better at competing for resources compared to r-strategists, but the latter is better at quickly adapting to an environment with less competition. r- and K-strategists are often referred to as generalists (r-selection) and

Table 1.1: Some key attributes for crowded versus uncrowded environments which affect fitness. Table reproduced from Andrews and Harris (1986).

Environment	Population density	Per capita food supply	Effect on potential growth inhibition or death acceleration		Potential death acceleration due to predation or parasitism
			Inadequate food	Toxic metabolites	
Uncrowded	Low	High	Low	Low	Low
Crowded	High	Low	High	High	High

specialist (K-selection) (Andrews and Harris, 1986). The former is doing 'many things indifferently' while the latter is 'doing one thing well'. According to Andrews and Harris the organisms are faced with different choices in crowded and uncrowded environments, and the adaptation is either classified as r- or K-related choice. Examples are high rate of acquisition of nutrients (r-strategist) or high affinity for nutrients (K-strategist); high stress resistance of spores (r-strategist) or high sensitivity of spores to stimulation (K-strategist). What is characterizing either a crowded or uncrowded environment is defined by Andrews and Harris and shown in Table 1.1.

This shows that where population densities are low and density-dependent growth factors are negligible, r-conditions are obtained over time. Regulation of populations in such an environment is typically through density-independent mechanisms like storms, extreme temperature changes or similar (Andrews and Harris, 1986), causing selection for r-strategists. Conversely, in crowded environments population densities are high and limited by density-dependent factors such as nutrient supply, predation and toxic metabolites. This environment is therefore selecting for K-strategists.

From this the conclusion can be made, that if a stable environment is obtained, it will select for K-strategists that will out-compete the generalist r-strategists. Currently, the strategy for the aquaculture is disinfectants to reduce the microbial load as much as possible, reasoning that it is necessary to reduce the opportunistic pathogen impact on the reared organism. Amongst methods that are used, ozonation and UV irradiation are some of the most common (Summerfelt (2003), Kasai et al. (2002)). According to the r- and K-theory, reducing the microbial load in the system might select for opportunistic pathogens making an uncrowded environment with a high supply of nutrients and minimal competition (De Schryver and Vadstein, 2014). In the article the authors mention selecting for generalists (K-selection) as a possible way of water quality management based on the theoretical microbial ecology. Since most pathogens are fast growing opportunists, water management like that mentioned above, will decrease the risk of pathogens present.

There are already papers suggesting that selection for K-strategists provide a healthier environment for the fry, in regards to less mortality, better growth and better appetite among fry (Skjermo et al. (1997); Skjermo and Vadstein (1999); Attramadal et al. (2014)).

These papers have one thing in common. Their basis in matured water, with a well established microbial community. This is in agreement with Mac Arthur's theory about the K-strategists dominating a stable environment with an equilibrium between available nutrients and the population densities. To develop a microbial management regime it is important to get an overview of where the microbial populations come from (e.g. the water, the fry itself, live feed), and it has been shown that a big part of the microbes are in fact coming from live feed (Vadstein et al., 2018).

1.3 Production of the copepod *Acartia tonsa*

This thesis was done in collaboration with C-Feed, a copepod production company that produces the species *Acartia tonsa*. Primarily C-Feed sells copepod eggs to the aquaculture and aquarium enthusiasts. These are disinfected before shipping, which means that most of the microbes from the water and outside of the eggs are removed. Secondly they sell live copepods. Figure 1.1 shows the difference between the nauplius stage and the copepodite stage of *A. tonsa* reared at C-Feed. For the live copepods it is not a well established disinfection method that can be used, without them being killed in the process.

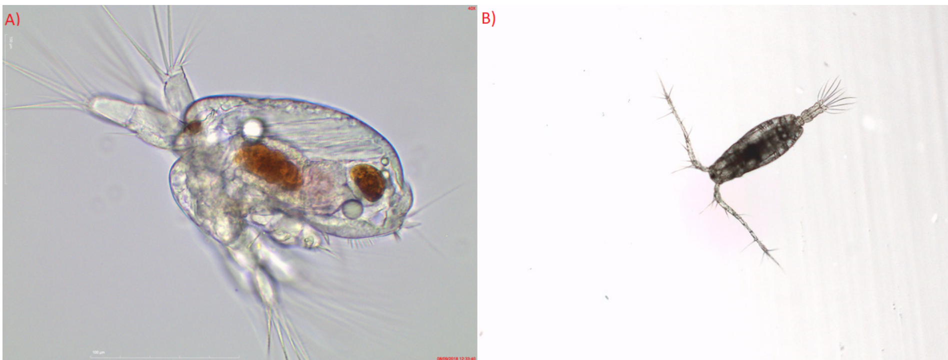

Figure 1.1: A) shows an *A. tonsa* nauplius in growth stage 3 (n3). B) shows an *A. tonsa* copepodite in growth stage 5 (c5). *Source: C-Feed.*

At C-Feed the production of *A. tonsa* is carried out in cycles of 14 days, from hatching to adult size. With 24 hours light cycles and a water temperature around 20 °C the growth rate of *A. tonsa* is approximatley one stage per day. The eggs are harvested and either prepared for sale or used to start a new production cycle. Developmental stages n4 (nauplius stage 4), n5, c1 (copepodite stage 1) and c2 are most commonly sold as live feed because of size. As feed for the copepods C-Feed uses the cryptophyte algea *Rhodomonas baltica*, that has been shown to be a suitable feed for *A. tonsa* (Støttrup and Jensen, 1990).

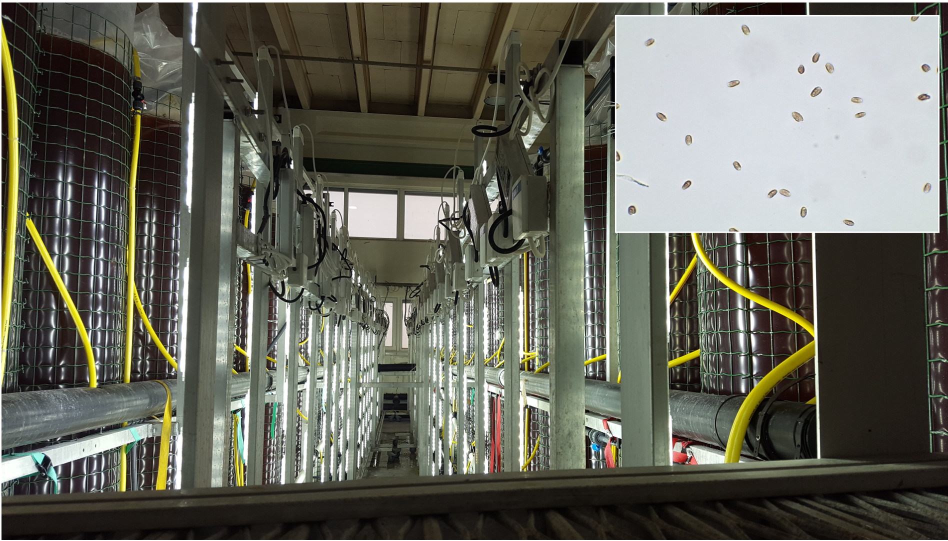

Figure 1.2: In addition to producing copepods, C-Feed also produces the algae *R. baltica* that is used as feed for the copepods. The figure shows the tanks where *R. baltica* is produced and a 400X magnification of *R. baltica*. *Source: C-Feed.*

As a cryptomonade, *R. baltica* has the abillity to perform phagocytosis (ingestion of bacteria) (Clay, 2015). The redish to brown colored algae absorb light at the following wavelengths: 435,638 og 676 nm (Neori et al., 1986). 435 nm corresponds to the spectrum of blue and green light, while the latter two are in the orange to red light part of the spectrum. In the production of *R. baltica* C-Feed starts with 1 L inoculums, upscaling firstly to 10 L tanks, then 40 L tanks and 450 L tanks before the algae gets transferred to the algae reservoir. All tanks from 1 L to 40 L are batch reactors. Each 450 L tanks are continuously supplying the algae reservoir with a rate of 8.4 L per day, and around 160 tanks are in use every day. The algae reservoir is close to a continuous reactor, constantly being fed with algae from the 450 L tanks, and supplying the copepod tanks with algae. The flow through water is started up two days after the eggs hatch, and have an increasing flow with increasing copepod growth.

1.4 Aim

The aim of this thesis was to describe qualitative and quantitative aspects of the microbiota associated with the production of copepods. This was done by solving the following tasks:

- Finding the total cell concentration in the water samples (algae reservoir, copepod water and in-water), and estimate percentage of cells with a high RNA content.
- Investigating nutrient availability by looking at the growth potential.
- Finding CFU concentration for all samples mentioned above, in addition to for the copepods. And using this data to estimate percentage of fast growing cells in the

samples.

- Investigate potential haemolytic activity present.
- Estimating growth rate and loss rate in the copepod water, as well as microbial cell supply from in-water and algae reservoir.
- Determining phenotypic- and genotypic fingerprinting of the communities present in the different samples.

These data would help determining the type of microbial environments present in the different samples. Based on the r-/K-theory the microbial water quality could be assessed. By linking the contribution of cells from different environments to the copepod water, a more qualitative assessment of the microbial water quality associated with the copepods could be done.

Solving this aim will provide new information about the microbiota associated with copepod rearing. How supplied microbial communities will affect the water the copepods are reared in, and help determining the stability of the microbial communities. This, will in turn, provide valuable information about chance of pathogens occurring, and breeding conditions for the copepods. Phenotypic- and genotypic fingerprinting will give a more detailed picture of the microbial communities present in the different processes. This, together with haemolytic activity detected, will help reinforce the conclusions made about microbial water quality. As well as assessing the safety of the copepods as live feed for marine larvae. The former being a basis for further optimization of copepod production, and the latter determining potential risks of feeding marine larvae with live copepods.

Chapter 2

Principals of analytical methods used

2.1 Determining percentage of fast-growing microbes

Counting colony forming units (CFU) on an agar plate is a widely used method to get an estimate of microbial concentration in a given sample. There are also other types of information that can be estimated from the colonies on a plate. Some examples are detecting presence of a particular microbe, diversity by colony morphology and investigating nutrient utilization in a microbial community. When considering the theory behind r- and K-selection, CFU can be used to estimate the percentage of fast-growing microbes, as presented in Salvesen and Vadstein (2000). Here the authors are showing how the maximum specific growth rate, (μ_{max}), of colonies on a plate is decreasing with time. From a high growth rate after one day of incubation ($\mu_{\text{max}} \geqslant 0.32\text{h}^{-1}$) to a much lower growth rate on day 47 (μ_{max} between 0.05 and 0.08 h^{-1}). As mentioned earlier, r-strategists are often called generalists, which means that they have a high μ_{max} when access to nutrients is good and competition is low (Pianka, 1970). Therefore, counting colonies on a plate early after plating, and then comparing this number to the number after an appropriate longer time, can give an estimate of the percentage of r-strategists present in a microbial community. The method have been used with success to investigate microbial communities in soil and roots (De Leij et al., 1994), *Artemia* (Verschuere et al., 1997) and in rearing of turbot fry (Skjermo et al., 1997).

This method is good for getting an estimate of the r-/K-selection in the microbial community in question. However, it is important to acknowledge the limitations of using growth on solid medium. For example, it is well established that many bacterial strains are unculturable on traditional agar plates (Salvesen and Vadstein, 2000), which means that a CFU count from a plate rarely or never will be representative for the total community present in the sample investigated.

2.2 Quantification of microbes with haemolytic activity

Blood agar plates have been used to investigate haemolytic microbes for decades, and even though ”blood agar” is not a defined term, it consists of agar with some type of mammalian blood infused in it (Buxton, 2005). Usually around five percent sheep’s blood, but also horse or rabbit blood have been used to investigate NAD-requiring organisms (organisms that require addition of nicotineamide adenine dinucleotide to preform enzymatic reactions (Gazzaniga et al., 2009)). The blood agar plates have historically mainly been used in the medical field for diagnosis or investigating already known pathogens (Wegner et al. (1992), Edwards and Larson (1974), Parveen et al. (2011)). But blood agar has its purpose in other fields as well. For example it is used in the food industry to investigate the presence of pathogens on food (Hausdorf et al., 2013) and to investigate bacterial communities on surfaces (walls, flooring e.g.) in hospitals (Johani et al., 2018). Common for the different fields, is that blood agar plates are used to either cultivate or detect the presence of potential pathogens by detecting haemolytic acitivity. The number of microbes with haemolytic activity is in regards to live feed, used as a quality measure. Less haemolytic microbes gives a higher quality live feed for the fry (Olsen et al., 2000).

Haemolysis, which is the disruption of the cell membrane of red blood cells (Johani et al., 1972), can be divided into three forms of hemolysis (Buxton, 2005).

- **alpha-haemolysis**: The reduction of the hemoglobin in red blood cell to methmeglobin. This turns up as a green or brown discoloration in the medium surrounding the colony. This is also called ”bruising” the cells and is in fact not a true disruption of the cell membrane. It is also called ”partial hemolysis”.
- **beta-haemolysis** is defined as true lysis of the red blood cells. This type of haemolysis causes destruction of the red blood cells.
- **gamma-haemolysis** is not really a hemolysis, since the red blood cells stay intact. The term is used for microbes that can grow on the blood agar without disruption or destruction of the red blood cells (Buxton, 2005).

2.3 Total cell count from flow cytometry

Flow cytometry is a method that originally was developed for use in the medical field for various types of analysis (Brown and Wittwer, 2000). For example immunophenotyping of cells from different tissues like; blood, bone marrow, serous cavity fluids, urine, solid tissue and cerebrospinal fluid. Since the development of this method, however, it has been shown to have a broader use than just medical analysis. The main advantage of flow cytometry is counting individual cells in a liquid sample quickly and efficiently. Secondly, the method can be used to determined different characteristics of the cells in a sample. E.g. size, cytoplasmic complexity, DNA or RNA content and membrane-bound and intracellular proteins (Mandy et al., 1995).

To run a flow cytometry analysis, first the cells in a sample are stained, using a fluorescent agent (Rong, 2019). This is unless the cells themselves produces a form of fluorescent

agent, like photosynthetic pigments in algae (Determann et al., 1998). After the staining of the sample, the flow cytometer can analyze up to 20.000 cells per second through a focused laser beam. The laser beam excites the fluorescent agent and light at a specific wave length is emitted, as shown in Figure 2.1.

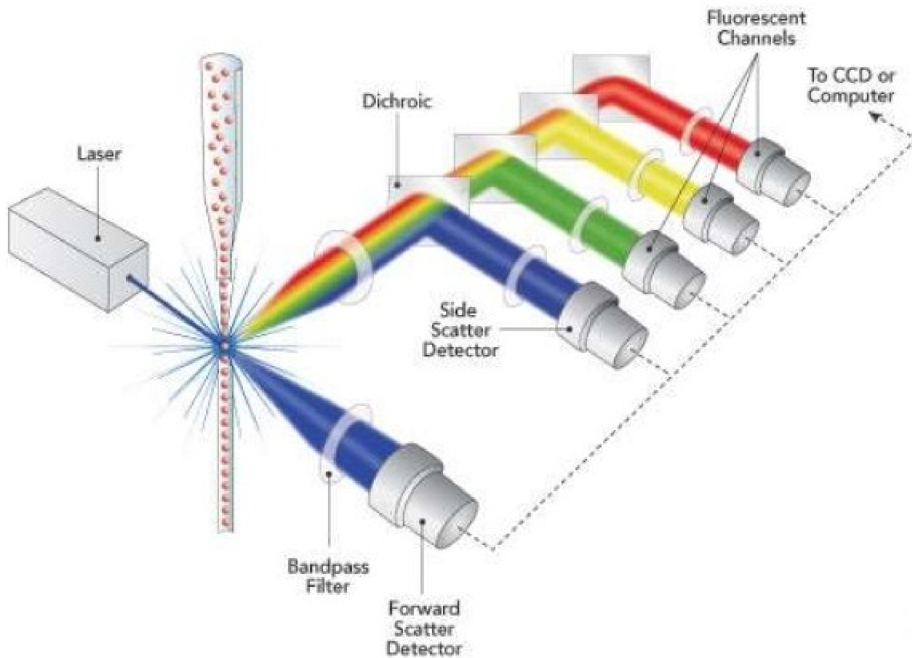

Figure 2.1: A schematic overview of a flow cytometer. For every cell the laser hit, emitted light will be detected by specific detectors that correspond to a given wavelength. Here this is illustrated in different colors. (*Illustration by IDEX Health & Science, Semrock*).

The emitted light is registered by designated detectors. In addition, the light scattering of each cell is measured. The intensity of the emitted light is proportional to the characteristics of the cell being measured. The cell size is measured by forward light scatter, while the complexity is measured by side scatter. Because flow cytometry counts all cells, dead and alive, culturable or not, this method gives a more realistic number of cells in a sample, compared to counting CFU on a plate. In the latter a limitation is dead cells and unculturable cells in the sample that are not reflected on the plate.

2.4 Using flow cytometry for diversity analysis

As mentioned earlier, flow cytometry is more than just a cell counting method. It provides a lot of additional data about the cells investigated (Mandy et al., 1995). These traits have been used to develop a method to analyze diversity within and between communities, by

flow cytometry (Props et al., 2016). The authors used two fluorescent signals to get information about nucleic acid content, as well as two scatter signals to obtain morphological traits of the cells. They used this information to make a diversity analysis of real and mock communities. The results were compared to a standard genotypic analysis (16S rRNA gene amplicon sequencing) for validation. The result was strikingly similar interpretation between the phenotypic and the genotypic fingerprint analysis. Even though this method is quite new, and little research have yet been done on the topic, the result of this study (Props et al., 2016) suggests exciting possibilities in terms of using flow cytometry for diversity analysis in the future.

2.5 Using RNA content to estimate percentage of fast - growing microbes

It is well known that RNA-content in cells is linearly correlated to growth rate. High RNA-content (specifically ribosomal RNA or rRNA) suggests a high growth rate, and low RNA-content corresponds to a lower growth rate (Kerkhof and Ward (1993), Kemp and LaRoche (1993), Kemp (1995), Benthin et al. (1991)). Even though there is not a universal mathematical formula that describes the exact relationship between rRNA-content and specific growth rate, applicable for all microbial species, it has been shown that most species investigated in the laboratory yields results that corresponds to this assumption. For example for *E. coli* grown at different growth rates, the RNA:DNA ratio is linearly correlated to the growth rate (Kemp and LaRoche, 1993). Also for marine species this correlation have been investigated, to see if it is applicable for marine microbial communities (Kerkhof and Ward, 1993). Even though there is still not enough knowledge to say that this is the rule for all microbial species, there is enough data to suggest that it is a general trend. Therefore, measuring the RNA-content of single cells in a microbial community, and separate it into high- and low content, is a good way to estimate the percentage of fast growing microbes present. Since flow cytometry counts individual cells and relies on a dye agent, an RNA dye can be used. The dye will then give a higher signal in the given light spectrum for cells with high RNA-content compared to cells with a lower content of RNA.

2.6 Estimating community composition and diversity by Illumina sequencing

Using methods to determine fast- and slow growing bacteria is a good way to get information about the microbial community in question. However, it does not say anything about the taxonomic composition in the community. Here Illumina sequencing can be used to estimate which kind of microbes are present in the community. Illumina sequencing is in this study based on the 16S rRNA gene. The latter is a useful sequence to investigate to determine taxonomy for a number of reasons; it appears in all prokaryotic cells, has an extreme sequence conservation, and a domain structure where variable evolutionary rates can be found (Tringe and Hugenholtz, 2008). This makes the 16S a well suited gene to

use to get more information about the microbial communities. The 16S gene consists of approximately 1500 base pairs (bp) and is divided into nine variable regions (V1 to V9) (Winand et al., 2020). In-between them more conserved regions are found. The 16S gene have historically been used to classify isolates, but more recently for more complex microbial samples, like those found in the human gut, soil and oceans (Andersson et al. (2008), Hermans et al. (2017), Kirchman et al. (2010)). The Illumina sequencing method performs what is called "short-read" sequencing. This means that short sequences of the 16S gene is obtained. Even though the sequences are too short to cover the entire 16S gene, it can sequence one or more of the variable regions. This makes the method able to differentiate between genus. (Winand et al., 2020).

The way the Illumina sequencing work is that an amplified amount of DNA from a sample (obtained using PCR) is labeled with adapter sequences on the 5'- and 3'-ends of the DNA strands (different adapter on each end) (*Source: Illumina, Inc*). Further on complimentary adapter sequences are fixed on a glass slide, in which the labeled DNA-strands are sticking to. A second PCR-reaction is then run, and bridges between the adapter sequences on the DNA-strands and their complementary adapter sequence on the glass is formed. This results in a collection of many copies of the same DNA-strand on the specific area of the glass slide, that then can be compared with sequences already obtained in a database. This is done by first clustering the DNA-samples into OTU's (operational taxonomic units) with a set percentage of similarity, and then comparing the clusters to those in the database. From this a richness of genus or species is obtained within a certain percentage accuracy (usually 97%) (Schloss and Handelsman, 2005).

There are errors in this method. Amongst other, it has been found that only up to 86% of bacteria can be reliably identified at a species level(Winand et al. (2020)). Other problematic aspects is that the 16S regions have been shown to vary in information contained across different species, genera and families, and varying copy number in different species. The database content is also a limiting factor for classification. Only genera or species already existing in the database are classified. On the taxonomic level of genus, the method seems to be more accurate. A problem however is bias when looking at the richness of the different genera. If there are one or more genera not represented in the result, the relative abundance of the other genera will be miss-represented. Errors when doing the sequencing is another problem.

Chapter 3

Material and methods

3.1 Sampling strategy and sample handling

The production cycle of *A. tonsa* was was investigated by analyzing three whole production cycles of the copepod from eggs hatching until fully grown. This was done in collaboration with C-Feed. A schematic drawing of C-Feed's copepod production cycle, including algae production, is shown in Figure 3.1

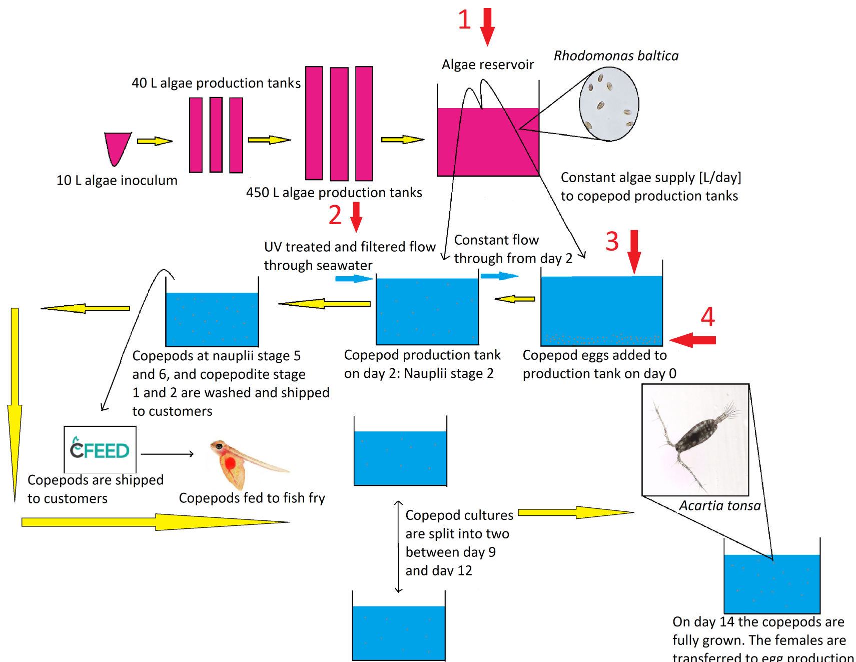

Figure 3.1: Schematic drawing of both algae and copepod production at C-Feed's facillity. The red arrows indicate sampling points in the production. 1) algae reservoir, 3) copepod water and 4) the copepods were sampled on day 0, 1, 2, 3, 5, 7, 9, 12 and 14. 2) in-water was sampled on day 3, 5, 7, 9, 12 and 14.

C-Feed monitored the following chemical and physical variables in the tanks with time: O_2-saturation, NH_4-nitrogen, pH and temperature. They also quantified the following information about the copepods: Survival (in percentage), average length and density (copepods per milliliter), as well as the rate of flow through water and algae into the copepod tanks (in liters per day). The total data set for this is found in the Appendix in Table 6.1, 6.2 and 6.3.

Four different types of samples were investigated throughout the experiment. 1) Water from the algae reservoir, a close to chemostat reactor, with constant in-water from the 450 L algae production tanks, and flow-over of algae to the copepod production tanks. 2) From the in-water; water collected from Trondheimsfjorden, in close proximity to C-Feed's facility. The in-water is filtered and UV-treated before going into the tanks. 3) The copepod water in the tanks, with copepods filtered out by C-Feed. 4) Samples containing copepods of the different stages throughout the cycles.

Samples where taken five times a week (no samples Tuesday and Sunday) for analysis through the whole cycle (14 days) for three consecutive cycles. The samples where taken in 20 ml sterile tubes every sampling day by C-Feed. The samples where then shipped with the speed boat to Trondheim with an estimated travel time of 1.5 hours before they reached the lab. Samples where shifting between being packed in Styrofoam boxes and plastic bags during transport, so transport temperature might be varying between the samples. When arriving in the lab, the samples where analysed immediately with approximately three hours of processing time. As far as possible samples were handled under sterile conditions.

3.2 Analytical methods

3.2.1 Plate counting of bacteria

All water samples where transferred to sterile eppendorf tubes and ten times dilution series were made using 80% filtered and sterile seawater. The copepods were washed with 80% filtered sterile seawater through a filter to get rid of bacteria in the water and loosely attached the copepods. Approximately ten copepods where then collected in an eppendorf tube, homogenized and diluted to a total volume of 1 ml. Further dilutions where done for the copepods as well. Using a laminar flow fume hood and sterile equipment the samples where plated out on M-65-medium plates (Salvesen and Vadstein (2000): 0.5 g yeast extract, peptone and tryptone; 10 g agar; 100 ml MilliQ-water; 800 ml filtered sea water; per liter medium). This is a general marine medium for heterotrophic bacteria. Several dilutions were tested to find the appropriate ones for each sample type to stay within 30-300 colonies per plate. A dilution of 1000 and 10.000 were found to be the best for the copeod water and algae reservoir, while a dilution of 10 and 100 were used for the copepods and in-water. In total four plates (two dilutions, two replicates per dilution) were plated out per sample. The plates where incubated in the dark at 18°C. The reason they were incubated in the dark was to limit algal growth, especially for the algae reservoir and copepod water samples. The plates were counted after two, three and ten days of incubation.

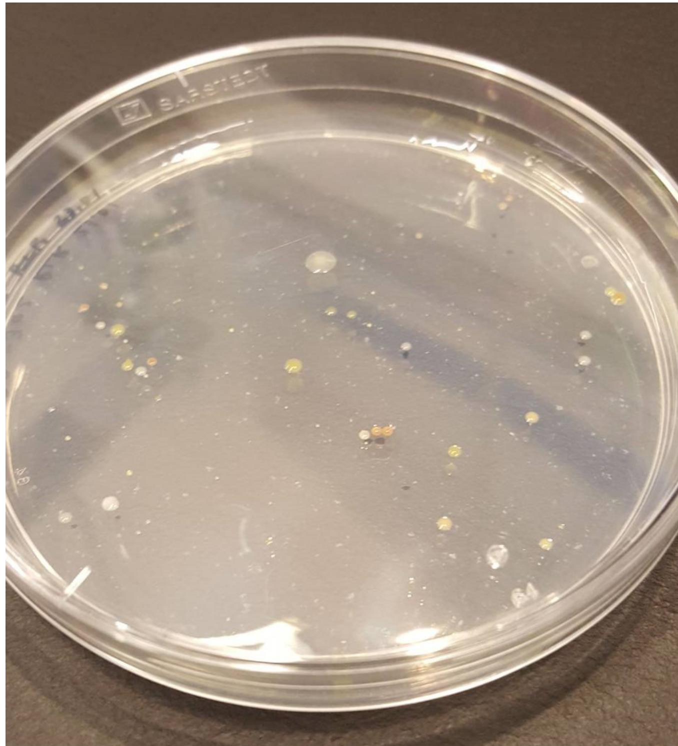

Figure 3.2: Example of colony growth on the M-65 medium after ten days.

For the first sample day of the first production cycle the plates where incubated at $\approx 24\,°\mathrm{C}$ for one day due to technical problems with the incubator. For the two first sampling days of round 1, a too big filter was used to wash the copepods, resulting in no copepods plated out on these days. This was adjusted for sampling day 3, round 1.

3.2.2 Haemolytic activity

10 to 15 days after plating, presence of haemolytic bacteria was investigated by transferring colonies from the M-65 plates to blood agar plates (replica plating). This was done using sterile nitrocellulose membranes (Whatman Protran nitrocellulose membranes, 0.45 μm). The membrane was lightly pressed down on the M-65 agar plate, using a sterile pair of tweezers, causing the colonies to leave residues on the membrane. The membrane was lifted straight up from the plate and put down on the blood agar plate and gently pushed down to transfer the residues to the blood agar. Afterwards, the membrane was gently removed. After transferring the colonies, the blood agar plates were incubated at 18°C for $\approx$ 20 hours before colonies where counted.

Figure 3.3 shows colonies formed on a blood agar plate.

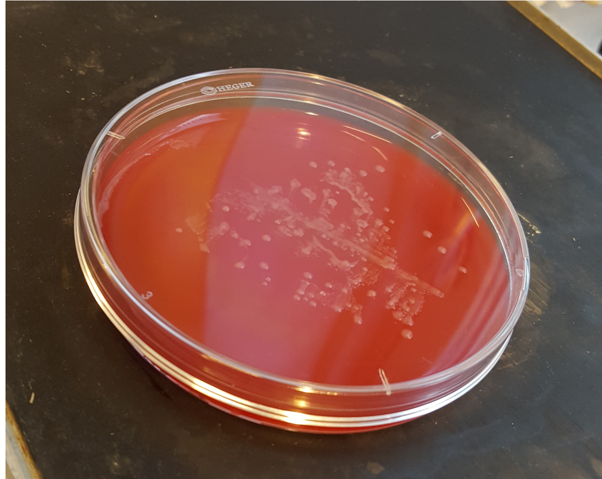

Figure 3.3: CFU's on a blood agar plate 20 hours after incubation.

I discovered rapid growth on the blood agar plates in a trial run, and therefore the M-65 plates with the least number of colonies (within the 30-300 range) where used. This is also why the incubation time was only 20 hours. One transfer was done per sample. Because of the shut down due to the corona virus pandemic, 18 samples were yet to undergo replica plating. Therefore, these plates were incubated for more than 6 weeks. This resulted in several plates drying up, and instead of choosing the plates with the lowest number of colonies, plates that were not dried out were used.

3.2.3 Flow cytometry for counting and phenotypic fingerprinting of bacteria

Preparing samples for flow cytometry

- To quantify cell densities and fraction of cells with a high RNA content, 1.8 ml of each water sample was transferred to a cryotube. To the cryotubes glutaraldehyde was added to a final concentration of 1% (70 μl 25% solution glutaraldehyde). The samples were mixed well and set aside for approximately 30 min to 1.5 hours to allow the cells in the samples to be fixated by the glutataldehyde. Thereafter, the samples were snap frozen in liquid nitrogen and stored at -20°C. Two parallels per sample were prepared.

- The growth potential in the different samples and sample types were also investigated. 5 ml of the samples were transferred to sterile 15 ml tubes with ventilation. The tubes were incubated at 18°C for three days, in a tilted position to increase the surface area and thereby the O_2-supply. On day three the samples were transferred to cryotubes and treated the same way as mentioned above. This was done to investigate the potential excess of nutrients in the sample. If the total cell concentration after three days of incubation, with no nutrients added, increases compared to the total cell concentration from the initial sample, this suggest an excess of nutrients in the sample.

Cell counting

Firstly the flow cytometer (BD Accuri™C6) was calibrated using the beads validation (as described in the protocol (BD Bioscience, 2012). The fluorescent dye SYBR Green II was used to stain the samples. SYBR Green II is mainly staining RNA and emits green light with a maximum at 521 nm (Source: Sigma Aldrich: SYBR® Green II RNA gel stain). This means that the dye is registered in the FL1-channel. SYBR Green II, staining RNA, was used to differentiate cells with high and low RNA content, as mentioned in Chapter 2.

According to the protocol (BD Bioscience, 2012) it is recommended to have less than 1000 events per μl for bacterial count. Therefore, all samples were diluted 1:100, except samples from the in-water. These had a low cell-content and were only diluted 1:10. 0.1x TE-buffer (filtered through a 0.2 μm filter) was used for dilution. Total volume after dilution was 1 ml. To this volume 10 μl diluted SYBR Green II was added (10 μl SYBR Green II and 490 μl MilliQ water). The samples were incubated for 15 min in the dark prior to analysis. The samples were ran with a tube of MilliQ water in every 3rd to 4th well to prevent residues from the former sample from contaminating the next sample. The parameters for the analysis was:

Time	2 min
Flow rate	35 μl / min
Treshold	Delete events less than 1000 on FL1-H
Agitate plate	1 cycle every 1 well

The statistical software RStudio was used to analyse the samples. Using the FlowCore package and the archsinh function the data were transformed to a pseudo-logarithmic scale, where small values were scaled linearly and large values had a logarithmic scale. The data were then plotted, and gates were fitted to denoise the data and separate the total cell count from the high RNA cell count, as shown in Figure 3.4.

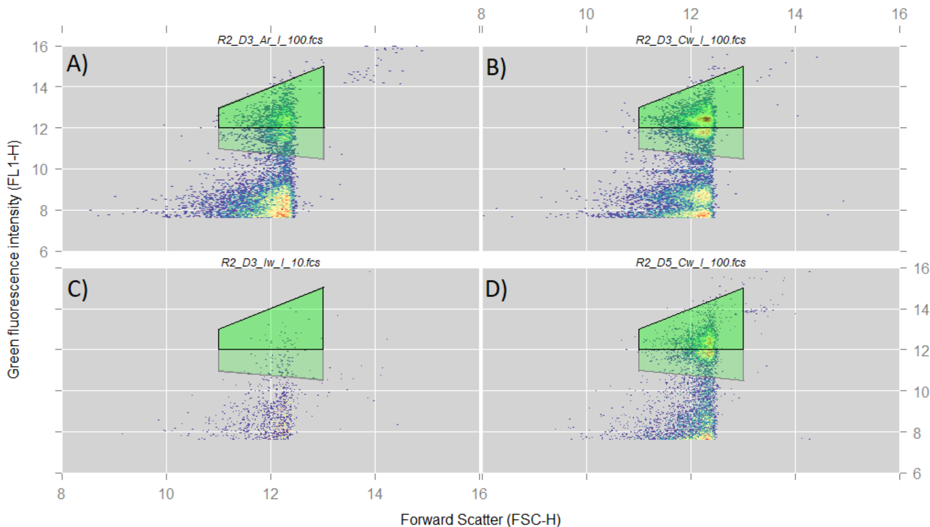

Figure 3.4: Four different gating plots, with two different gates (total cell count and cells with high RNA content) in each plot are shown as an example, for the FL1-channel (y-axis) and FSC-channel (x-axis). R2 means round 2 or tank 2, Ar is the algae reservoir, Cw is the copepod water, Iw is the in-water and I means initial sampling. The numbers corresponds to dilution (100 is 1:100 dilution with TE buffer and 10 is 1:10 dilution.

Finally, the counts inside the gates were corrected for volume and dilution, to estimate a final cell concentration. The cell concentrations were then checked for normality, using the Shapiro-Wilk test and compared for statistical significance using the Kruskal-Wallis test.

Phenotypic fingerprinting

A phenotypic fingerprinting analysis, or phenotypic diversity analysis, was also done using RStudio. Firstly the noise was removed from the cell count data, to make sure only the cells inside the gates were assessed. These data were then normalised to the [0,1] range using the FlowCore package, and a microbial fingerprint was made using a 128 bivariate binning grid. A binning grid is used to assign density estimations to a grid (Props et al., 2016). The flowBasis function was used to calculate the phenotypic finerprint.

Alpha diversity was calculated using the rf.fbasis function, with R = 100. R is here spec-

ifying the number of bootstraps, or how many times the the function runs through all the samples to analyze and check for consistency. From this calculation, the alpha diversity was plotted. (Props et al. (2016), Lucas et al. (2017)). Alpha diversity was plotted for the three Hill orders 0, 1 and 2. Hill number metrics are interpretable as 'effective number of species'. Order 0 take into account only richness of the microbial community. The individual abundance of species is taken into account in an increasing degree with increasing order (Props et al., 2016). Generally, Hill orders 0 through 2 is considered sufficient for a thorough interpretation of alpha diversity.

To assess the beta diversity, a PCoA (Principal coordinate analysis) was done to compare community fingerprints, based on the Bray-Curtis dissimilarity metric (Props et al., 2018).

3.2.4 Illumina sequencing

The copepods life stages n4/n5 (nauplius stage 4 and 5) and c1/c2 (copepod stage 1 and 2) are the stages C-Feed sells their live copepods in. Therefore, the samples for Illumina sequencing was taken on these sampling days. A total volume of 3.6 ml was taken from each of the water samples, while the copepods were up-concentrated using a filter, then rinsed in 80% sterile seawater and a total volume of 3.6 ml was taken out, also here. The samples were stored at -20°C until analysis. For the first production no water samples were taken.

Before analysis, the DNA had to be extracted. First the tubes were spun down for 10 min at the maximum speed (21.500g) on the centrifuge. The liquid was then carefully removed, leaving the cell cluster in the tube. The DNeasy PowerSoil Kit was used to extract the DNA. First solution C1 was added to the sample, which was vortexed and then added to the powerbead solution. From this point on the protocol was followed step by step (QIAGEN, 2017).

PCR was ran on the samples to amplify the desired regions of the 16S rRNA gene. A mastermix consisting of the primes ill338F and ill805R was used to target the V3 and V4 regions (Nordgård et al., 2017). The remaining components and ratios in the mastermix is shown in Appendix, Table 6.5. The first round of PCR was done using the Bio Rad T100™ Thermal Cycler PCR with the following program: warming up the samples to 95°C, 1 min at 98°C, 15 seconds at 95°C, 20 seconds at 55°C, 20 seconds at 72°C. The last three steps were ran in a cycle 36 times, before the samples were ran at 72°C for five minutes and cooled down to 4°C for 1 minute. The samples were then checked for desired product using gel electrophoresis with 1% w/v agarose (5 μl Gel Red Nucleic Acid Gel Stain (Biotium) was added to 100 ml liquid agarose). 5 μl of the GeneRuler 1 kb Plus DNA ladder (Thermo Fisher Scientific) was added to the first well. 4 μl of each sample was added to a well after mixing with 1 μl DNA Gel Loading Dye (6X) (Thermo Fisher Scientific). The gel electrophoresis was ran for 50 minutes at 115 V. Finally, a picture was taken of the gel using UV light, to check for desired product.

After confirming PCR product with correct length and sufficient strength, normalizing the samples were done using Sequal Prep Normalization plate Kit, Invitrogen. After normalization, indexing was done in a second round of PCR. The recipe for the second master-

mix is found in Appendix, Table 6.6. Indexing PCR was done with the following cycle conditions: 98°C for 1 min; 98°C for 15 seconds, 50°C for 20 seconds, 72°C for 20 seconds (step 2 trough 4 were ran in 10 cycles); 72°C for 5 minutes and finally 4°C for 1 minute. A second round of gel electrophoresis was done to check if the indexing had worked. A new normalization of the PCR product was done according to the protocol (Thermo Fisher Scientific, 2008).

Finally, all the samples were merged and a final gel electrophoresis was ran. The gel was labeled with amount of product, bp number of product and bp number of the two closest bands to the product shown on the ladder. The gel was sent to the Norwegian Sequencing Centre at UiO (Oslo University) for analysis.

Data analysis and statistics

The Illumina data were processed using USEARCH pipeline (version 11). Firstly, pair reads were merged, primer sequences were trimmed and all reads shorter than 400 bp were filtered out. Further on, quality filtering and demultiplexing was done, with an expected error threshold of 1. OTU clustering was performed at a 97% similarity level. This was done by implementing the UPARSE algorithm (Edgar, 2013), that also remove chimera sequences and singletons simultaneously. Taxonomic assignment with the Ribosomal Database Project (RDP) was done using a confidence value threshold of 0.8 and the Sintax command (Edgar, 2016).

The OTU data was further normalized based on the sample containing the lowest sum of reads (13 300 reads). This caused 127 OTU's to be removed. The data was sorted and plots containing the percentage of OTU's for phylum and class was made. For phylum all were plotted. For class, the 16 classes with the highest average percentage was presented. The remaining 9 was placed in an 'others' category. The OTU table was also analysed for alpha and beta diversity using PAST (Hammer et al., 2001). For alpha diversity, Hill order 0, 1 and 2 was estimated for each sample (Hill, 1973) , as well as the evenness (Equation 3.1

$$E = \frac{D1}{D0} \tag{3.1}$$

Here E is the evenness, $D1$ is alpha diversity of Hill order 1 and $D0$ is alpha diversity of Hill order 0. The latter is also defined as richness of species in the sample. From the combination of the non-parametric estimator Chao 1 and the taxa sequences, a percentage of sequence coverage was estimated. The sequence coverage was found by dividing the number of taxa sequences of each sample on the predicted number of taxa sequences (Chao 1). Beta diversity was also estimated using PAST. A multivariate PCoA plot was created, as well as a PERMANOVA statistical analysis to look at significance levels between sample types. The PCoA was based on Bray-Curtis and Dice-Sørensen distances.

3.3 Calculations

To calculate the percentage of fast growing microbes, first the concentration in CFU per milliliter for each plate within the 30-300 range was calculated with respect to the dilutions. The copepod samples were additionally divided by 10 to get the unit of CFU per copepod (it was approximately ten copepods per milliliter sample). This was done for CFU's counted on day 3 and day 10. The plates outside the 30-300 range were dropped from the calculations, except where none of the plates were within the range for a specific sample. In this case the plate(s) with the CFU count closest to 30 or 300 were chosen. The average concentration was found for all samples at day 3 and day 10 and by dividing the average concentration from day 3 on day 10, the percentage of fast growing microbes were found. To find the growth potential, the total cell concentration for the growth potential samples were divided by the total cell concentration of the corresponding initial samples.

A standard deviation (SD) calculation was done for the CFU counts, assuming Poisson distribution. First the coefficient of variation (CV) was calculated as shown in Equation 3.2.

$$CV = \frac{\sqrt{CFU_{\text{tot}}}}{CFU_{\text{tot}}} \tag{3.2}$$

Here CFU_{tot} is the total amount of CFU's for all the plates used to calculate the concentration for the respective sample and CV is the coefficient of variation. Further on, the standard deviation was calculated as shown in Equation 3.3

$$SD = CV \cdot C \tag{3.3}$$

Here SD is the standard deviation and C is the cell concentration in the sample.

To easily compare the growth potential with other types of data, the doubling numbers in cell density was calculated, using Equation 3.4.

$$DN = log_2(\frac{\text{Cell count from growth potential}}{\text{Total cell count}}) \tag{3.4}$$

Here DN is the doubling number, cell count from growth potential is the total cell count from the water samples that where incubated for three days, and the total cell count is the cell count obtained from the initial samples.

To find out how things changes in the copepod water, the specific loss rate (L), specific net rate of change (R) and specific supply rate (S) were estimated. Firstly, the loss factor (l) was found for each day using the following equation:

$$l = \frac{F_{\text{Ar}} + F_{\text{In}}}{V_{\text{Cw}}} \tag{3.5}$$

Here F_{Ar} is the flow of algae into the copepod tank [L/day], F_{In} is the flow of in-water into the copepod tank [L/day] and V_{Cw} is the volume of water in the copepod tank [L]. The specific loss rate (L) was found calculating the average of l for all days. The supply factor (s) for each day was found as shown in Equation 3.6.

$$s = \frac{C_{\text{Ar}} + C_{\text{Iw}}}{C_{\text{Cw}}} \tag{3.6}$$

Here, C_{Ar} is the concentration of algae into the tank [cells/day,L], C_{Iw} is the concentration of cells in the in-water [cells/day,L] and C_{Cw} is the concentration of cells in the tank [cells/L] for each specific day. The specific supply rate (S) was found calculating the average of s for all days.

The net change factor (r) was found by first calculating the logarithm of the copepod water concentration per day for all three rounds (because of the relationship shown in Equation 3.7). The obtained values were plotted with time and a regression line for each round was made. The slope for each round was found and the average of this represented the specific net rate of change (R). From the calculated factors, the concentration of cells supplied, lost and net change was calculated using the following equation:

$$X_t = X_0 \cdot e^{y \cdot t} \tag{3.7}$$

Here, X_t is the cell concentration after time t, X_0 is the cell concentration at time 0 and y represents either -L, R or S. The changes in concentration for loss, supply and net change were calculated and plotted as a function of time. The estimated average constants L, R and S were used to calculate the specific growth rate of cells in the copepod tank, using the following Equation:

$$\mu = R + S - L \tag{3.8}$$

Chapter 4

Results

The results are presented for each of the different groups of samples separately, for all three production cycles. Sample types are illustrated in 3.1.

4.1 Algae reservoir

4.1.1 Microbial density: CFU and total cell count

CFU

The CFU concentration with time for the algae reservoir is shown in Figure 4.1.

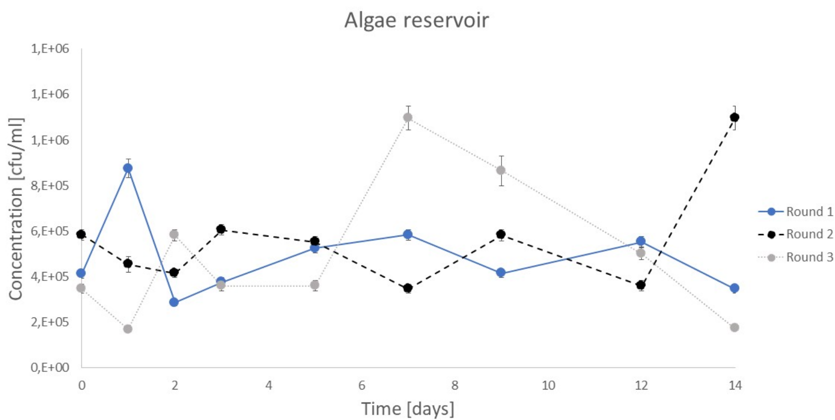

Figure 4.1: Cell concentration [cfu/ml] from CFU's of the algae reservoir (y-axis) as a function of time (x-axis) for the three different replicates. Standard deviation for each sample point is given.

The cell concentration in the algae reservoir was relatively stable with time within the magnitude of 10^5 CFU's per milliliter (the average of all rounds were $5.1 \cdot 10^5 \pm 2.6 \cdot 10^4$ CFU's per milliliter). Within the three replicate cultures variations could be observed, but no apparent trends were found.

Total cell count

From flow cytometry total cell concentration was obtained (Figure 4.2).

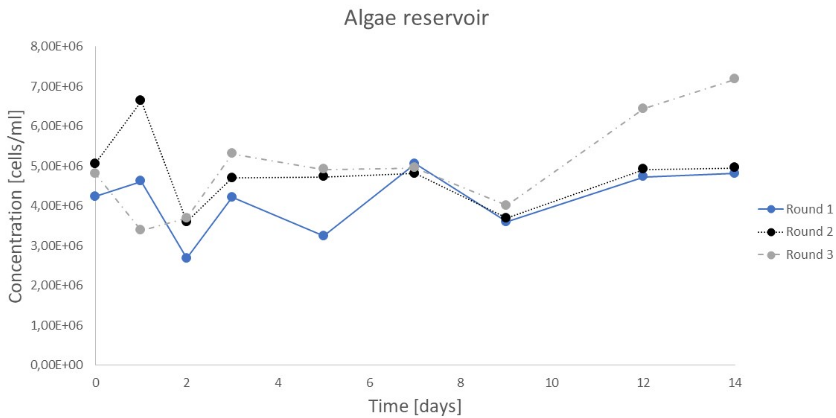

Figure 4.2: Total cell concentration, obtained from flow cytometry, [cells/ml] (y-axis) as a function of time (x-axis) for the three replicates from the algae reservoir.

It was observed a similar trend as for the CFU counts with a cell concentration that varied little with time (the average total cell count was $4.6 \cdot 10^6 \pm 6.9 \cdot 10^5$ cells per milliliter). For the total cell count, however, the concentration was approximately 10 times higher than for CFU's. All data points from total cell concentration was within $2 \cdot 10^6$ and $7 \cdot 10^6$ cells per milliliter, suggesting a more stable cell concentration than for the CFU counts.

Comparison of average cell concentration from CFU counts and flow cytometry is shown in Figure 4.3, where the average from each of the three replicates, for the two analysis, was calculated.

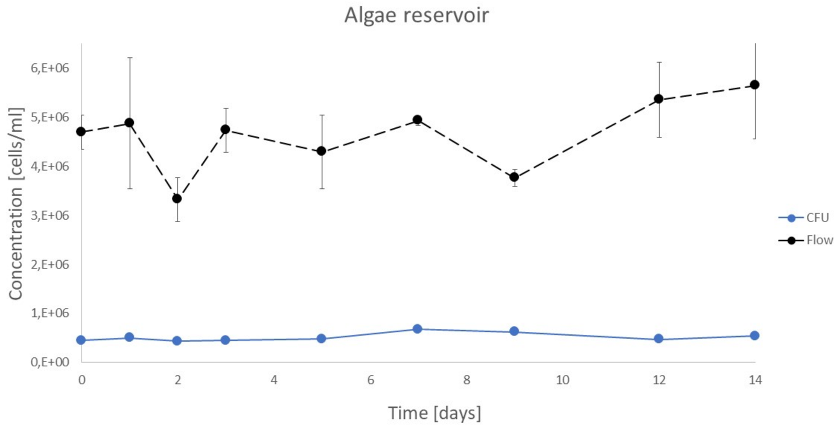

Figure 4.3: Average total cell concentration (from flow cytometry and from CFU's) [cells/ml] (y-axis) as a function of time (x-axis) are shown for the algae reservoir.

The average from both analysis methods supported the observations from the individual replicates in terms of having a stable cell concentration with time. The average cell concentration from each of the replicates from CFU counts and total cell concentration is shown in Figure 4.4.

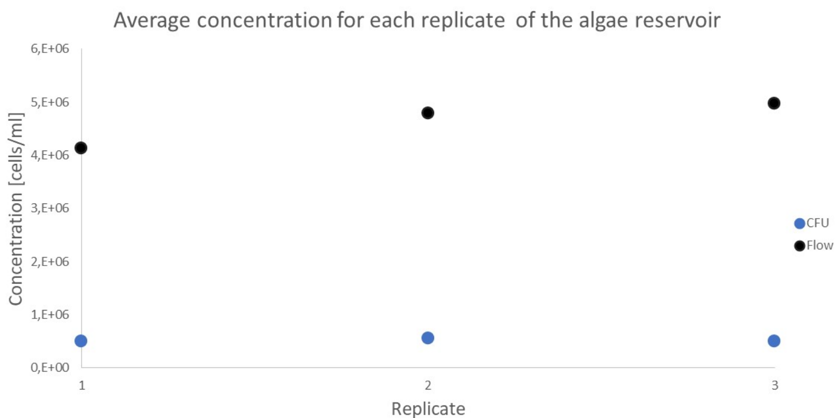

Figure 4.4: Average total cell concentration (from flow cytometry and from CFU's) [cells/ml] (y-axis) for each of the three replicates (x-axis) are shown for the algae reservoir.

The distance between CFU counts and total cell concentration for all three rounds was relatively similar. The CFU count was within the magnitude of 10^5 cells per milliliter, while the total cell count was within the magnitude of 10^6 cells per milliliter.

4.1.2 Percentage of fast growing microbes

From CFU counts the percentage of fast growing microbes was calculated (Figure 4.5).

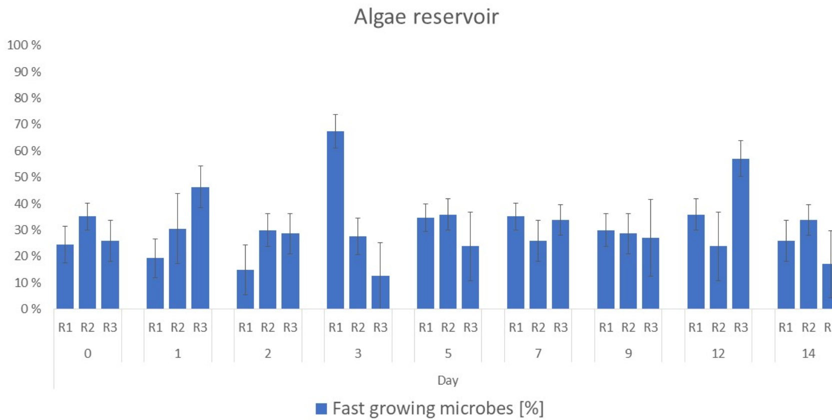

Figure 4.5: Percentage of fast growing microbes calculated from the CFU's, as percentage of visible colonies on the plates after three days of incubation, is shown for the algae reservoir.

An observed trend was an overall stable percentage of fast growing microbes (average of $31\% \pm 8\%$). Except for a couple of data point (round 1 day 3 was close to 70% and round 3 day 12 was close to 60%). The trend seemed to be independent of time and cultivation round.

4.1.3 Growth potential

To assess the nutrient availability in the algae reservoir, the doubling number for the growth potential data was plotted against time, as shown in Figure 4.6.

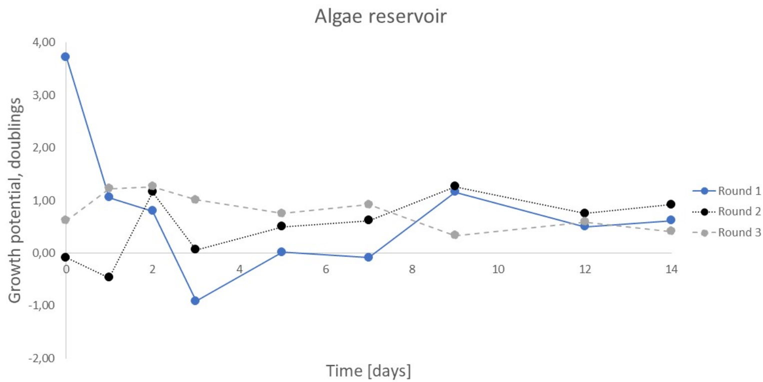

Figure 4.6: Doubling number for growth potential, obtained as shown in Equation 3.4 (y-axis) plotted against time (x-axis).

The growth potential was varying between 4 doublings and 0 doublings on day zero, but the trend seemed to be a stabilization with time. The average doubling number was 0.69 ± 0.37, suggesting nutrient availability for roughly 0.5 to 1 doubling in cell concentration.

4.1.4 Percentage of microbes with high RNA content

Cells with a high RNA content was counted and for the algae reservoir the percentages are shown in Figure 4.7.

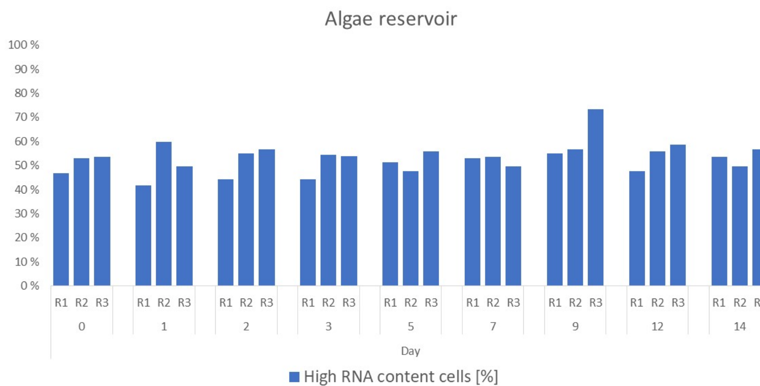

Figure 4.7: Percentage of microbes with a high RNA content obtained from the flow cytometry analysis are shown for all three replicates as a function of time (x-axis) for the algae reservoir.

The percentage of cells with high RNA content was stable throughout the production cycle (average of $53\% \pm 3\%$). There was little variability, both in between replicates and as a function of time.

4.2 In-water

4.2.1 Microbial density: CFU and total cell count

CFU

The CFU concentration with time for the in-water is shown in Figure 4.8. Because the in-water was started up during day 2 of the production cycle, the sampling, and therefore, the measurements started on day 3.

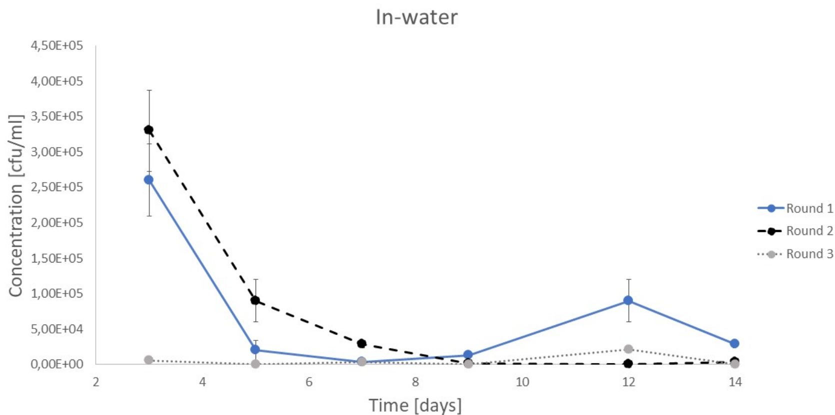

Figure 4.8: Cell concentration [CFU/ml] obtained from CFU's on the in-water plates (y-axis) as a function of time (x-axis) is shown for the three different replicates. Standard deviation for each sample point is given.

From Figure 4.8 it was observed a big variation in CFU concentration. Ranging from a few hundred CFU's per milliliter up to a magnitude of 10^5 CFU's per milliliter. The measuring points on day 3 of round 1 and round 2 had the highest concentration, but CFU count dropped drastically between day 3 and day 5 for the two rounds. The average concentration was found to be $(7.2 \pm 1.7) \cdot 10^4$ CFU's per milliliter.

Total cell count

From flow cytometry the total cell concentration was obtained (Figure 4.9).

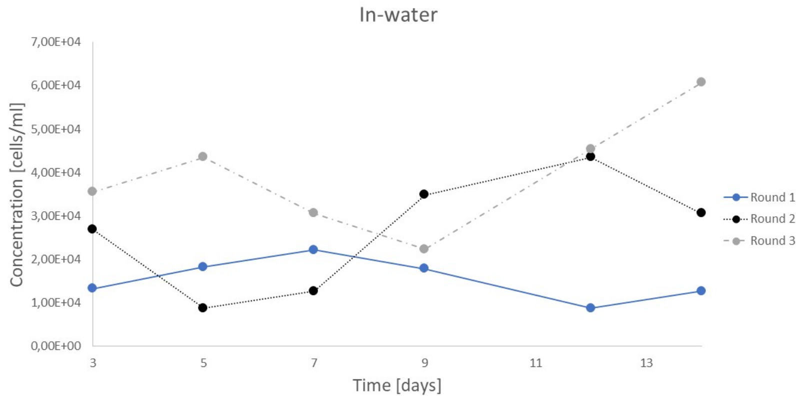

Figure 4.9: Total cell concentration, obtained from flow cytometry, [cells/ml] (y-axis) as a function of time (x-axis) for the three replicates from the in-water.

The cell concentration seemed to be within the magnitude of 10^4 cells per milliliter with an average of $2.7 \cdot 10^4 \pm 4.8 \cdot 10^3$ cells per milliliter. However, there were variations with time and between rounds, without any apparent trends observed. Comparison of average cell concentration from CFU counts and flow cytometry is shown in Figure 4.10, where the average from each of the three replicates for the two analysis was calculated.

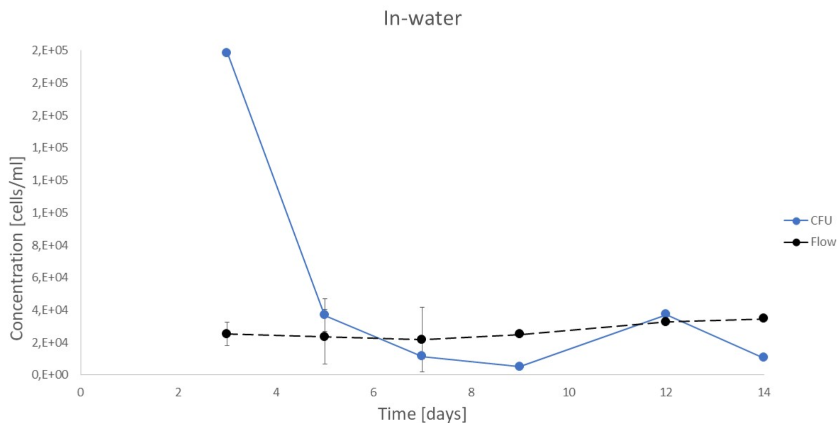

Figure 4.10: Average total cell concentration (from flow cytometry and from CFU's) [cells/ml] (y-axis) as a function of time (x-axis) are shown for the in-water.

The CFU count on day 3 was quite different from the rest of the data points, corresponding to about 1000 cells more per milliliter. Aside from this data point, the concentration

seemed to be more stable. Especially for the total cell count samples. Here the concentration was varying between $2{\cdot}10^4$ - $4{\cdot}10^4$ cells per milliliter. When day 3 (the extreme outliner) was discarded from the average calculation, the concentration from CFU ($2.0 \cdot 10^4 \pm 6.0 \cdot 10^3$ CFU's per milliliter) became significantly closer to the total cell count ($2.7 \cdot 10^4 \pm 4.8 \cdot 10^3$ cells per milliliter).

The average cell concentration from each of the replicates from CFU counts and total cell concentration is shown in Figure 4.11.

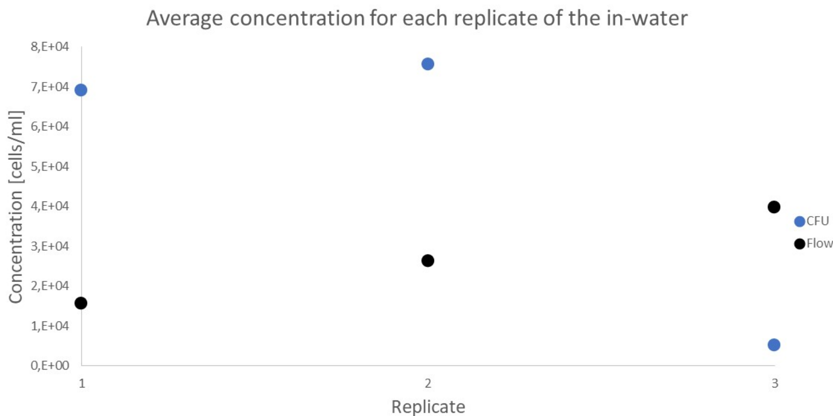

Figure 4.11: Average total cell concentration (from flow cytometry and from CFU's) [cells/ml] (y-axis) for each of the three replicates (x-axis) are shown for the in-water.

For round 1 and 2 the average CFU count was higher ($7{\cdot}10^4$ to $8{\cdot}10^4$ cells per milliliter) than for the average total cell count ($1{\cdot}10^4$ to $2{\cdot}10^4$ cells per milliliter). Only for round 3 the average total cell count was higher than the average CFU count (around $4{\cdot}10^4$ and $3{\cdot}10^3$ cells per milliliter, respectively).

4.2.2 Percentage of fast growing microbes

From CFU counts the percentage of fast growing microbes was calculated (Figure 4.12).

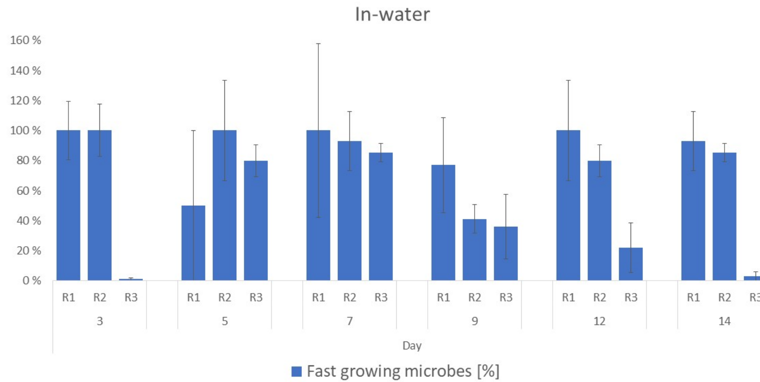

Figure 4.12: Percentage of fast growing microbes calculated from the CFU's, as percentage of visible colonies on the plates after three days of incubation, is shown for the in-water.

In general the in-water had quite high percentages of fast growing microbes. Several measurements had close to a 100%. However, big variations, both with time and between cultivation rounds were observed. For round 3, day 3 and 14, the percentage was close to 0. Overall, the average was 69% ± 20%. No apparent trends were found, neither with time, nor between cultivation rounds.

4.2.3 Growth potential

To assess the nutrient availability for the in-water, the doubling number for the growth potential data was plotted with time, as shown in Figure 4.13.

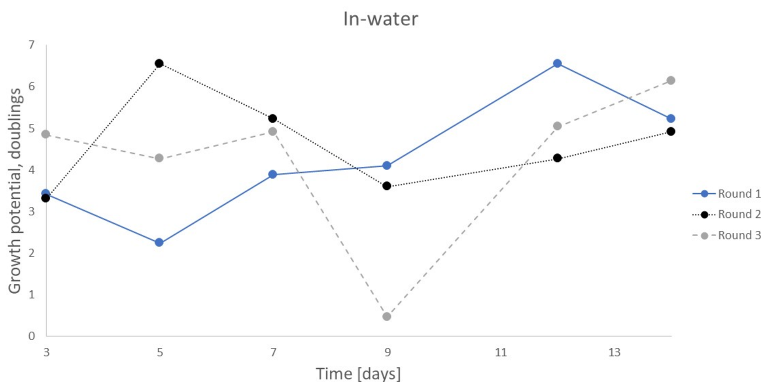

Figure 4.13: Doubling number for growth potential, obtained as shown in Equation 3.4 (y-axis) plotted with time (x-axis).

All samples had a positive doubling number, suggesting nutrient availability in the in-water. The average was found to be 4.38 ± 0.92, meaning that most samples had 3 to 5 doublings in cell concentration. No apparent trends were observed with time or between cultivation rounds, but individual variations were found.

4.2.4 Percentage of microbes with high RNA content

Cells with a high RNA content was counted and for the in-water the percentages are shown in Figure 4.14.

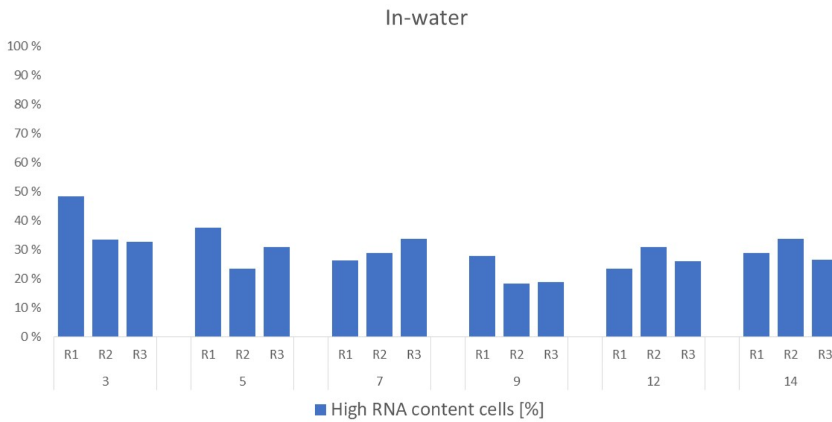

Figure 4.14: Percentage of microbes with a high RNA content obtained from the flow cytometry analysis are shown for all three replicates as a function of time (x-axis) for the in-water.

The percentage of cells with high RNA content was overall stable ($29\%\pm5\%$). No apparent trends or variations were observed between cultivation rounds or with time.

4.3 Copepod water

4.3.1 Microbial density: CFU and total cell count

CFU

The CFU concentration with time for the copepod water is shown in Figure 4.15.

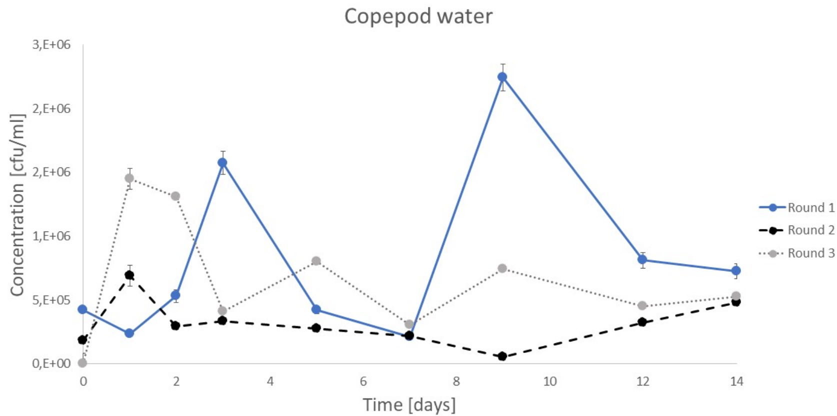

Figure 4.15: Cell concentration [cfus/ml] from CFU's of the copepod water (y-axis) as a function of time (x-axis) for the three different replicates. Standard deviation for each sample point is given.

From Figure 4.15 it was no apparent trend in cell concentration development over time. Variations between rounds and with time were found. However, it looked like the CFU count was approximating a stabilization in CFU count on the last two sample days (day 12 and 14). The average CFU count for all rounds was $5.9 \cdot 10^5 \pm 3.8 \cdot 10^4$ CFU's per milliliter.

Total cell count

From flow cytometry total cell count was obtained for the copepod water (Figure 4.16)

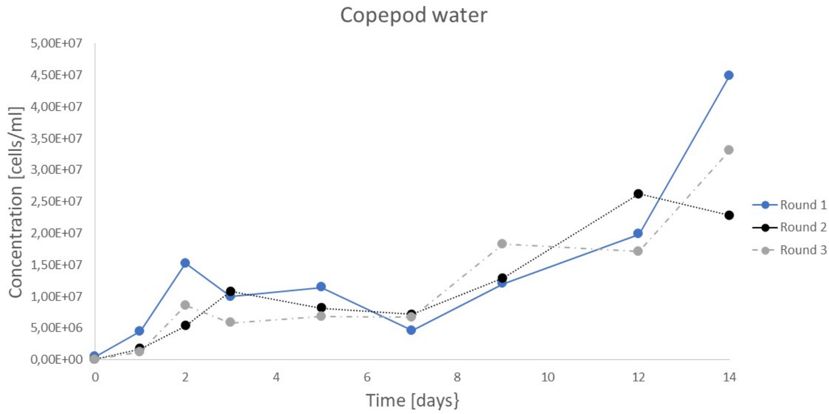

Figure 4.16: Total cell concentration, obtained from flow cytometry, [cells/ml] (y-axis) as a function of time (x-axis) for the three replicates from the copepod water.

Contrary to the CFU counts, a clear trend was observed for the total cell count. Increasing cell concentration with time was observed for all three cultivation rounds, with little variation in between rounds. The average cell concentration was $1.2 \cdot 10^7 \pm 9.7 \cdot 10^6$ cells per milliliter. Comparison of cell concentration from CFU counts and flow cytometry is shown in Figure 4.17, where the average from each replicate was calculated.

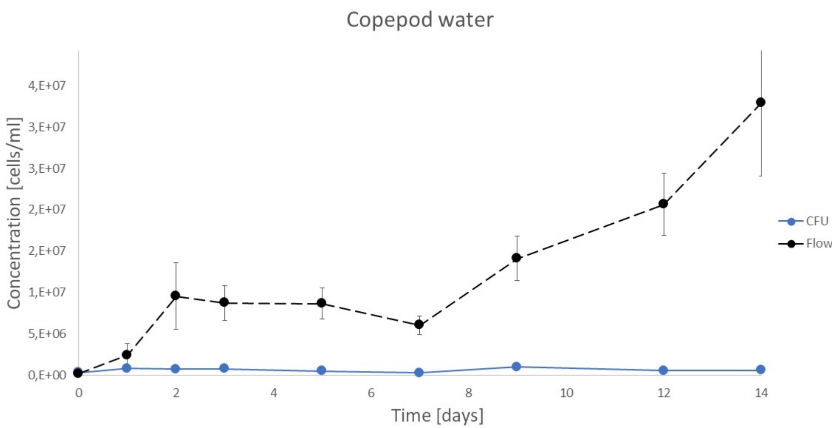

Figure 4.17: Average total cell concentration (from flow cytometry and from CFU's) [cells/ml] (y-axis) as a function of time (x-axis) are shown for the copepod water.

Here, the increasing cell concentration for total cell count was even more clear. Especially from day 7, where there is a clear increase observed for every sampling day. The total cell count was for most data points within the magnitude of 10^7 cells per milliliter. Meanwhile, the CFU count was stable within the magnitude of 10^5 CFU's per milliliter through the whole production cycle. The average cell concentration from each of the replicates from CFU counts and total cell concentration is shown in Figure 4.18.

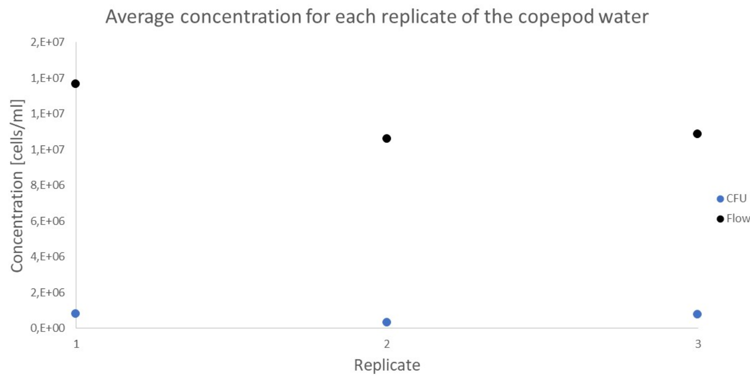

Figure 4.18: Average total cell concentration (from flow cytometry and from CFU's) [cells/ml] (y-axis) for each of the three replicates (x-axis) are shown for the copepod water.

For all three cultivation rounds the average total cell count was higher than the average CFU count. The average total cell count was within the magnitude of 10^7 cells per milliliter, while for the CFU's the concentration was within the magnitude of 10^5 CFU's per milliliter.

4.3.2 Percentage of fast growing microbes

From CFU counts the percentage of fast growing microbes was calculated (Figure 4.19).

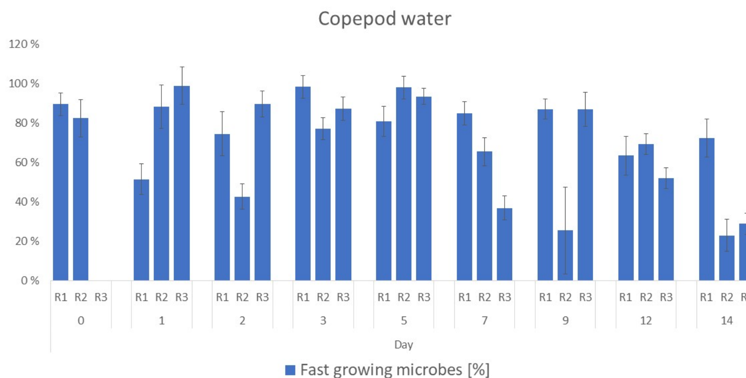

Figure 4.19: Percentage of fast growing microbes calculated from the CFU's, as percentage visible colonies on the plates after three days of incubation, is shown for the copepod water.

Overall, a high percentage of fast growing microbes was observed (average of $69\% \pm 7\%$). Between cultivation rounds no apparent trend was observed. Relatively big individual differences were found. However, with time it seemed like a slight decreasing trend was present. In Figure 4.19 it can also be observed that one data point (round 3, day 0) had 0% of fast growing microbes. This sample point had no CFU's on the agar plate, and naturally the percentage of fast growing microbes was zero as well.

4.3.3 Growth potential

To assess the nutrient availability in the copepod water, the doubling number for the growth potential data was plotted with time, as shown in Figure 4.20.

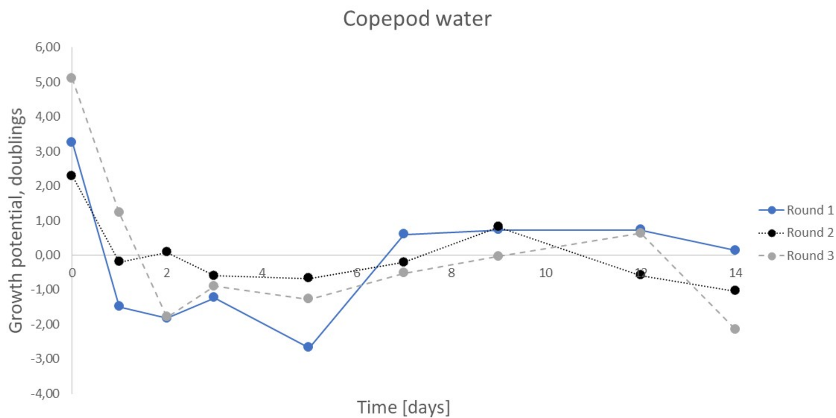

Figure 4.20: Doubling number for growth potential, obtained as shown in Equation 3.4 (y-axis) plotted with time (x-axis).

All three cultivation rounds had a high doubling number on day 0 (between 2 and 5 doublings). After day 0 the growth potential seemed to alternate between 2 halves and 1 doubling. The average doubling number was -0.06 ± 1.43, suggesting variations between decreasing cell concentration and increasing cell concentration for the growth potential. This means that the copepod water was varying between having nutrients available for the microbes, and no nutrient availability. There was no apparent trend found with time.

4.3.4 Percentage of microbes with high RNA content

Cells with a high RNA content was counted and for the copepod water the percentages are shown in Figure 4.21.

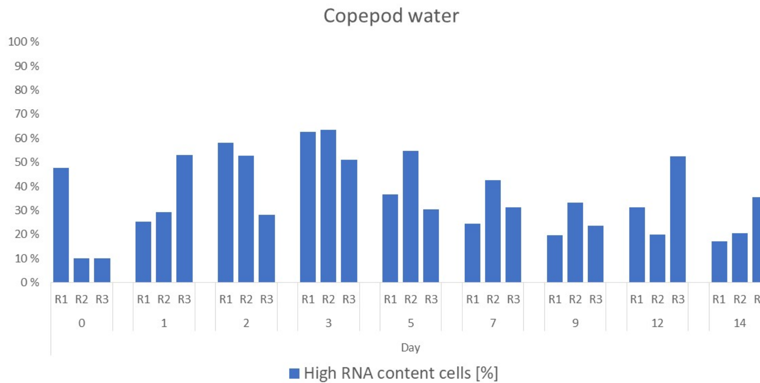

Figure 4.21: Percentage of microbes with a high RNA content obtained from the flow cytometry analysis are shown for all three replicates as a function of time (x-axis) for the copepod water.

As an overall trend it seemed like the percentage was increasing from day 0 and reached a peak on day 3, before the percentage decreased from day 3 and towards the end of the cycles. The average percentage of microbes with high RNA content was $36\% \pm 11\%$. However, there were variations, both between cultivation rounds and with time.

4.4 Copepods

4.4.1 Microbial density from CFU's

The CFU concentration over time for the copepods is shown in Figure 4.22. On day 0 the tanks mainly consisted of copepod eggs. Because of this the sampling of copepods started on day 1.

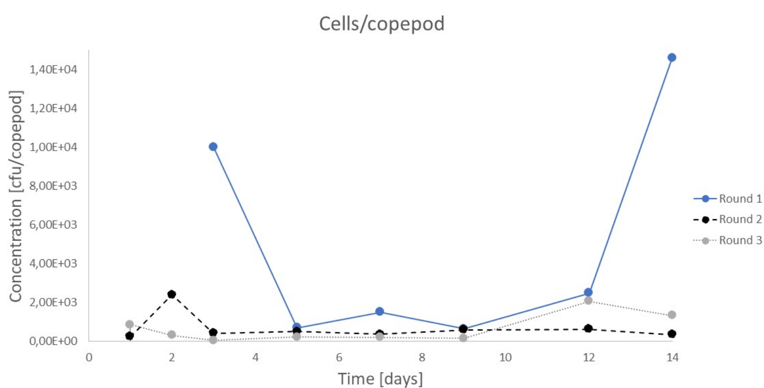

Figure 4.22: Cell concentration [cells/copepod] obtained from CFU's of the copepod plates (y-axis) as a function of time (x-axis) is shown for the three different replicates. Standard deviation for each sample point is given.

Overall, the CFU seemed to be stable and under $2 \cdot 10^3$ CFU's per copepod. However, cultivation round 1 had two outliners (day 3 and 14). The average CFU count for round 2 and 3 was 666 ± 66 CFU's per copepod. For round 1 this number was 5000 ± 813 CFU's per copepod, a magnitude of 10 times higher than for the two other rounds. From Figure 4.22 it can also be observed that the data points for round 1, day 1 and 2 are missing, for reasons explained in Chapter 3.

4.4.2 Percentage of fast growing microbes

From CFU counts the percentage of fast growing microbes was calculated (Figure 4.23).

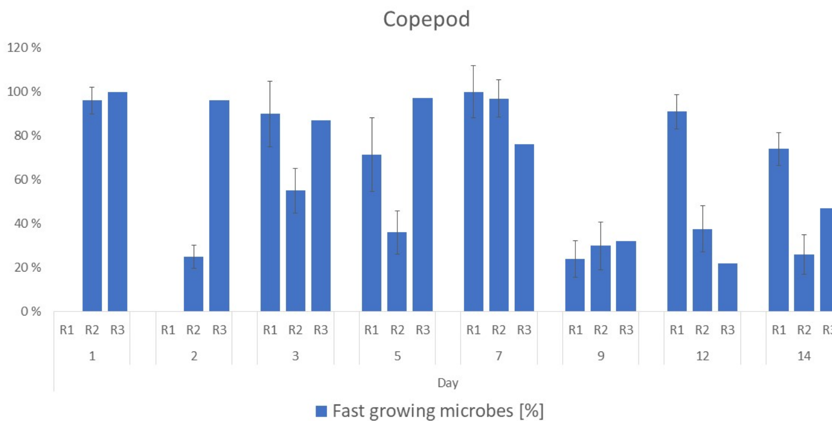

Figure 4.23: Percentage of fast growing microbes calculated from the CFU's, as percentage of visible colonies on the plates after three days of incubation, is shown for the copepods.

A big variation was observed, both between rounds and with time, for the percentage of fast growing microbes. A rough trend could be that the percentage was decreasing after day 7. The average percentage was $65\% \pm 15\%$.

4.5 Haemolytic activity

For several of the blood agar plates, for all sample types, colony growth was observed. However, none of the plates showed signs of haemolysis, e.g. there was no discoloration on the medium surrounding the colonies, for any of the samples.

4.6 Statistical comparison of cell densities between sample types and cultivation rounds

To assess the cell densities obtained from total cell count, the data first had to be checked for normality. This was done using the Shapiro Wilk test and a confidence interval of 95% was chosen. From the Shapiro Wilk test it was determined that the data was not normally distributed ($p = 3.021 \cdot 10^{-9} < 0.05$). Therefore, the non-parametric Kruskal Wallis test was used to check if the different sample types had statistically different cell densities. Testing all three sample types against each other showed that they were significantly different ($p = 2.084 \cdot 10^{-8}$). The sample types were also checked for equality in cell densities in pairs (algae reservoir against in-water: $p = 7.7 \cdot 10^{-8}$, algae reservoir against copepod water: $p = 0.004$ and copepod water against in-water: $p = 6.5 \cdot 10^{-7}$). This showed that all three sample types were significantly different from each other in terms of cell density.

The sample types were also checked for equality between rounds, to determine if there were big differences between cultivation rounds of the same sample type (algae reservoir: p = 0.002, copepod water: p = 0.008, in-water: p = 0.04). All sample types were found to have significantly different cell densities between rounds, suggesting individual differences. However, if a confidence interval of 99% was chosen, the cell densities of the in-water would be regarded as equal.

4.7 Microbial cell contribution to copepod tanks

To more easily see the differences between the different sample types analyzed, the average values of the quantities investigated are presented in Table 4.1.

Table 4.1: Average values of investigated quantities for all sample types, with standard deviation.

	Algae reservoir	**In-water**
Total cell count	$4.60 \pm 0.69 \cdot 10^6$ cells/ml	$2.70 \pm 0.48 \cdot 10^4$ cells/ml
CFU	$5.10 \pm 0.26 \cdot 10^5$ CFU's/ml	$5.0 \pm 1.1 \cdot 10^4$ CFU's/ml
Fastgrowing microbes	$31 \pm 8\%$	$69 \pm 20\%$
High RNA content	$53 \pm 3\%$	$29 \pm 5\%$
Growth potential (doubling)	0.69 ± 0.37	4.38 ± 0.92
	Copepod water	**Copepod**
Total cell count	$1.20 \pm 0.97 \cdot 10^7$ cells/ml	-
CFU	$5.90 \pm 0.38 \cdot 10^5$ CFU's/ml	$2.10 \pm 0.32 \cdot 10^3$ CFU's/ml
Fastgrowing microbes	$69 \pm 7\%$	$65 \pm 15\%$
High RNA content	$36 \pm 11\%$	-
Growth potential (doubling)	-0.06 ± 1.43	-

To find out which processes were contributing to the cell concentration in the copepod tank, the relative contribution from the algae reservoir compared to the in-water was calculated (Figure 4.24).

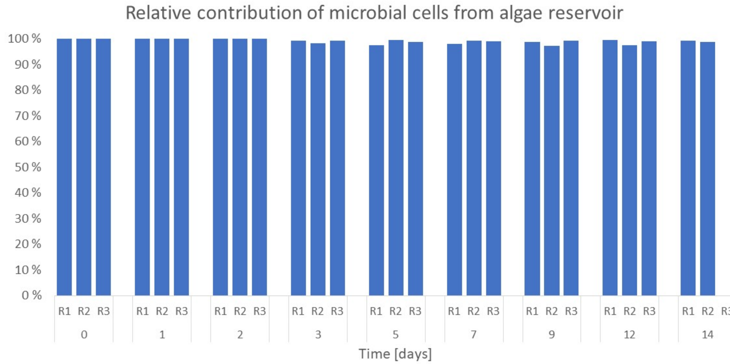

Figure 4.24: The percentage contribution of microbial cells to the copeod rearing tanks from the algae reservoir relative to the in-water.

The algae reservoir was responsible for between 97 and 99% of the microbial cell contribution to the copepod rearing tank for all sample data except day 14, round 3. Here, the tank was not fed algae on day 14, making the in-water the only contributor (Appendix, Table 6.3). This clearly show that the algae fed to the copepod tanks is the major cell supplier. The flow of in-water was about 2-3 times higher than the flow of algae into the copepod tanks (Appendix, Table 6.1, 6.2 and 6.3). As the microbial cell density is magnitudes higher for the algae reservoir than the in-water, the relative contribution of algae into the copepod tanks were still much higher than for the in-water.

The different rates of change within the copepod tanks were also found (Figure 4.25).

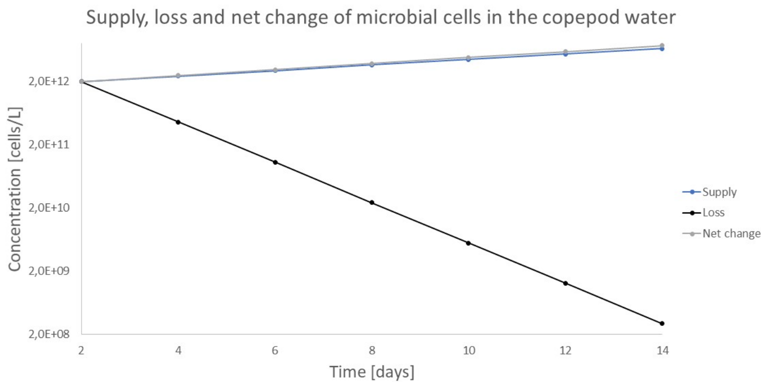

Figure 4.25: The cell concentrations in terms of net change, loss and supply are plotted with time. Benchmark, or start concentration was set to $2.0 \cdot 10^{12}$ cells per milliliter to give the same starting point for all three average concentration rates. Because of little consistency in the start up of the copepod tanks, day 2 was chosen as the starting point.

As the net rate of change and supply rate was quite similar, a new plot was made without the loss rate, to more easily visualize the other average rates (Figure 4.26).

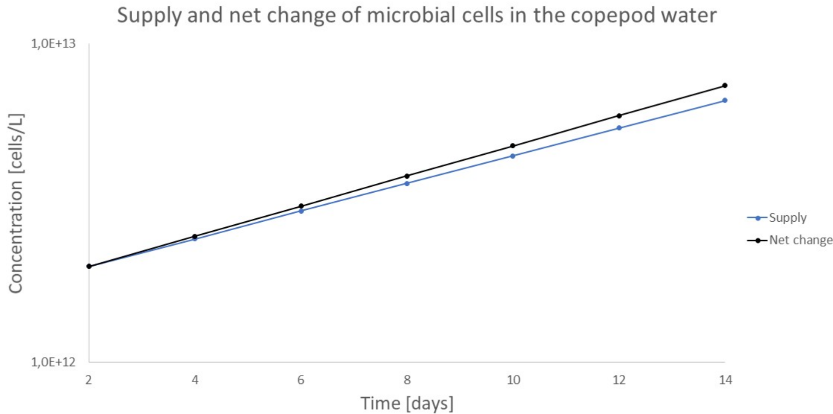

Figure 4.26: The cell concentrations in terms of net change and loss are plotted with time. Benchmark, or start concentration was set to $2.0 \cdot 10^{12}$ cells per milliliter to give the same starting point for both average concentration rates. Because of little consistency in the start up of the copepod tanks, day 2 was chosen as the starting point.

From Figure 4.26 it was observed that the microbial cell concentration supplied, and net concentration was quite equal the first couple of days. However, with time, the net concentration was increasing more rapidly than the supplied concentration. The following rate constants found were calculated from the data:

- **Specific net change rate:** $0.1\ \mathrm{d}^{-1}$
- **Specific loss rate:** $0.7\ \mathrm{d}^{-1}$
- **Specific supply rate:** $0.1\ \mathrm{d}^{-1}$

From these rates, the average specific growth rate, μ, for the microbial community in the copepod tank was found ($\mu = 0.7\ \mathrm{d}^{-1}$). Growth rate is a measure of rate of increase in biomass. An average specific growth rate of $0.7\ \mathrm{d}^{-1}$ means a 0.7 times increase in biomass per day. On a one-cell basis, one cell in average produces 0.7 new cells per day.

4.8 Diversity analysis based on phenotypic fingerprinting

4.8.1 Alpha diversity

Alpha diversity, or the average diversity within the different samples, was estimated. Figure 4.27 shows the result for the three different water samples.

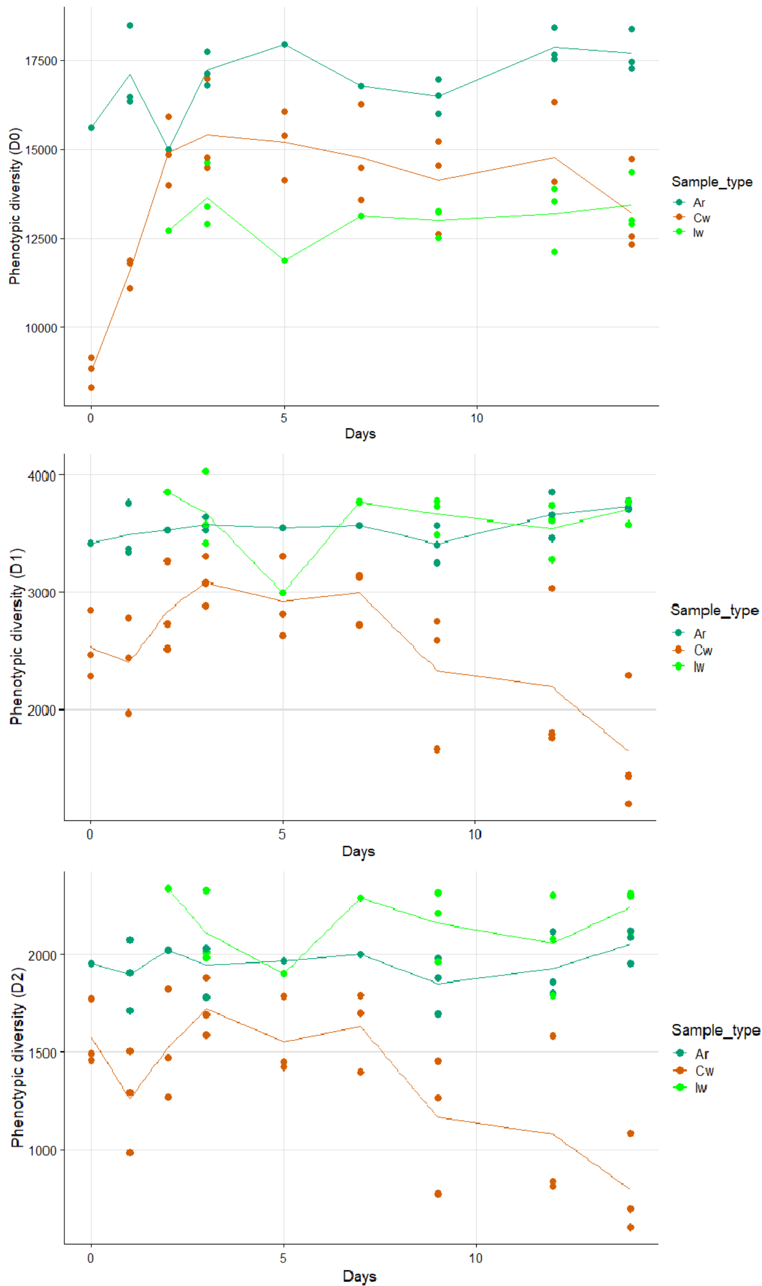

Figure 4.27: Development in Phenotypic diversity with time for the different sample types are shown for Hill orders 0, 1 and 2. Abbreviations Ar, Cw and Iw corresponds to algae reservoir, copepod water and in-water, respectively. The line for each sample type represents the mean value at the specific day.

From the Hill order 0, it was found that the algae reservoir had the highest richness of species. The in-water had the lowest, and the copepod water had a rapid increase in species richness from day 0 to day 3, before it stabilized. However, a slight decrease seemed to happen between day 12 and 14. From an increasing Hill order (increasing weighting on abundance) the in-water seemed to have a higher abundance, or community evenness than the two other sample types. With more emphasis on abundance, the phenotypic diversity of the copepod water seemed to decrease rapidly from day 7 and onward (both for Hill order 1 and 2). The phenotypic diversity of the algae reservoir seemed to be stable for all three Hill orders.

4.8.2 Beta diversity

The beta diversity was estimated from the same data set as the alpha diversity. The beta diversity found, or the ratio between the within sample average and between sample average is shown in Figure 4.28.

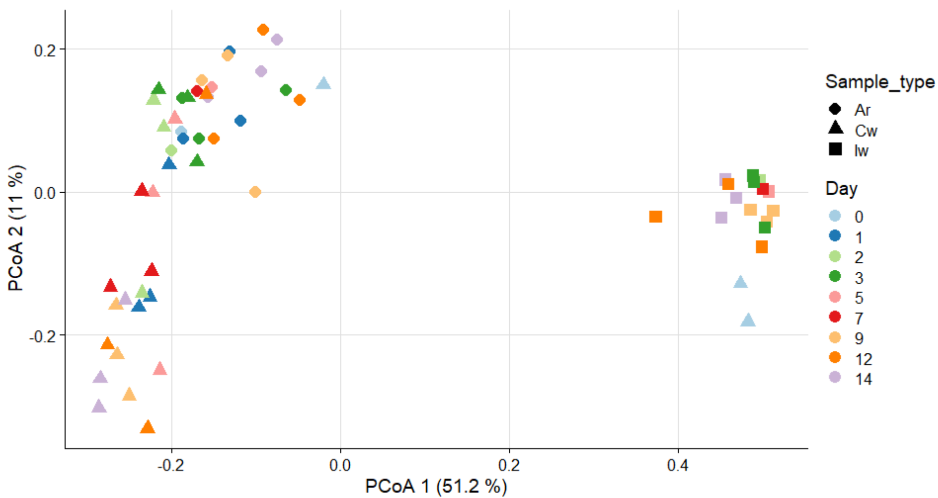

Figure 4.28: Principal Coordinates Analysis (PCoA) plot, based on Bray-Curtis metrics, showing beta diversity. The different symbols corresponds to sample types (Ar: algae reservoir, Cw: copepod water and Iw: in-water). The colors corresponds to the sampling day. In total 62.2% of the variance are explained by this plot.

The beta diversity analysis resulted in three distinguished clusters in the plot. The most apparent trend was all in-water samples clustered together, quite far from the two other sample types. Also, the samples from the algae reservoir clustered together, clearly separated from the two other clusters. The copepod water was also clustered separate from the two other clusters. However, several samples (day 1 and day 3) were found in the same cluster as the algae samples. Copepod water samples for day 5 and day 7 were varying between the copepod water cluster and the algae reservoir cluster. The most striking result, was that two of the copepod water samples on day 0 were quite close to the in-water cluster. The third sample point was found closer to the algae reservoir cluster. Since the x-axis

is explaining more than 50% of the variation, while the y-axis is only explaining 11%, the in-water samples were more different from the other sample types, while the algae and copepod water was more similar. The fact that two of the copepod water samples (day 0) were close to the in-water cluster, while the third sample (day 0) was close to the algae samples, suggests a large individual variation between these samples.

4.9 Illumina sequencing

After the first round of PCR, gel electrophoresis was used to check the samples for product by taking a UV photo of the gel. The result are shown in Figure 4.29.

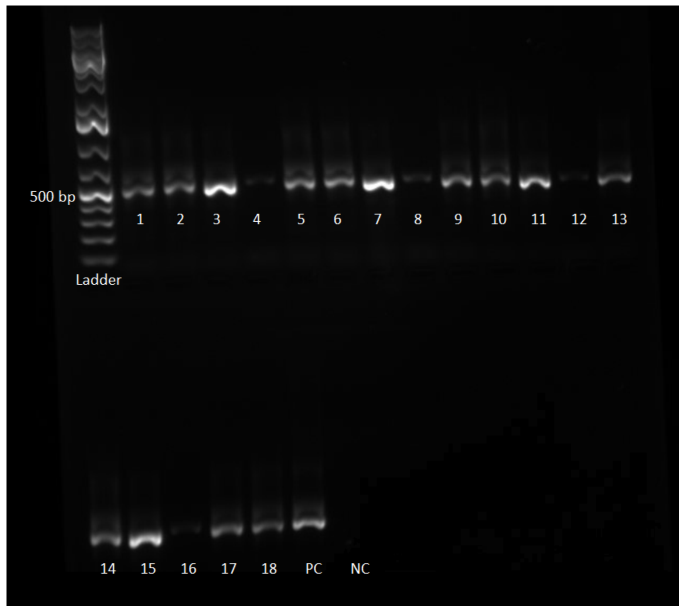

Figure 4.29: The DNA fragment size of the products is according to the ladder approximately 500 bp. The ladder used was GeneRuler 1 kb plus DNA ladder (Thermo Fisher Scientific). Sample 1, 2, 5, 6, 10, 14 and 18 are from the copepods; sample 2, 7, 11, and 15 are from the algae reservoir; sample 4, 8, 12 and 16 are from the in-water; sample 5, 9, 13 and 17 are from the copepod water. PC represents the positive control and NC represents the negative control. Additional information about the individual samples are found in Appendix, Table 6.4

4.9.1 Taxonomy

An average sequence coverage for all the samples were calculated to 87%, meaning that approximately 87% of the predicted number of taxonomic sequences were found. The genotypic fingerprinting of samples taken on day 5 and day 7 are shown on the taxonomic level of phylum in Figure 4.30.

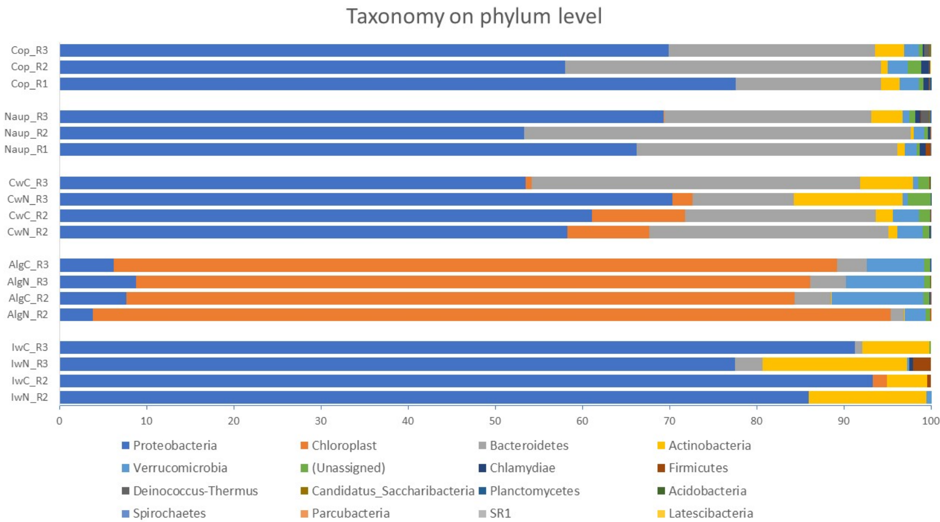

Figure 4.30: Taxonomy on phylum level, for the samples taken on day 5 and 7 of the cycle for all three production cycles, in percentage. Cop and Naup means *A. tonsa* at copepodite and nauplii stage, respectively. Cw is the copepod water, Iw is the inn-water and Alg is water from the algae reservoir. C and N corresponds to the water samples associated with either the copepodite (C) or the nauplii (N), and R1, R2 and R3 corresponds to production round 1, 2 and 3, respectively.

At the taxonomic level of phylum the *Proteobacteria* was dominating all sample types, except the water samples from the algae reservoir. Here the *Chloroplasts* was dominating. The *Chloroplasts* were also easily observed in the copepod water, though more for round 2 than round 3. The second most abundant phylum (excluding the *Chloroplasts*) was *Bacteriodetes*. Found in all sample types, but with the highest percentage for the *A. tonsa* samples and the copepod water. The inn-water had a quite low percentage of *Bacteriodetes*. The fourth most abundant phylum was *Actinobacteria*. Especially in the inn-water, and to some degree in the copepod water. For the algae reservoir, the *Actinobacteria* was not noticeable from Figure 4.30.

The taxonomic composition of samples on the class level is shown in Figure 4.31.

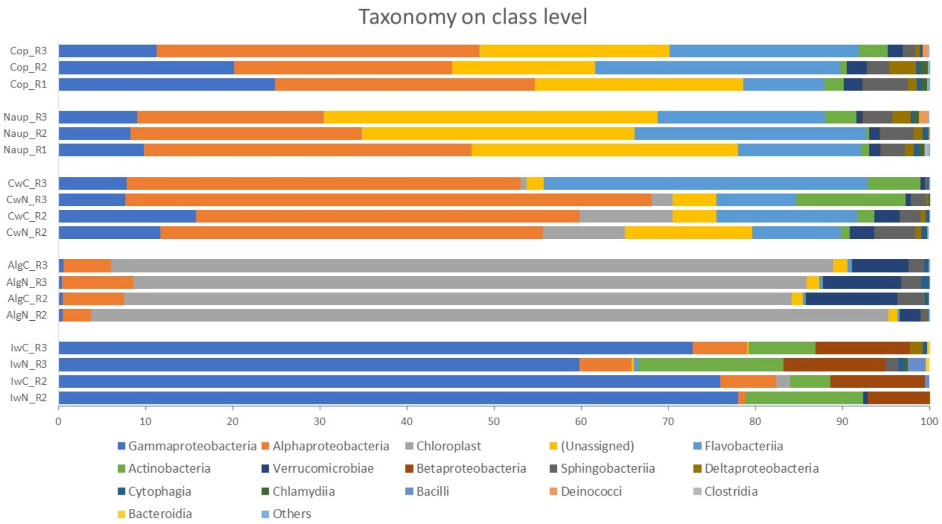

Figure 4.31: Taxonomy on class level, for the samples taken on day 5 and 7 of the cycle for all three production cycles, in percentage. Cop and Naup means *A. tonsa* at copepodite and nauplii stage, respectively. Cw is the copepod water, Iw is the inn-water and Alg is water from the algae reservoir. C and N corresponds to the water samples associated with either the copepodite (C) or the nauplii (N), and R1, R2 and R3 corresponds to production round 1, 2 and 3, respectively.

On the taxonomic level of class the *Alphaproteobacteria* was dominating both the *A. tonsa* samples and the water samples from the copepod production cycles. Some examples of *Alphaproteobacteria* with a high percentage was the genuses *Hyphomonas*, *Nautella* and *Lentilitoribacter*. These samples also had a high occurrence of *Gammaproteobacteria*, *Flavobacteriia* and unassigned OTU's. From the *Flavobacteriia* class, the genera *Flavobacterium* and *Tenacibaculum* had a high sum of reads in the OTU table for the mentioned samples. *Gammaproteobacteria* was dominating the inn-water. Examples of *Gammaproteobacteria* genera with a high percentage was *Vibrio*, *Kangiella* and *Pseudoalteromonas*. *Alphaproteobacteria*, *Actinobacteria* and *Betaproteobacteria* also had a high occurrence in the inn-water. The *Chloroplasts* were dominating the water samples from the algae reservoir. Which was as expected, as the *R. baltica* was not filtered out before sequencing. Aside from the *Chloroplasts*, the *Alphaproteobacteria* and *Verrucomicrobiae* had the highest occurrence.

4.9.2 Alpha diversity

The alpha diversity calculated from OTU's is shown in Table 4.2 for Hill order 0, 1 and 2, as well as evenness (E).

Table 4.2: Alpha diversity in terms of Hill order 1, 2 and 3 and evenness (E) for all sample types, separated by production cycles (R).

	Nauplii			**Copepodite**			**Copepode water**	
	R1	R2	R3	R1	R2	R3	R2	R3
D0	245	220	242	242	267	204	209	145
D1	31.0	26.2	22.2	28.2	40.5	26.0	39.1	12.1
D2	1.08	1.09	1.18	1.10	1.05	1.10	1.05	1.21
E	0.13	0.12	0.09	0.12	0.15	0.13	0.19	0.08
	Algae reservoir water			**Inn-water**				
	R2	R3		R2	R3			
D0	189	190		21	35			
D1	14.7	11.8		8.0	14.9			
D2	1.24	1.26		1.28	1.15			
E	0.08	0.06		0.38	0.43			

As seen in Table 4.2, the *A. tonsa* samples had the highest alpha diversity in terms of Hill order 0. A measure of species richness. Meaning that the *A. tonsa* samples had the highest amount of different species present. The water associated with the copepods, as well as the algae reservoir had almost as high species richness as the *A. tonsa* samples. However, it can be observed that the species richness of the copepod water varied heavily between round 2 and 3. Suggesting variations between production cycles. The sample with the lowest species richness by far, was the inn-water. The inn-water had a species richness ten times lower than the rest of the sample types. In addition to this, the inn-water had the highest evenness, suggesting few species present with a similar amount of each. None of the other samples had particularly high evenness, with the algae reservoir having the lowest. Also for the evenness, the copepod water had high variations between rounds (round 2 had over two times the evenness of round 3). The nauplii and copepodite samples had quite similar evenness between each other and between rounds.

4.9.3 Beta diversity

The principal coordinate analysis (PCoA) of beta diversity, done with Bray-Curtis and Dice-Sørensen distances, are shown in Figure 4.32.

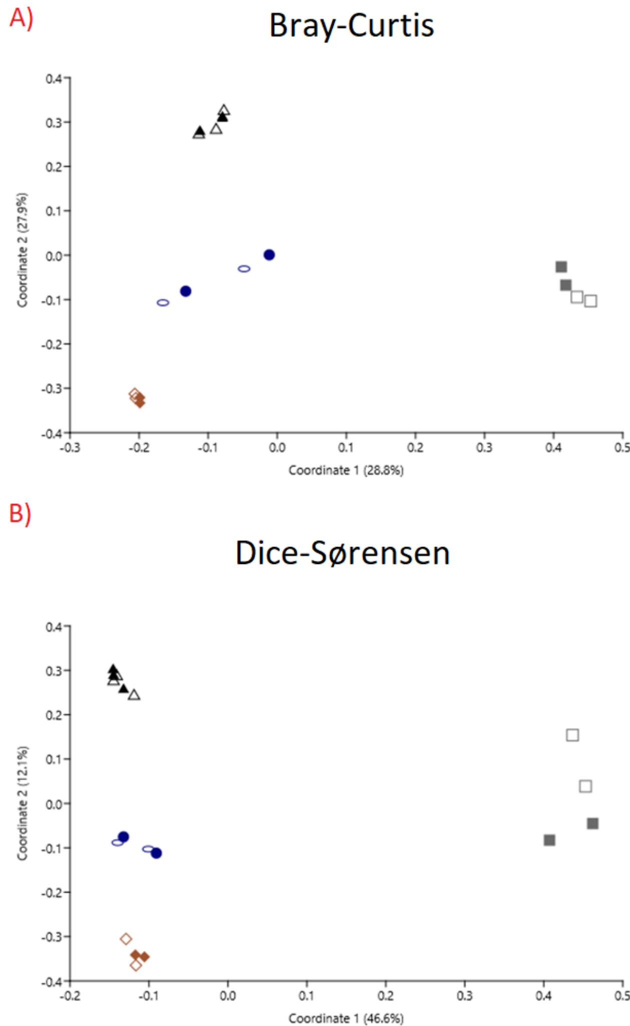

Figure 4.32: A) shows the Bray-Curtis distance (56.7% of the variance explained) and B) shows the Dice-Sørensen distance (58.7% of the variance explained). For both plots the filled symbols corresponds to samples associated with the copepodites and the other symbols corresponds to samples associated with the nauplii. The triangles are the copepod samples, circles are the copepod water, diamonds are the algae reservoir samples and squares correspond to the inn-water.

In both the Bray-Curtis and Dice-Sørensen plot, the inn-water was clustering far away from the other samples on the x-axis. Especially for Dice-Sørensen, where the x-axis is explaining 46.6% of the total variance. Meaning that the inn-water samples were very different from the rest of the samples. Along the the y-axis a smaller amount of the total variance was explained (only 12.1% for Dice-Sørensen). Meaning that even though the sample types were separating clearly, they were more equal to each other than the inn-water. From the Bray-Curtis plot the variance was more evenly explained by the two axis (27.9% on the y-axis and 28.8% on the x-axis). However, the same trend was observed. The inn-water cluster was located far away from the three other sample types on the x-axis. For both plots, all four sample types were clearly separated from each other. However, the copepod water was closer to the algae reservoir samples, than the inn-water and copepods.

From the PERMANOVA analysis, all the sample types were significantly different from each other at a 95% confidence interval. Both for Bray-Curtis and Dice-Sørensen, and uncorrected and sequenctial Bonferroni corrected p-values.

Chapter 5

Discussion and conclusion

5.1 Discussion

5.1.1 Total cell concentration and RNA content

Overall, the water in the copepod cultures had the highest total cell concentration ($1.20 \pm 0.97 \cdot 10^7$ cells/ml). About ten times higher than for the algae reservoir ($4.60 \pm 0.69 \cdot 10^6$ cells/ml), and a magnitude of 10^3 higher than the inn-water ($2.70 \pm 0.48 \cdot 10^4$ cells/ml). That the inn-water had such a low total cell concentration makes sense, considering cells are both filtered away and undergoing UV treatment. The latter preventing further cell growth (Laroussi and Leipold, 2004). The inn-water was also the sample type with the most varying cell concentration. Both with time, and between production cycles. This low cell concentration and high variability might indicate an uncrowded environment (Andrews and Harris, 1986).

The copepod water had both a supply of microbial cells, and potential nutrients from the algae reservoir and inn-water, as well as an increasing load of fecal matter from the copepods with time. The nutrient availability might explain why the total cell concentration was higher for the water in the copepod cultures. From copepods in the wild, it has been found that the microbial concentration associated with fecal matter was within the magnitude of 10^8 cells/ml (Tang, 2005). Several magnitudes higher than for typical marine microbial populations. This supports the assumption that fecal matter from the copepods contributed to nutrient availability. Another observation made, was that the copepod water was the only sample type with a clear increase in cell concentration with time. An explanation for this could be a combination of increasing algae supply, and increasing amount of fecal matter produced by the copepods. The fact that the cell concentration was increasing with time suggested that the water in the copepod cultures was an uncrowded environment, due to nutrient availability and little competition (Andrews and Harris (1986), Mac Arthur and Wilson (1967)).

The algae reservoir had the most stable total cell concentration with time. Being the sample

type closest to a chemostat (Smith and Waltman, 1995), with continuously algae supply and flow over, the logical assumption would be stable conditions, independent of time. This fits well with the observed cell concentration with time (Figure 4.2). It also suggests factors limiting the growth present. Several things could explain this. Firstly, the *R. baltica* has the abillity to do phagocytosis (Clay, 2015), suggesting predation present. Secondly, a limitation in nutrient availability could have limited the cell growth. The stable cell concentration and possible predation present indicates the algae reservoir being a crowded environment (Andrews and Harris (1986), Mac Arthur and Wilson (1967)).

Both the copepod water and inn-water had a composition of cells with a high RNA content, corresponding to approximately 30% (Table 4.1). The algae reservoir, on the other hand, had over 50% of high RNA content-cells. Since high RNA content is linearly correlated to growth rate (Benthin et al., 1991), a natural assumption is that over half of the cells had a high growth rate. A stable crowded environment should have been selecting for slow growing cells according to theory ((Andrews and Harris, 1986)). Since it was the opposite case for the algae reservoir, something must have caused the high percentage of cells with a high RNA content. One explanation could be the predation exercised by the algae (Clay, 2015). On one hand, predation keeps the cell concentration at a stable point. On the other hand, the algae might force the microbes to evolve towards a higher growth rate to prevent them from being obliterated.

That both the water in the copepod cultures and inn-water only had 30% high RNA content-cells suggests an overall lower growth rate for these two sample types. As both the copepod water and inn-water was assumed as uncrowded environments, an access to nutrients would have selected for a higher amount of microbes with a high growth rate. One explanation might be poor access to nutrients in these sample types. For the inn-water another explanation might be water temperature. Even though the inn-water have a temperature of 22°C when entering the copepod tanks, it is retrieved from the Trondheimsfjord. The fjord generally have a much lower temperature, and the sampling was done during winter and spring. When the temperature in the fjord is at its lowest (3-5°C). Since growth rate is increasing exponentially with increasing temperature (Ratkowsky et al., 1982), this is a possible explanation for the low percentage of cells with a high RNA content.

5.1.2 Nutrient availability

The inn-water also had the highest growth potential by far, with a doubling number of 4.38 ± 0.92. This suggested that without added nutrients, the cell concentration for the inn-water could double over 4 times in three days. A strong indication for nutrient availability in the inn-water samples. Both the algae reservoir (0.69 ± 0.37) and water in the copepod cultures (-0.06 ± 1.43) had a lower growth potential. However, the copepod water had the clearest trend in swinging between doubling and halving the concentration, compared to initial concentration. Suggesting a widely varying access to nutrients. The algae reservoir had approximately half to one doubling from initial concentration. As nutrients are added to promote algal growth, a higher growth potential would have been a natural assumption. However, the algae was not filtered out before incubation. An explanation for the low

growth potential may therefore be that the algae was performing phagocytosis while the samples were incubated (Clay, 2015).

5.1.3 CFU and fast growing microbes

Contrary to the total cell concentration, the CFU count of the algae reservoir and copepod water was of the same magnitude. 10^5 CFU's/ml (Table 4.1). Both being lower than the the total cell concentration. This corresponds to the theory of limitations associated with the plate counting method (Salvesen and Vadstein, 2000). The lacking trend of increasing CFU concentration with time for the copepod water (Figure 4.17), suggests only a certain amount of microbes able to grow on solid medium present.

The inn-water had the lowest CFU concentration of the water samples ($5.0 \pm 1.1 \cdot 10^4$ CFU's/ml). However, compared to the total cell concentration, the average CFU concentration was higher, which does not correspond with the theory (Salvesen and Vadstein (2000), Brown and Wittwer (2000)). A misrepresented CFU concentration, due to using a too low dilution (as mentioned in Chapter3), for the first sampling day of round 1 and 2 may explain this. In Figure 4.8 it can be observed that round 3 did not follow the same trend of high CFU concentration on day 1. Reinforcing this assumption.

Of all the sample types, the copepods had the poorest growth on plates ($2.10 \pm 0.32 \cdot 10^3$ CFU's/copepod). For round 1, there were two outliners impacting the CFU count significantly. Averaging on $5.00 \pm 0.81 \cdot 10^3$ CFU's/copepod, compared to $6.70 \pm 0.67 \cdot 10^2$ CFU's/copepod for round 2 and 3. Suggesting a big variation in concentrations between production cycles. An explanation could be the use of a too high dilution for the first production cycle, causing misrepresentation of CFU concentration. When compared to *A. tonsa* living in the wild, the CFU concentration seemed to be in the lower end of the reported range (Tang, 2005). An explanation for this might be the limitations associated with the plate counting method, as mentioned earlier (Salvesen and Vadstein, 2000). Another explanation was that a general medium for marine heterotrophs was used. Microbes associated with copepods might have other growth requirements, limiting CFU's on this general medium. Since no total cell concentration was found for the copepods, this will remain an assumption.

All samples, except the algae reservoir, had close to 70% fast growing microbes in their samples. The algae reservoir had a 50% fast growing microbes composition. Fast growing microbes are associated with opportunists (Andrews and Harris (1986), Mac Arthur and Wilson (1967)), which are not favorable in a microbial water management point of view. Pathogens are often associated with opportunists (Vadstein et al., 2018). Of the water samples, the inn-water had both the highest percentage and variability. Suggesting a less than favorable microbial composition, selecting for opportunists (r-strategists). The copepods also had a high percentage of fast growing microbes. However, as mentioned above, a general growth medium was used. As fast growing microbes, or r-strategists, are known for being generalists, a fair assumption is that these were over-represented on the plates due to poor growth conditions for the K-strategists (Andrews and Harris, 1986). The fact that the algae reservoir had the lowest percentage of fast growing microbes, is an indication of

selection for K-strategists in this process.

5.1.4 Haemolytic activity

An important trait with live feed is that it needs to be safe for the fry to eat (e.g. no pathogens present). Even though r- and K-selection can be used to predict the possibility of opportunistic pathogens present (Vadstein et al., 2018), the haemolytic activity have been used to assess the live feed quality of *Artemia* (Olsen et al., 2000). This was also done here. Both for the copepods and all the water sample types, with an uplifting result for C-Feed. No haemolytic activity was found in any of the sample types investigated. This suggested that the copepods had a good live feed quality. Especially compared to the *Artemia* investigated by Olsen et al. (10% of the total CFU count had haemolytic activity) (Olsen et al., 2000). The fact that none of the water samples had haemolytic activity dramatically decrease the risk of microbes with haemolytic activity being transferred to the copepods, either from the inn-water, algae reservoir or the water surrounding the copepods. Increasing the safety of the copepods as live feed.

5.1.5 Growth rate and cell contribution

The average specific growth rate (0.7 d^{-1}) for the water in the copepod cultures was found to be equal to the specific loss rate. Meaning as many cells were formed per day, as lost in the out-water. Since a steady increase in cell concentration was seen for the copepod water, something else must have contributed to the cell concentration. This being the average specific supply rate (0.1 d^{-1}). Or the rate of supplied microbial cells to the copepod water. However, from Figure 4.26 it is clear that the net change in microbial cell concentration was increasing faster than the microbial cells supplied, with time. Suggesting that the specific growth rate might be increasing with time. This fits well with the total cell concentration increasing more and more rapidly with time (Figure 4.16). Even though the specific growth rate was seven times bigger than the supply rate, it is a reasonable assumption that the cells supplied from the algae reservoir and inn-water could impact the microbial community in the copepod tank.

Since the algae reservoir and inn-water had very different characteristics for their microbial communities (strong indications of K-selection and r-selection, respectively), which process contributed the most is a valid factor. As seen in Figure 4.24, there is no doubt the algae reservoir contributed most to supplied cells (97-99%), relative to the inn-water. Even though the inn-water had characteristics of r-selection, the chance of transferring opportunistic pathogens to the copepod water was extremely small, considering how small the relative contribution was compared to the algae reservoir. The latter having the characteristics of K-selection suggests an overall good microbial water quality in the supplied cell concentrations to the copepod water.

5.1.6 Phenotypic fingerprinting

A phenotypic diversity analysis was done to evaluate species richness and abundance of the microbial communities present in the water samples (Props et al., 2016). The algae reservoir had the highest species richness, but with higher emphasise on abundance the inn-water had the highest phenotypic diversity (Figure 4.27, D2 on the Hill order). As the algae reservoir was assumed a K-selecting environment, high species richness and low abundance fits well with this assumption from r- and K-selection theory (Andrews and Harris (1986), Mac Arthur and Wilson (1967)).

The inn-water was assumed an r-selecting environment. With the lowest species richness, and a higher species abundance this also fits into the r- and K-selection theory. K-selecting environments are crowded with microbial populations adapting to a narrow range of substrates, and are limited by substrate availability and competition. Adaptation to specific substrates indicate species richness (under the assumption of a range of substrate options), and competition keeps the abundance low. Conversely, in r-selecting environments, little competition indicates lower species richness and high substrate availability promotes species abundance.

For the copepod water varying phenotypic diversity was observed. Supporting the assumption of variable conditions in the rearing tank. The phenotypic diversity had a rapid increase in species richness from day 0 to day 2 (Figure 4.27, D0 on the Hill order), before it stabilized, and a slow decrease in species richness was observed with time. With more emphasise on abundance (D2 on the Hill order), the phenotypic diversity reached a peak on day 3, before it decreased more and more rapidly with time. This supports an assumption of the copepod rearing tanks initially being an r-selecting environment, with a slow shift towards K-selection with time. However, no stabilizing trend was found for the alpha-diversity. Suggesting that the microbial community in the copepod water had not reached any form of equilibrium.

From between samples comparison (beta diversity) it was clear that the inn-water and algae reservoir had significantly different microbial community compositions. Especially, seeing as they had a distinct separation on the x-axis. The latter explaining 51.2% of the total variance. The fact that the algae reservoir and inn-water samples were all located in their respective cluster, gives a strong indication of little variance within sample types.

For the copepod water, two of the initial samples were clustered close to the inn-water samples. As the disinfected eggs are put to hatching in filtered and UV-treated seawater (same as the inn-water), it makes sense that the microbial communities of the copepod water initially had a similar community composition as the inn-water. However, one of the initial copepod water samples (day 0) was clustered with the algae reservoir samples. This suggests that the sample was more similar to the community composition of the algae reservoir. Meaning a different microbial community composition within replicates on the initial sampling day. Several other samples for the copepod water were clustered with the algae reservoir samples as well. A reasonable assumption is that the algae supply contributed to the microbial community composition in the copepod water.

An explanation for the difference observed in microbial communities between the initial copepod water samples, can be that two of the samples were taken before algae supply was started (for round 2 no algae was fed to the copepod tank on day 0, Appendix: Table 6.2). While the third initial sample was taken after algae supply was initiated to the tank. Even though several copepod water samples were clustered with the algae reservoir, several were also clearly separated in a cluster along the y-axis (11% of the variance explained, Figure 4.28). Supporting the assumption made about variability in microbial community composition in the copepod water. However, a rough trend was observed in that the copepod water samples seemed to separate more and more from the algae reservoir samples with time. This leads to the assumption that a distinct microbial community was slowly developing with time for the copepod water.

5.1.7 Genotypic fingerprinting

From the taxonomic evaluation it was clear that all sample types (disregarding the *Chloroplasts* from *R. baltica*) was dominated by *Proteobacteria*. A phylum which contains more than a third of all characterized species of *Bacteria* (Madigan et al., 2015). Making this phylum quite common, and expected in the samples. The class *Alphaproteobacteria*, that was dominating the *A. tonsa* samples and associated water, generally consists of oligotrophs, preferring to grow in low nutrient concentration (Madigan et al., 2015). E. g. not consistent with r-strategists. However, one of the genera with high reads found, *Hyphomonas*, have been linked to pathogenic species causing disease in seaweed (Li et al., 2014). Illustrating the point that pathogens also can be found in this class. The class *Flavobacteriia* also made up a high percentage of the *A. tonsa* samples and associated copepod water. Two OTU's with a high read for these samples corresponded to the genera *Flavobacterium* (Touchon et al. (2011), Urdaci et al. (1998), Laanto et al. (2011)) and *Tenacibaculum* (Habib et al. (2014), Avendano-Herrera (2006)). Genera associated with several fish pathogens. The species level was not investigated, and determination of fish pathogens could not be done. However, presence of genera that includes several fish pathogens was worrisome.

Usually *Alpha*- and *Betaproteobacteria* are common in marine environments. In this case, however, the *Gammaproteobacteria* was by far the dominating class of the inn-water. It also had a high percentage for the copepod samples. The *Gammaproteobacteria* class contains many well known pathogenic species (Madigan et al., 2015). In the inn-water samples the genus *Vibrio* had a high OTU read. The *Vibrio* genus consists of over 60 species, in which several are pathogenic. They are, however, a common marine genus. Another high OTU read found for the inn-water was the genus *Pseudoalteromonas*, which have some interesting properties. Amongst other, it is known for having antimicrobial properties (Bowman, 2007). Bowman also writes that the *Pseudoalteromonas*: 'influence settlement, germination and metamorphosis of various invertebrate and algal species; and may also be adopted by marine flora and fauna as defensive agents'. The antimicrobial properties might be beneficial for C-Feed. However, the influence on invertebrate and algal species, like *A. tonsa* and *R. baltica*, could be problematic.

From the genotypic alpha diversity analysis, much of the same could be observed as from

the phenotypic diversity analysis, in terms of species richness. The inn-water had a species richness about a tenfold lower than the other sample types. Here the difference in species richness for the copepod water and algae reservoir water was not as pronounced as for the phenotypic analysis. However, the same trend in stable species richness and varying species richness for the algae reservoir and copepod water, respectively, was found (Table 4.2). The *A. tonsa* samples had the highest species richness. This could be explained by the fact that these samples were the only samples that were up-concentrated before sequencing. However, from the OTU table, the *A. tonsa* samples also had the highest percentage of unassigned classes, which may have influenced the species richness. This is most likely due to a lack of thorough mapping of the *A. tonsa* microbiome.

The evenness comparison showed that the algae reservoir had the lowest evenness, suggesting that the species were far from evenly distributed in terms of number of each species. As the algae *R. baltica* contributed with *Chloroplasts* to the OTU table, it is not unreasonable to assume that the high *Chloroplast* amount contributed to the low evenness. The fact that the inn-water had the highest evenness, suggests that not one or a very few species were dominating the community. The *A. tonsa* samples had a stable low evenness, with no major change observed between the nauplii and copepodite stage. Suggesting a stable environment between the two days investigated. The high species richness and stable evenness may support the assumption of r-strategists being over-represented on the CFU's. High species richness and small changes with time and between cycles suggests a K-selecting environment. However, the low evenness suggests one or a few dominating species, which is typical in r-selecting environments. As the genotypic fingerprinting was only done for two days in the production cycle, this is not sufficient to say anything about changes with time.

Through beta diversity analysis it was found that the inn-water was the sample type that was most different. In agreement with the findings from the phenotypic beta diversity analysis. The fact that the copepod water samples were found between the algae reservoir samples and the *A. tonsa* samples, slightly closer to the former, supports the assumption that the copepod water had more similar community characteristics to the algae reservoir. This is in agreement with the findings from the phenotypic analysis. That the copepod water share similarities in microbial community comsposition with the algae reservoir for the first few days of the production cycle. As the sample types were still clearly separated, this indicates a development of a distinct community composition with time.

5.2 Conclusion

The water in the copepod tanks had the highest microbial cell concentrations of all sample types. It was also the sample type having the clearest trend of increasing cell concentration with time. Both the qualitative measures of microbial cells with a high RNA content and fast growing microbes, as well as a the nutrient availability, showed big variations with time and between samples. Leading to the conclusion that no apparent selection regime was found for this sample type. However, a slight decrease in both microbial cells with a high RNA content and fast growing microbes, as well as a reduced variation in

nutrient availability with time suggested stabilization. The water in the copepod cultures was most likely transitioning from an r-selected environment to a K-selected environment with time. However, no actual stabilization was found, suggesting that 14 days is too short of a running time to get stable microbial water conditions in the tanks. This was supported by the phenotypic diversity analysis, where both the species richness and abundance of species decreased with time. As well as the change in microbial community, from having likeness with the inn-water, to being more like the algae reservoir and then distancing itself from both sample types with time. The genotypic diversity analysis supported this assumption, as the copepod water samples showed a higher likeness to the copepods and algae reservoir, than the inn-water.

Both the inn-water and algae reservoir had clear selection environments in terms of r- and K-theory. The inn-water had a low and variable microbial cell concentration, high nutrient availability and a high percentage of fast growing microbes. As well as a low species richness and high species abundance. All of this leading to the conclusion that the inn-water was selecting for r-strategists. Finding a high sum of reads from the OTU table for the pathogen associated *Vibrio* genus reinforced this conclusion. Conversely, the algae reservoir had a high and stable microbial cell concentration, low nutrient availability and a low percentage of fast growing microbes. As well as a high species richness and a low species abundance. All the mentioned factors are in agreement with selection for K-strategists.

As K-selection is favorable (pathogens are usually r-strategists), the majority of supplied microbial cells to the copepod water had a desired microbial community composition. Leading to the conclusion that the chance of supplying potential pathogens from the inn-water was extremely small.

As for the copepods, severe limitations in quantitative analysis methods lead to little knowledge about the microbial community associated with the copepods. The plate counting method had several weaknesses, and the medium used was a general medium for marine heterotrophic bacteria. Leading to the assumption that the percentage of fast growing microbes (generalists) was over-represented in the CFU count. However, the fact that these CFU's were used for replica plating to detect haemolytic activity, without finding any, only reinforce the conclusion that the copepods were a good quality live feed option. No haemolytic activity was found for any of the other samples either, leading to the conclusion that the chance of transferring pathogens from the water to the copepods were extremely small. From this the conclusion can be made, that the copepods had a high live feed quality, and posed a low risk of pathogen transfer, when being fed to marine larvae. However, the taxonomic analysis of both the copepods, and the associated water showed reason to concern. Two OTU's had a high sum of reads, corresponding to the genera *Flavobacterium* and *Tenacibaculum*. Both having several fish pathogen species included. As the taxonomy was not evaluated at species level, no determination of pathogen presence could be done. The findings suggests, however, that the chance of pathogens transferred from the copepods to marine larvae could not be entirely excluded.

5.3 Further research

As the quantitative analysis of the copepods was quite limiting in this thesis, using / developing other methods could be useful to look at the microbial cell concentrations associated with the copepods. This may contribute to a broader understanding of microbes associated with copepods, as well as providing an estimate of environment selection. As for the rearing conditions of the copepods, doing an experiment with matured water, rather than disinfected water, could provide more information about optimal rearing. Looking at the hatching percentage, survival percentage and size as parameters. The phenotypic diversity analysis gave consistent result for this thesis. However, this is still a method in development. Using RNA staining for this method have not been done before. Therefore, more research into the quality and robustness of this method is needed.

Chapter 6

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Appendix

A Raw data parameters for the tanks

For round one 715 million eggs where divided into 12 different tanks. The volume of each tank was 4500 liters for day 0, 5000 liters on day 1 and then constantly 6800 liters for the remaining 12 days of the cycle. The salinity was 30% on day 0 and 31% for the rest of the cycle. On day 9 the copepod cultures where split into two cultures. In Table 6.1 the rest of the data is presented.

For round two 600 million eggs where divided into 6 different tanks. The volume of each tank was 4000 liters for day 0, 5500 liters on day 1, 6000 liters on day 2 and then constantly 6800 liters for the remaining 11 days of the cycle. The salinity was 30% on day 1 and 31% for the rest of the cycle. On day 8 the copepod cultures where split into two cultures. In Table 6.2 the rest of the data is presented.

Table 6.1: Raw data for rearing of the copepods during the production cycle in Round 1.

Day	Density [nauplii/ml]	Survival [%]	Average length [μm]	O_2 [%]	Flow through [L/day]	Algae in [L/day]	Temperature [°C]	pH	NH_4-N [mg/L]
0	-	-	-	99.9	0	576	22	8.13	Low
1	118	80	115	99.8	0	1123	22.1	8.07	Low
2	102	94	148	97.4	3564	1123	21.2	8.02	0.67
3	108	100	183	90.5	3450	1340	21.4	7.77	1.16
5	81	75	234	82.4	3542	820	21.1	7.62	1.76
7	82	76	372	82.2	3450	800	21.2	7.57	1.95
9	67	62	408	76.5	3456	1380	21.4	7.47	2.55
12	27	50	530	82.2	3900	1380	21.3	7.69	1.92
14	20	37	582	76.5	3900	1300	21.5	7.7	2.01

Table 6.2: Raw data for rearing of the copepods during the production cycle in Round 2.

Day	Density [nauplii/ml]	Survival [%]	Average length [μm]	O_2 [%]	Flow through [L/day]	Algae in [L/day]	Temperature [°C]	pH	NH_4-N [mg/L]
0	-	-	-	-	-	-	-	-	Low
1	58	57	111	97.3	0	950	20.3	7.96	Low
2	87	94	130	91.9	3456	1120	19.8	7.76	0.18
3	82	100	155	91.9	3300	1080	20.8	7.62	1.6
5	77	94	215	90.8	3700	1300	20.9	7.52	1.27
7	75	91	248	89	3456	1225	21.1	7.56	1.63
9	27.5	67	405	93	3744	1300	21.5	7.92	1.61
12	21.5	52	538	84.5	3900	1440	21.7	7.69	1.86
14	25.5	62	635	82.7	3900	2100	22.4	7.66	2.99

For round three 585 million eggs were divided in 10 tanks. On day 0 the volume of the tanks were 4000 liters, on day 1 4600 liters and on day 12 4300 liters (here the cultures were split in 2). The rest of the 14 day cycle the total volume was 6800 liters. The salinity was constant at 31%. In Table 6.3 the rest of the data is presented.

Table 6.3: Raw data for rearing of the copepods during the production cycle in Round 3.

Day	Density [nauplii/ml]	Survival [%]	Average length [μm]	O_2 [%]	Flow through [L/day]	Algae in [L/day]	Temperature [°C]	pH	NH_4-N [mg/L]
0	-	-	-	100	0	430	22.7	7.88	Low
1	96	74	105	101	0	260	22.3	7.89	Low
2	98	98	139	97.2	2900	1440	22	7.61	0.55
3	88	100	177	93.5	2900	2440	21.7	7.55	1.04
5	84	95	231	73.3	2750	1870	21.6	7.52	1.88
7	84.5	96	354	64.3	2880	1730	22.4	7.42	2.38
9	72	82	399	59.1	3000	2440	22.2	7.41	2.99
12	42.5	61	530	66.2	3000	2160	21.2	7.33	1.82
14	25	57	606	67.6	3000	0	21.8	7.48	2.6

B Illumina 16S rRNA sequencing

B.1 Sample information

The samples mentioned in Table 6.4 were the ones analyzed by Illumina 16S rRNA sequencing. The numbers and corresponding samples are given.

Table 6.4: Samples and corresponding number analyzed by Illumina 16S rRNA sequencing.

Sample	Number
Nauplii stage 5 and 6 for tank 1	1
Copepodite stage 1 and 2 for tank 1	2
Algae reservoir for tank 2 (nauplii stage)	3
in-water for tank 2 (nauplii stage)	4
Copepod water for tank 2 (nauplii stage)	5
Nauplii stage 5 and 6 for tank 2	6
Algae reservoir for tank 2 (copepodite stage)	7
in-water for tank 2 (copepodite stage)	8
Copepod water for tank 2 (copepodite stage)	9
Copepodite stage 1 and 2 for tank 2	10
Algae reservoir for tank 3 (nauplii stage)	11
in-water for tank 3 (nauplii stage)	12
Copepod water for tank 3 (nauplii stage)	13
Nauplii stage 5 and 6 for tank 3	14
Algae reservoir for tank 3 (copepodite stage)	15
in-water for tank 3 (copepodite stage)	16
Copepod water for tank 3 (copepodite stage)	17
Copepodite stage 1 and 2 for tank 3	18

B.2 Mastermix for first round of PCR

The volumes and components used in the mastermix for 1st round of PCR analysis is shown in Table 6.5.

Table 6.5: Recipe for mastermix of a total volume of 25 μl.

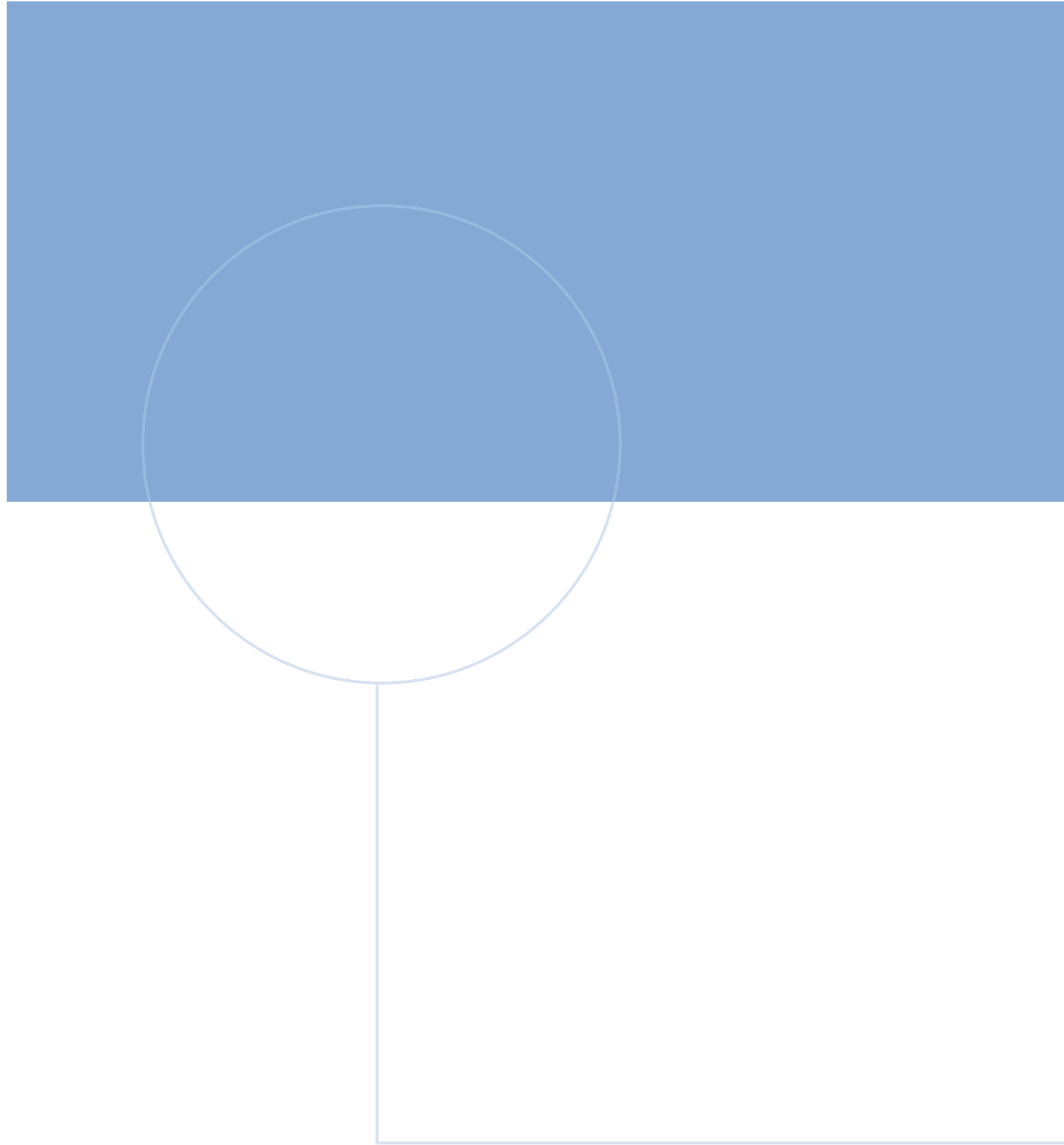
Component	Volume
PCR grade water	16.4375 μl
5x Phusion buffer HF(7.5 mM $MgCl_2$)	5 μl
ill338F (10 μm)	0.75 μl
ill805R (10 μm)	0.75 μl
dNTP (10 mM each)	0.625 μl
$MgCl_2$ (50 mM)	0.25 μl
Phusion Hot Start DNA polymerase	0.1875 μl
Template	1 μl

B.3 Indexing and mastermix for second round of PCR

The recipe for a total volume of 25 μl of the indexing mastermix is shown in Table 6.6

Table 6.6: Recipe for mastermix of a total volume of 25 μl.

PCR grade water	11.437 μl
5x Phusion buffer HF(7.5 mM $MgCl_2$)	5 μl
dNTP (10 mM each)	0.625 μl
$MgCl_2$ (50 mM)	0.25 μl
Phusion Hot Start DNA polymerase	0.188 μl
Index 1	2.5 μl
Index 2	2.5 μl
Template (normalized from 1st round PCR	2.5 μl



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