Aquaculture 560 (2022) 738439

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Microbial water quality of the copepod *Acartia tonsa* in cultures for use as live feed☆

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ARTICLE INFO

Keywords:
Microbial water quality
Acartia tonsa
Water management
Microbial management
Live feed for marine larvae

ABSTRACT

Live feed is important for rearing of marine larvae in the aquaculture industry and copepods have shown some superior qualities compared to *Artemia* and rotifers. Whereas the nutritional composition is one factor contributing to this, the mechanisms behind better performance are not fully known. Microbial conditions could be an important contributor. We characterized the microbiota associated with intensive rearing of the calanoid copepod *Acartia tonsa* by culture-dependent and independent methods. During three independent 14-days production cycles, qualitative and quantitative aspects of incoming water, algal cultures (*Rhodomonas baltica),* and copepod production tanks were measured using flow cytometry with RNA-staining, plate counts, 16S-rDNA amplicon sequencing and hemolytic activity. Assessment of the microbial quality was done based on r/K-theory, presence/absence of hemolytic activity and taxonomic characterization. None of the sample types showed detectable bacteria with hemolytic activity. The incoming water was dominated by opportunistic r-strategists, whereas the algae reservoir was dominated by K-strategist. The production tank had dominance of r-strategists. Abundance of genera with known pathogenic members were not detected in any samples, except the incoming water samples. However, the incoming water contributed with only 1–3% of the cells to the production tank. Because of this, the intensively reared *A. tonsa* cultures appear to have a good microbial water quality as live feed for marine larvae, and the microbial water quality may contribute to the good results obtained when using copepods during first feeding.

1. Introduction

Copepods are considered to be the most abundant multicellular organisms in the world (Hammervold et al., 2015). They are an important food source for numerous species across the higher trophic levels, both in fresh- and saltwater. As copepods form the base of many natural food-chains, they are assumed to be a good choice of live feed for reared fish larvae (Støttrup, 2008). In particular for larvae that are challenging to rear with high survival (Højgaard et al., 2008). Aspects like size, behavior, nutritional quality and digestibility makes the copepods superior as live feed, compared to more traditional feed like *Artemia* and rotifers (Melianawati et al., 2015). The superiority is manifested by the following factors: Improved growth, survival, stress, tolerance and normal pigmentation, as well as reduced deformity rates for fish larvae fed copepods either as their sole food source or as supplements with other diets (Conceição et al., 2010; Evjemo et al., 2003; Karlsen et al., 2015; Liu and Xu, 2009; Øie et al., 2017). The mechanisms for this increased performance are not fully understood, and while the microbial aspect is often overlooked it is possibly a contributing candidate.

The newly hatched fish larvae are often just a few millimeters in length and have organs that are not fully developed at the time of hatching, making larval rearing a complex matter. In addition to the nutritional composition of the feed being of great importance, research published during the last 2–3 decades has documented that microbial conditions play a major role in performance during the first feeding of marine larvae. The research shows that this is more related to dysbiosis than due to obligate pathogens (De Schryver and Vadstein, 2014; Vadstein et al., 2018a). Live feed cultures may serve as a source for detrimental bacteria due to their way of cultivation, often operating at high biomass and a high organic load from overfeeding and the enrichment process (Benavente and Gatesoupe, 1988). Thus, with live feed there is always a risk of transferring pathogens to the vulnerable fish larvae

☆ This paper is dedicated to our coauthor Yngve Attramadal, who died too early during the process of publishing this paper.

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https://doi.org/10.1016/j.aquaculture.2022.738439
Received 11 October 2021; Received in revised form 21 May 2022; Accepted 29 May 2022
Available online 7 June 2022
0044-8486/© 2022 Published by Elsevier B.V.

(Lahnsteiner et al., 2009). *Artemia* and rotifers are well established live feed options, but studies show that microbial communities associated with these cultures often are unstable and harbor detrimental bacteria (Austin and Allen, 1982; Benavente and Gatesoupe, 1988; Zheng et al., 2016), which increase the possibility of transferring pathogens to the larvae. Whereas several studies have been done, both on cultivated copepods (Berggreen et al., 1988; Drillet and Lombard, 2015; Jepsen et al., 2021) and on copepods in natural ecosystems (Castonguay et al., 2008; Peterson et al., 1991), little is known about microbiota associated with copepod production and about the microbial quality of copepods as live feed. To optimize the use of copepods as live feed for marine larvae, more research into these aspects is needed to ensure further improvement of larval rearing.

Microbial water quality in the rearing environment has received increasing attention during the last decade (De Schryver and Vadstein, 2014; Ge et al., 2016; Roalkvam et al., 2019; Vestrum et al., 2018), but so far no generally accepted measurements exist. The microbial water quality is conceptually difficult to define as it has both a quantitative and a qualitative aspect. Traditionally, the focus has been on the quantitative aspect, and antibiotic use and disinfection are well known methods used to decrease the microbial load in a system (Jorquera et al., 2002; Kasai et al., 2002; Summerfelt, 2003). The qualitative aspect has in the meantime often been ignored, even though it has been found to be more important (Vadstein et al., 2018b). The possibility to evaluate qualitative aspects is limited except for the presence of well-known pathogens, but the microbial problems in rearing of marine larvae is often not associated with known obligate pathogens (Vadstein et al., 2004). A concept that can be used to evaluate microbial water quality is the r/K-theory. This concept has been successfully applied to analyze and steer microbial conditions in larval rearing, and r-strategic, opportunistic species seems to be the ones mainly causing parasitic fish-microbe interactions (reviewed by Vadstein et al., 2018a). r-strategists are defined by having a high growth rate in uncrowded environments with excess nutrient availability. Opportunistic pathogens are often associated with this group of microbes. On the other end of the scale are the K-strategists. They have a slower growth rate, are found in more crowded environments with high resource competition, and tend to form more stable communities (Andrews and Harris, 1986). In such environments there is little room for r-strategists to take hold, and this limits the risk for outbreak of opportunistic pathogens. From this concept, creating a stable microbial community dominated by K-strategists within the rearing system will be beneficial, whereas the use of antimicrobial agents and disinfection will benefit conditions for opportunistic r-strategist by creating open niches. Traits based on r- and K-selection may therefore be used as a basis for developing criteria for microbial water quality (Skjermo et al., 1997). Thus, for production facilities it should be important to characterize the microbial communities associated with the larval rearing tanks. Live feed is one important input of microbes, but so far this aspect has not gotten much attention, outside of trying to limit them altogether by disinfection.

In this study we characterized the development of the microbiota associated with the calanoid copepod *Acartia tonsa* throughout three production cycles, with the aim to measure qualitative and quantitative aspects of microbial water quality and to determine which factors contributes to the microbiota during the culture process. As a consequence of this, crucial points of microbial management will be uncovered, and the microbial water quality of the *Acartia tonsa* cultures will be determined.

2. Materials and methods

2.1. Experimental design, sampling strategy and sample handling

Three independent production cycles of *A. tonsa* reared from disinfected eggs to adult stage (14 days) were investigated. The disinfection procedure consists of treating the copepod eggs to a NaOCl-bath (4 ml 14% NaOCl-solution with a 10-minute contact period) followed by neutralization using sodium thiosulfate. Thorough method development has found this treatment to eliminate bacterial growth from the surface of the copepod eggs (testing performed by qPCR on specific pathogens, TCBS-agar and Marine Agar, unpublished results). Initial density of the cultures was approximately 100 copepods/ml (see supplementary data) and the culture temperature was kept at 21 °C. The copepods were fed the microalgae *Rhodomonas baltica* (CCAP979/9), keeping a concentration > 500 μg C/L through continuously adding algae to the culture system (see supplementary data). There was no water exchange on day 0 and day 1. From day 2 the daily water exchange was gradually increased to 75% throughout the culture period, by continuous influx of new water.

Samples were collected five days a week throughout each production cycle. For each sampling, four sample types were analyzed to map the contributors to the microbiome in the *A. tonsa* production tank: 1) Filtered (0.2 μm) and UV-treated influx water entering the tank (in-water). 2) Water from the reservoir of continuously flow cultures of *R. baltica* (algae reservoir). 3) Water from the *A. tonsa* production tank (production tank). 4) Up-concentrated samples of *A. tonsa* (*A. tonsa*). The samples were transferred to sterile 20 ml tubes and processed within 3 h of sampling.

2.2. Analytical methods

2.2.1. Qualitative and quantitative water quality

To quantify colony forming units (CFU) and the percentage of fast growing bacteria, dilution series were made for each water sample that was plated on M-65 medium plates (0.5 g yeast extract, peptone and tryptone; 10 g agar; 200 ml MilliQ-water; 800 ml filtered seawater; per liter medium) (Salvesen and Vadstein, 2000). The *A. tonsa* samples were filtered and washed with a mixture of 80% sterile seawater and 20% MilliQ-water, before being transferred to a petri dish containing the same water mixture. 10 individuals were transferred to a sterile Eppendorf tube, homogenized, and diluted to a total volume of 1 ml using the same water mixture. The samples were plated out as described for the water samples. All plates were incubated at 18 °C and in the dark to avoid algal growth. The CFU counting was done after three and ten days of incubation. CFUs visible after three days were considered fast-growing and counts on day ten represented total CFU. Two technical replicates were made per dilution.

To quantify the number of hemolytic bacteria, blood agar with 1.5% salt was used. After between 10 and 15 days of incubation on M-65 plates, replica plating was done by using a Whatman Protran nitrocellulose membrane to transfer the colonies from M-65 plates to the blood agar plates. The blood agar plates were incubated at 18 °C for approximately 20 h, before they were checked for hemolysis. One replicate was made per sample.

Flow cytometry (BD Accuri™ C6) was used for quantification of total cell densities, determining fractions of cells with high RNA content and phenotypic fingerprinting of bacteria in the water samples. Samples (1.8 ml) were transferred to cryotubes and fixated to a final concentration of 1% glutaraldehyde. The samples were stored for 30–60 min at room temperature, before being snap frozen in liquid N_2 and finally stored at −20 °C until analysis.

Before analysis, the thawed samples were diluted 100× with 0.2 μm filtered 0.1× TE-buffer, to a total volume of 1 ml. The in-water samples were only diluted 10×. To the total volume 10 μl of 2% CYBR Green II (staining mainly RNA) was added. The samples were incubated in the dark for 15 min.

A maximum of nine samples were run through flow cytometry at the same time (due to the instability of the dye/RNA-complex) and for every 3rd or 4th sample, a wash with MilliQ water was used to limit contamination between samples. The data was collected for 2 min at a flow rate of 35 μl/min, and a threshold of minimum 1000 events/ μl (to minimize noise) on the FL1-H channel for the CYBR-RNA fluorescence.

To quantify the growth potential of bacteria, the water samples (5 ml) were incubated at 18 °C for three days in sterile 15 ml tubes. The tubes had ventilation and were incubated in a tilted position to ensure aerobic conditions. After three days the samples were fixated and snap frozen (as explained above), and the cell densities were counted using flow cytometry (same setup as for the initial samples).

2.2.2. Bacterial community composition by amplicon sequencing

Bacterial community composition was determined by Illumina sequencing of 16S rRNA gene amplicons for *A. tonsa* (nauplius stage 4/5 and copepodite stage 1/2) and associated water samples. These are two cultured life stages of *A. tonsa* that are used as live feed for ballan wrasse, Atlantic cod and Atlantic halibut (pers. Com. CFEED; Øie et al., 2017). The *A. tonsa* samples were up-concentrated and washed with 80% seawater and 20% MilliQ-water. Then a total of 3.8 ml of *A. tonsa* and associated water samples were snap frozen in liquid N_2 and stored prior to analysis.

Thawed samples were centrifuged at 21.500 *g*, and the supernatant was carefully removed. The protocol for high-throughput sequencing of 16S-rRNA PCR amplicons followed Fossmark et al. (Fossmark et al., 2020), except that the cells in the samples were concentrated using a centrifuge, rather than a sterile filter. In brief, the DNeasy PowerSoil Kit was used to extract DNA. PCR was performed using the primers ill228F and ill805 that target the V3 and V4 regions of the 16S rRNA gene. Gel electrophoresis (1% *w/v* agarose, 50 min, 115 V) was performed to confirm correct length and strength of the PCR product. For normalization, the Sequal Prep Normalization plate Kit from Invitrogen was used. The products were normalized, indexed, normalized again to make a pooled sample, and sent to the Norwegian Sequencing Centre at University of Oslo for analysis.

2.2.3. Data analysis and statistics

The flow cytometry data was analyzed using RStudio, and the FlowCore package(Ellis et al., 2019). For the counts of bacterial cells based on flow cytometry, two gates were made for the total and high RNA-content cell counts, respectively. As the counts were not normally distributed (Shapiro-Wilk test), statistical comparison was done using the Kruskal-Wallis test for independent samples.

A phenotypic fingerprinting analysis was performed using the flow cytometry data (Props et al., 2018). Noise was removed based on gating to exclude non-bacterial events. The data was normalized using the arcsine hyperbolic function to the range of [0,1] for all variables. The density estimates obtained from the normalization were assigned to a 128 bivariate binning grid (Props et al., 2016). This method is based on the hypothesis that phenotypic changes in the microbial communities are reflected by changes in event counts in the bins in a multidimensional space (Buysschaert et al., 2018). The binning grid created the flowBasis function that was used to calculate the phenotypic fingerprint of the samples. Alpha diversity was calculated using the rf.fbasis function, with $R = 100$ bootstraps, which means that the function was running through the samples to check for consistency 100 times.

The Illumina data was processed using the USEARCH pipeline (version 11). Pair reads were merged, primer sequences trimmed, and all reads shorter than 400 bp were filtered out. This was followed by quality filtering and demultiplexing, with an expected error threshold of 1. OTU clustering was performed at a 97% similarity level, by implementing the UPARSE algorithm (Edgar, 2013). This also removed chimera sequences and singletons. 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 normalized based on the sample containing the lowest sum of reads (13.300 reads, resulting in removal of 127 OTUs). Illumina sequencing data are deposited at the European Nucleotide Archive (accession number ERS7182688 -l ERS7182706).

For both the phenotypic fingerprinting and the amplicon sequencing data, alpha diversity indices were calculated as Hill numbers (Hill order 0, 1 and 2) (Hill, 1973). The beta diversity was visualized by ordination based on Principal Coordinates Analysis (PCoA), using both Bray-Curtis (abundance based) and Dice-Sørensen (presence/absence) distances. A PERMANOVA test was used to test for statistical differences between groups of samples.

2.2.4. Bacterial mass balance calculations

To assess how the bacterial cell concentration (C, cells/L) in the production tank varied relative to cell input, cell loss and growth during the production cycle, a mass balance was set up with the following parameters: Specific supply rate (S) from incoming water and supply of algal culture, specific loss rate (L) due to dilution, specific growth rate in the production tank and specific net rate of change (R) (net change in microbial cell concentration in the production tank per time). All rates are normalized to C and are given with dimension day^{-1}.

$$\frac{dC}{dt} = (S + \mu - L)\ C => \frac{dC}{dt}\frac{1}{C} = R = S + \mu - L \quad (1)$$

S was calculated using Eq. (2).

$$S = \frac{C(Ar) \bullet F(Ar) + C(Iw) \bullet F(Iw)}{C} \quad (2)$$

Here, *C(Ar)* and *C(Iw)* are the concentrations of bacteria [cells/L] in the algae reservoir and in-water pumped into the tank, respectively. F *(Ar)* and F*(Iw)* are the respective flow rates [L/day]. L was set equal to C multiplied with the dilution rate (D) of the production tank. D was calculated as the daily sum of flow rates from the algae reservoir and in-water into the culture divided by the volume of the culture.

The specific net rate of change (R) was estimated by linear regression of the logarithm of cell concentration in the production tanks (C) against time. R was estimated independently for each experiment. The specific growth rate (μ) of bacterial cells in the production tank was calculated independently for each experiment using Eq. (3).

$$\mu = R - S + L \quad (3)$$

Based on the estimated parameters a simulation was created to show the relative importance of the different supply and loss processes, assuming a start concentration of $9.0 \bullet 10^6$.

We thus assumed no mortality for bacteria in the production tank. For predation this is realistic as even nauplii cannot impose significant predation rates on cells as small as bacteria (Ingrid et al., 1996), whereas for mortality due to phage infections we have no data to validate this assumption.

All rates were calculated for each sampling day. Averages +/− SD or SE are reported below.

3. Results

3.1. Qualitative and quantitative evaluation of cell counts

There was little variation in total counts from flow cytometry and CFU counts between the three experiments, indicating high predictability in microbial quantities. Consequently, the averages and standard deviations for the three biological replicates are presented to evaluate variation with time for total cell counts and CFU counts (Fig. 1, a and b).

Both methods showed that the production tank had the highest cell concentration. For total cell counts (flow cytometry) it was the only sample type with a clear increase in concentration with time. The in-water had a concentration of 1 to 2 orders of magnitude lower than the production tank. The algae reservoir had a stable cell concentration over time. Fast-growing CFU's (a) and cells with a high RNA content (b) are presented in Fig. 2 as a function of time.

The production tank had the highest cell concentration, both for total cell count and CFU count, corresponding to Fig. 1. The difference in concentration between the algae reservoir and production tank is smaller for the CFU counts than for the total cell counts. The in-water

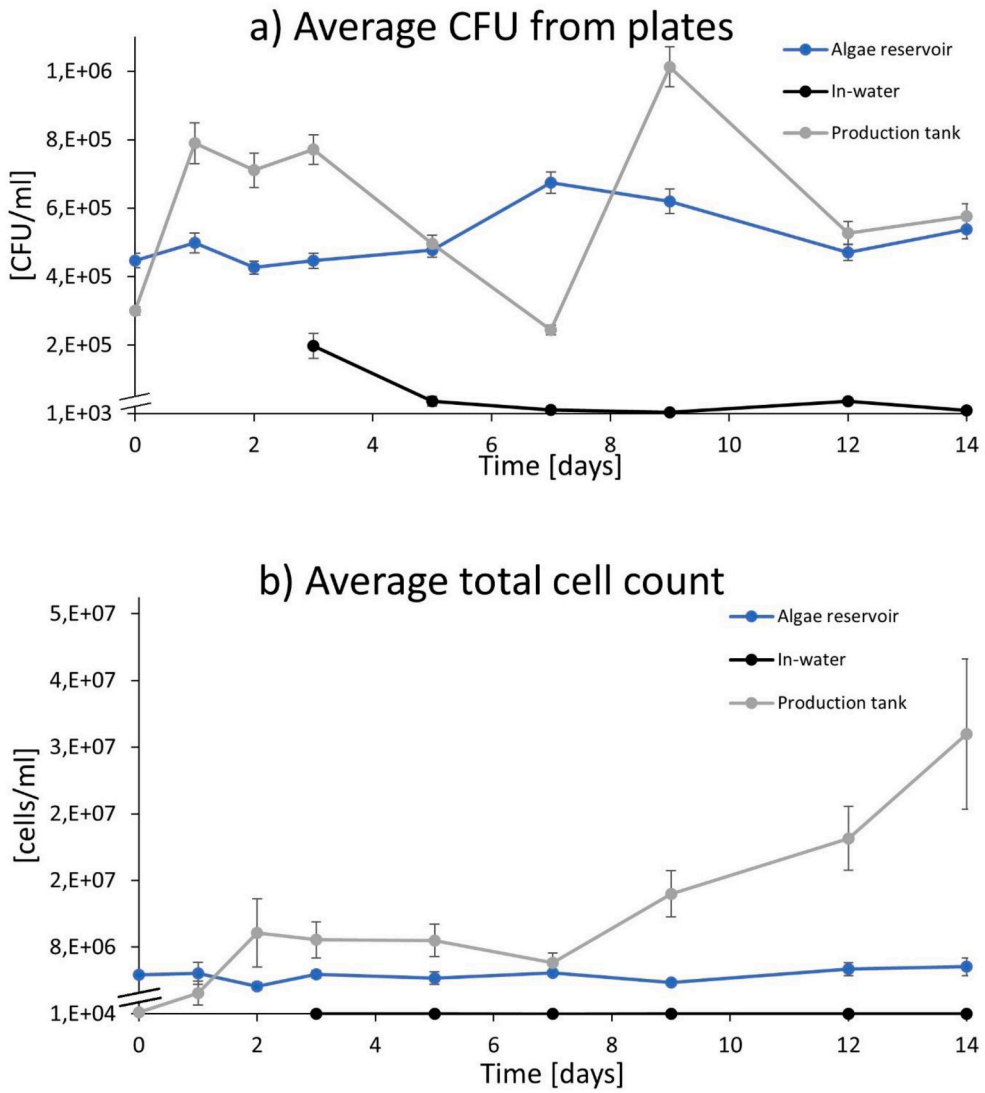

Fig. 1. Average CFU count (a) and total cell count from flow cytometry (b) from the three biological replicates of each sample type is shown in the plot. SDs for each of the three experiments are indicated. Lack of data for In-water is missing the first days due to no water exchange.

had the lowest cell concentration of the water samples, but of all sample types, *A. tonsa* had the lowest CFU counts per milliliter.

The production tank and in-water had the highest percentage of fast-growing microbes (see supplementary), but the fluctuations were higher for the in-water. *A. tonsa* had almost the same percentage of fast-growing microbes as the production tank and in-water, whereas the algae reservoir had less than half. The algae reservoir had the highest percentage of cells with a high RNA-content. In comparison, the in-water and the production tank had approximately 20% less cells with a high RNA-content.

Both the algae reservoir and the production tank had a low growth potential, ranging from −1 (one halving of cell concentration) and 1 (one doubling of cell concentration) (Fig. 3). The in-water differed significantly compared to the other water samples, with a growth potential of 4 doublings. It also had the highest variation.

Overall, the algae reservoir had the most stable growth potential, varying between 0 and 1 doubling while the in-water had the highest growth potential of the sample types. The production tank started out with a high growth potential (>3 doublings) but stabilized around zero. Thus, whereas there was hardly any organic matter for growth of heterotrophic bacteria in the algae reservoir and in the copepod production tank, there was enough organic matter in the in-water to support between 4 and 5 doublings in bacterial density (>16× increase). It must, however, be kept in mind that the initial cell densities in the in-water were low, providing more organic matter per cell. Microbial growth was observed on the blood agar plates, but no colonies had indications of hemolytic activity. Thus, no hemolytic bacteria were detected in any of the sample types, including the *A. tonsa* samples.

3.2. Microbial abundance budget in the production tanks

The change in microbial cell concentration (net change) in the production tank, dependent on supply, loss and growth of cells is shown in Fig. 4 with the mock concentration $9.0 \bullet 10^6$ cells/L as the basis.

The net cell concentration in the production tank had a steady increase with time (Fig. 4) and it was observed minimal variations between biological replicates (with one decimal accuracy all three rates were 0.1 d^{-1}). Specific net change rate was 0.109 ± 0.013 d^{-1}. The rate of supplied cells also increased with time for the first few days. However, variations between replicates were observed. The supply factor increased up until day 5–7, before decreasing. This is shown as a less steep increase for the supply rate (Fig. 3). The specific supply rate was 0.100 ± 0.052 d^{-1}. The specific loss rate (0.735 ± 0.088 d^{-1}) showed a steady loss of cells with time. From Eq. (3). the average specific growth

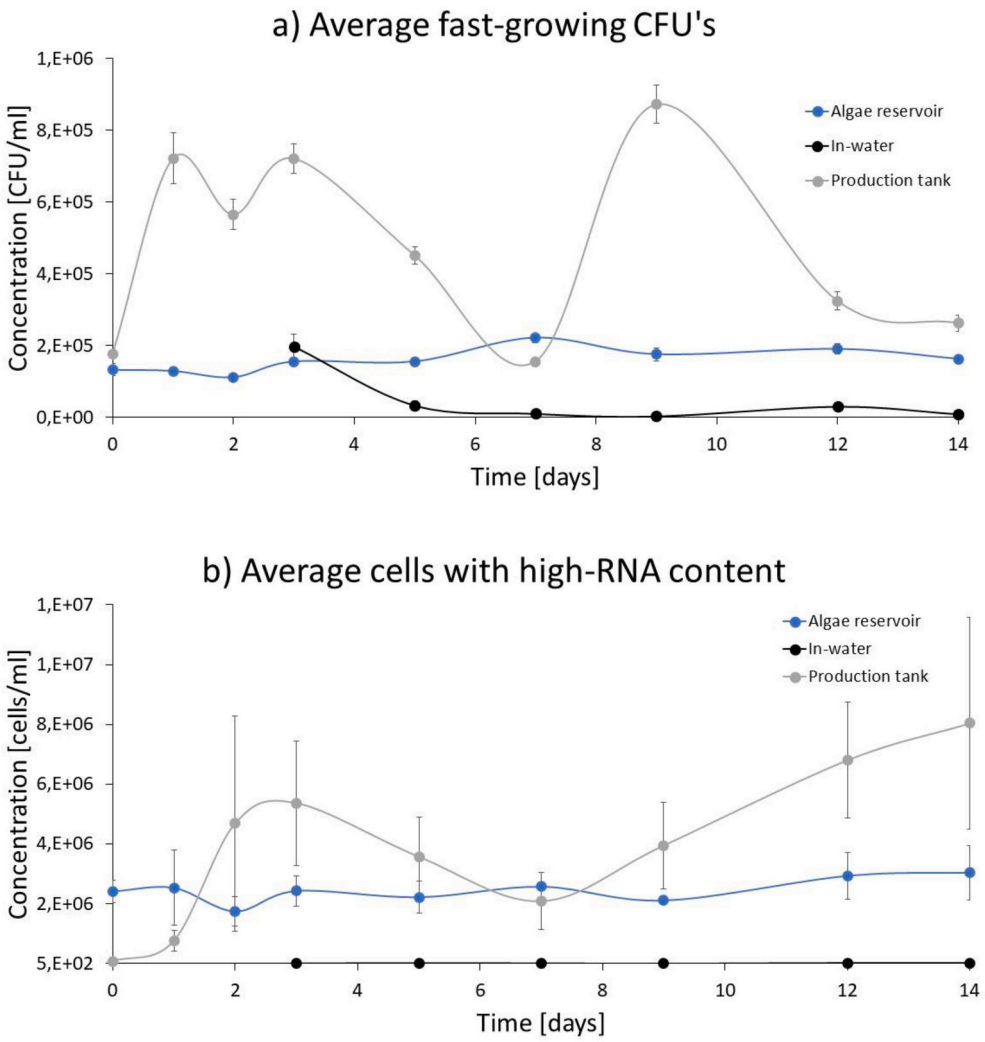

Fig. 2. Average concentration of fast-growing CFU's (a) and cells with a high RNA content (b) from the three biological replicates of each sample type are shown in the plot as a function of time. SD for each of the three experiments are indicated. Lack of data for In-water is missing the first days due to no water exchange.

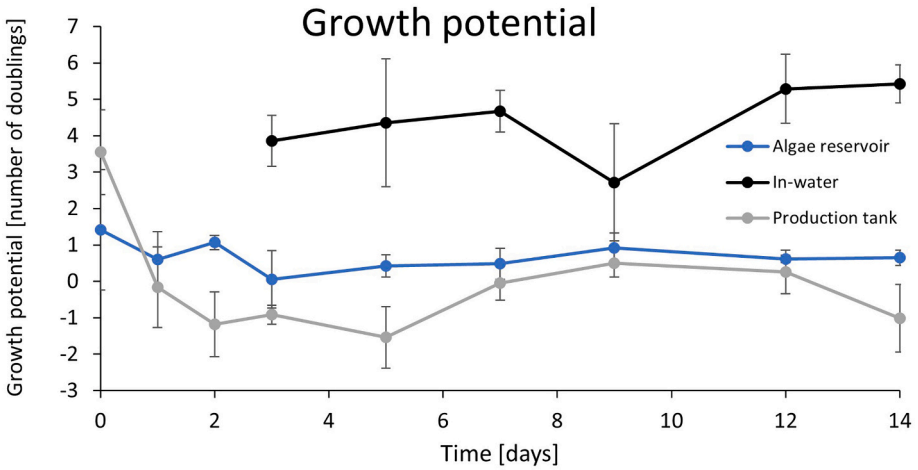

Fig. 3. Average growth potential quantified as number of doublings, for the three biological replicates of each sample type as a function of time. The SDs associated with each sample type are indicated.

rate (μ) was estimated to $0.744 \pm 0.097\ d^{-1}$. Meaning that the microbial biomass in the production tank doubled per day on average. The growth in the production tank was 7 times bigger than the supply rate, which was mainly from the algae culture. Of the supplied cells, the relative contribution of the algae reservoir was 97–99% compared to the in-water.

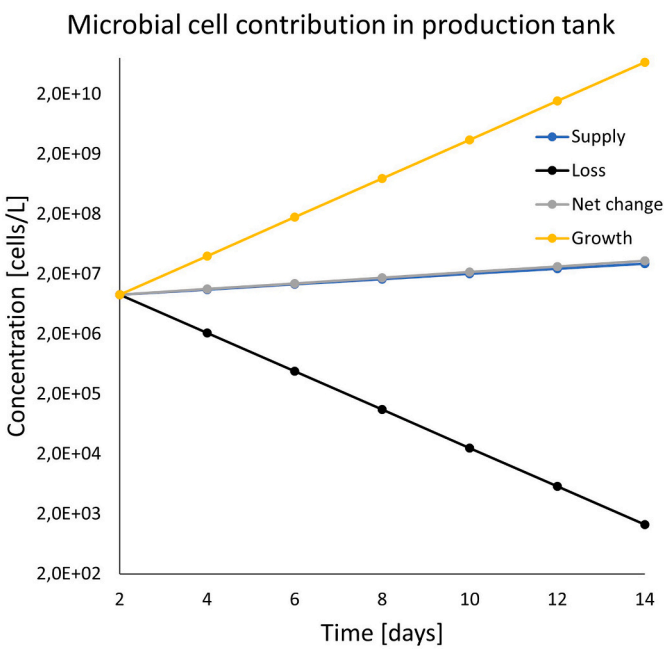

Fig. 4. The supply, loss, growth, and net change in cell concentration, based on the assumption of an ideal model of constant rates, as a function of time is shown. A mock concentration of $9.0 \bullet 10^6$ cells/L, with the basis of the cell concentration in the production tank, was chosen to secure the same basis for the four rates of change. Day 2 of the production cycle was chosen as the starting point to give the system time to stabilize.

3.3. Bacterial community composition within the *A. tonsa* production system

An average sequence coverage for all samples were calculated to 97% for the samples. The community composition at class level is presented for the 16 most abundant classes in Fig. 5.

The three most abundant genera in the production tank water were *Lentibacter*, *Polaribacter* and *Donghicola* (23, 2.6 and 2.3%, respectively). For the algae reservoir it was *Alphaproteobacteria, Verrucomicrobia* and *Sphingobacteriia* that dominated in the samples (adding up to 86% of the total read). The three most abundant genera were *Donghicola*, *Lentibacter* and *Hyphomonas* (5, 1.8 and 1.4%, respectively). For the in-water proteobacteria constituted >87% of the read, and included *Alphaproteobacteria, Gammaproteobacteria* and *Betaproteobacteria*. The three most abundant genera found were *Ralstonia*, *Vibrio* and *Nautella* (34, 5 and 3.4%, respectively). For the *A. tonsa* samples, *Alphaproteobacteria, Gammaproteobacteria*, *Flavobacteria* and a class of unassigned OTU's were the most abundant classes (Fig. 5). They typically added up to 90% of the total read. The three most abundant genera associated with *A. tonsa* were *Nautella*, *Flavobacteriia* and *Tenacibaculum* (4.5, 4 and 3%, respectively), but the three most dominating genera had a surprisingly low abundance. The water in the production tank had a composition similar to the *A. tonsa* samples. However, *Alphaproteobacteria* made up a higher percentage of the OTU's in the water (52% compared to 30% for *A. tonsa*), and the percentage of unassigned OTU's was lower (7% compared to 27% for *A. tonsa*). Even though there were some variations, overall, the biological replicates showed the same trend for each sample type.

3.4. Analysis of phenotypic and genotypic diversity

Both phenotypic and genotypic analysis showed that the algae reservoir had the highest alpha diversity in terms of richness, followed

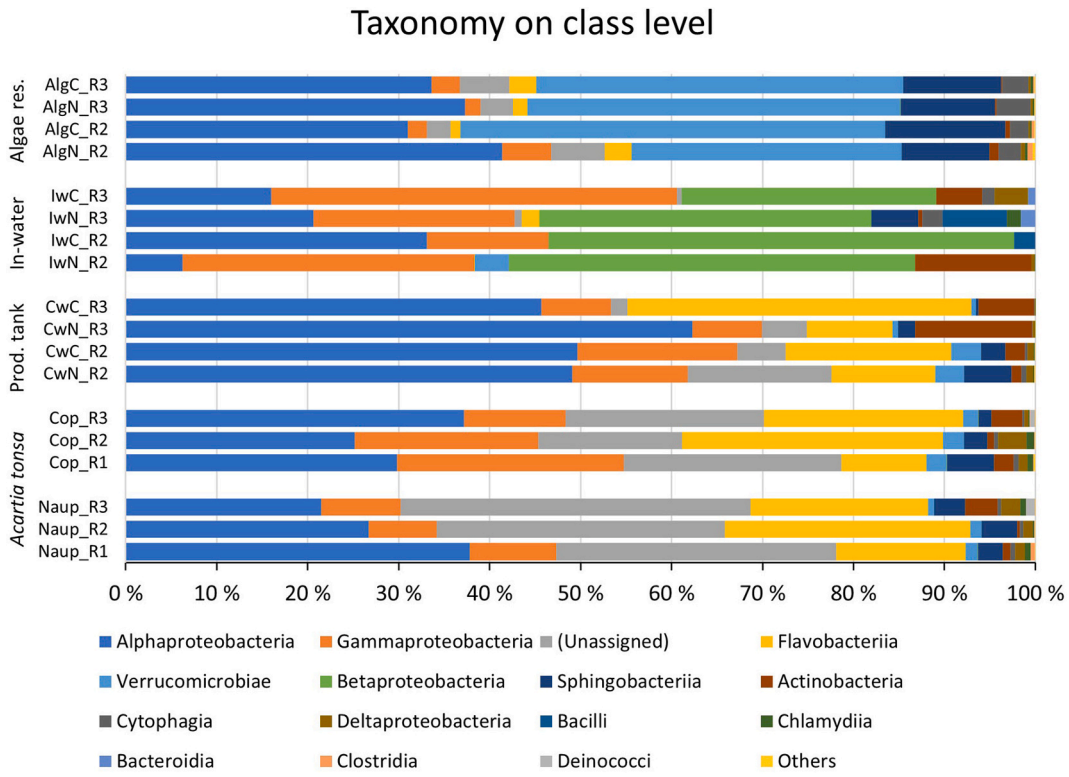

Fig. 5. Taxonomy on class level for the *A. tonsa* and water samples at nauplius stage 4/5 (Naup) (day 5) and copepodite stage 1/2 (Cop) (day 7) for each of the three replicated experiments (Rx). IwN and IwC are in-water samples corresponding to the nauplius stage and copepodite stage, respectively. Similarly, AlgN and AlgC, and CwN and CwC are the algae reservoir and production tank samples, respectively, that corresponds to the associated *A. tonsa* growth stages.

by the production tank and the in-water (Hill order 0; Fig. 6 and Table 1).

With more emphasis on abundance (Hill order 2) the in-water had the highest phenotypic alpha diversity, followed by the algae reservoir and the production tank. Whereas for genotypic alpha diversity the algae reservoir had a higher abundance than the in-water. Both were higher than for the production tank.

The in-water had the highest evenness, both genotypic and phenotypic (Table 1). The algae reservoir had the lowest genotypic evenness whilst the production tanks had the lowest phenotypic evenness. The phenotypic evenness of the different samples was more similar than the genotypic evenness (a difference of 0.09 between highest and lowest evenness, compared to 0.27 for the genotypic analysis).

Genotypic diversity analysis was also applied to *A. tonsa*, which had a higher species richness (Hill order 0) than any of the water samples (Table 1). For Hill order 2 *A. tonsa* had the same alpha diversity as the water in the production tanks. Except for the algae reservoir, *A. tonsa* had the lowest evenness.

To assess the phenotypic beta diversity for the algae reservoir, in-water and in the production tank, Bray-Curtis similarities and ordination was used (Fig. 7).

Three distinct clusters were found with the in-water samples furthest away along the PCoA 1-axis (51% of the variance). The algae reservoir and the production tank were more similar, with dispersion along the PCoA 2-axis (11% of the variance). The algae reservoir samples were also in a defined cluster, but slightly more spread out than the in-water. Initially (at day 0), the production tank water was similar to the in-water for two out of three replicates. However, already on day 1 the production tank adapted more of the characteristics of the algae reservoir. Roughly between day 3 and 5 the production tank started forming a distinct cluster through separation from the algae reservoir along principal coordinate 2.

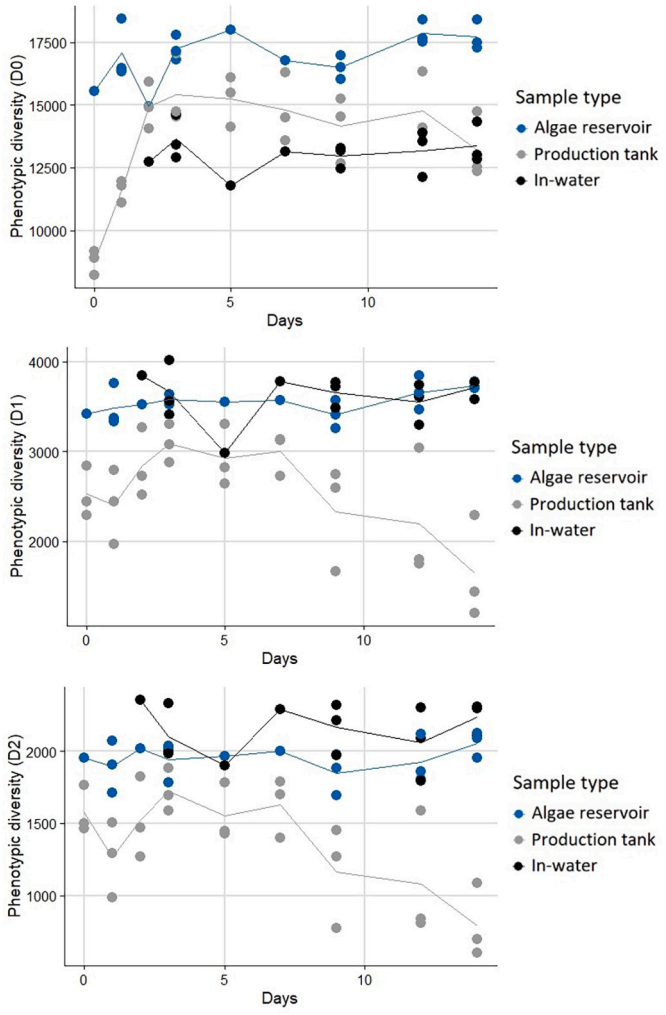

Fig. 6. Phenotypic alpha diversity for the water samples: Algae reservoir, production tank and in-water as a function of time. The diversity is presented for Hill order 0 (D0), 1 (D1) and 2 (D2), respectively. The lines represent mean values between replicates of each sample type per day.

Table 1
Comparison of average genotypic (genetic characteristics; Geno) and phenotypic (physical characteristics; Pheno) alpha diversity as Hill numbers of order 0, 1 and 2, and evenness (E), calculated based on D1/D0, for all sample types. n.a. = not available.

	Algae reservoir	In-water	Production tank	*A. tonsa*
	Geno / Pheno	Geno / Pheno	Geno / Pheno	Geno / Pheno
D0	190 / 17093	28 / 13145	177 / 13649	237 / n.a.
D1	13 / 3564	11 / 3624	26 /2548	29 / n.a.
D2	1.3 / 1946	1.2 / 2151	1.1 / 1367	1.1 / n.a.
E	0.07 / 0.21	0.41 / 0.28	0.41 / 0.28	0.12 / n.a.

For the genotypic diversity analysis at day 5 and 7, both Bray-Curtis and Dice-Sørensen distances were used to assess the beta diversity (Fig. 8 and supplementary data). The same clusters were found from both types of distances. This dataset is smaller but includes samples for bacteria associated with *A. tonsa*.

Four different clusters were formed for the respective sample types, indicating differences in community composition between all of them (Fig. 8). Along the 1st coordinate the in-water samples differed clearly from other sample types, which in turn were close to each other on Coordinate 1. On the 2nd coordinate the in-water is close to the production tank, whereas the *A. tonsa* samples and algae reservoir were less similar. The same trend is found in both plots. Bray-Curtis is based on abundance of species, whereas Dice-Sørensen (supplementary) is based on presence/absence of species (McCormick et al., 2000). This suggest that a considerable part of the variation between the samples is due to changes in the OTU inventory, and not only changes in relative abundance.

A PERMANOVA test based on the genotypic data confirmed that the microbial communities of *A. tonsa* were significantly different from that found in the water samples (Algae reservoir: $p = 0.0042$, production tank: $p = 0.0051$ and in-water: $p = 0.0053$). The water samples were also significantly different (algae reservoir vs production tank: $p = 0.029$, algae reservoir vs in-water: $p = 0.031$ and production tank vs in-water: $p = 0.027$). For the phenotypic data all sample types were significantly different ($p < 0.001$), with and without the two outliers from the production tank on day 0 (Fig. 6).

4. Discussion and conclusion

The production tank had the highest microbial cell concentration. The density was ten times higher than that of the algae reservoir and 10^3 times higher than the in-water. The production tank also had a clear increase in cell concentration with time, as the only sample type. As the production tanks were supplied with microbial cells from both the in-water and algae reservoir, some of the increase could be explained by this. However, the tank also had a high flow-through of water due to water exchange, which causes loss of microbial cells. Because of the clear concentration increase, a reasonable explanation is that the cell growth over-compensated for the cell loss. This indicates a presence of dissolved organic matter (DOM), produced by copepods (fecal matter) and algae (exudates) that can be utilized by the bacterial community (Vadstein et al., 1989). In natural systems microbial concentrations of 10^8 cells/ml have been associated with fecal matter from copepods (Tang, 2005). Copepods also produce a substantial amount of DOM by

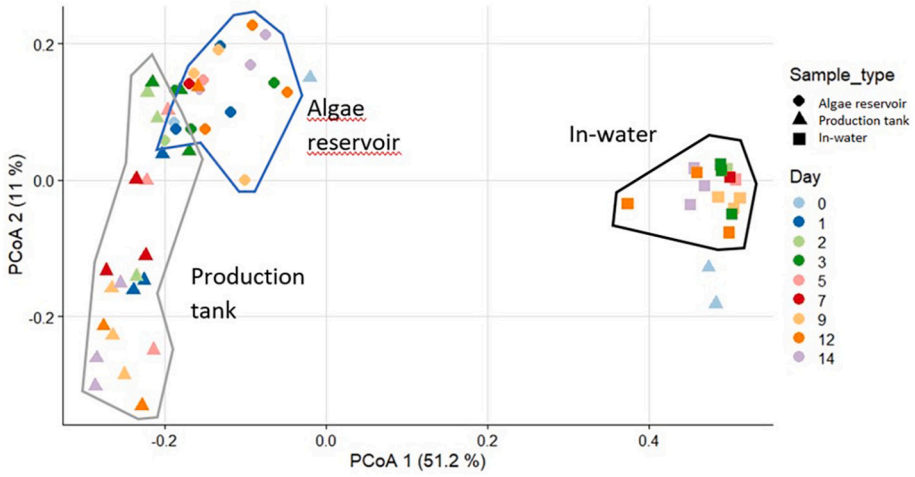

Fig. 7. PCoA plot based on Bray-Curtis similarity, showing the phenotypic beta diversity for the three independent experiments: Algae reservoir (circles), Production tank (triangles), In-water (squares). The colors indicate sampling. Variance explained by each principal coordinate is given in parenthesis in the axis titles (a total of 63% is explained by this plot). Poly-nomes marks sample types (data for the production tank is excluded for day 0).

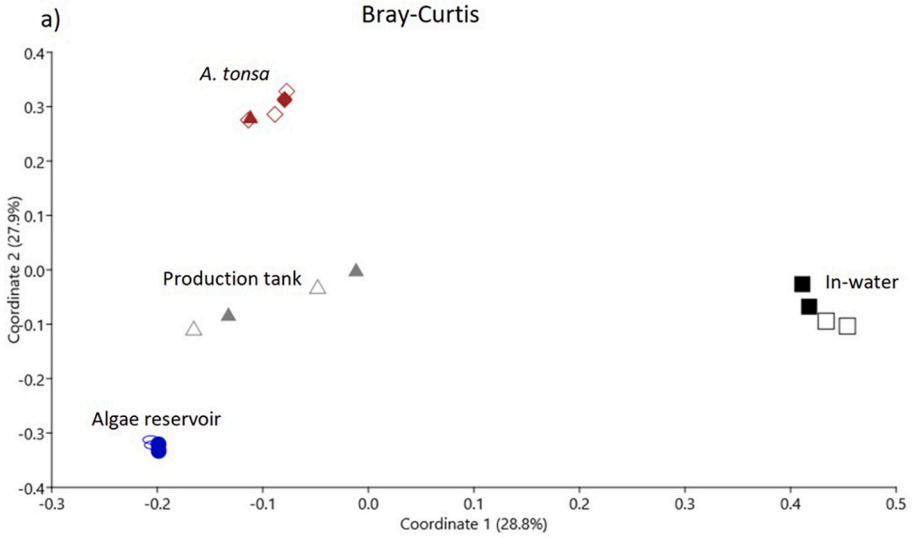

Fig. 8. PCoA plots based on Bray-Curtis similarity, showing the genotypic beta diversity for the four independent experiments (56.7% of the variance explained). The symbols represent sample types: *A. tonsa* (diamonds), production tank (triangles), algae reservoir (circles), in-water (squares). Open symbols represent samples on day 5 of the and closed symbols represents day 7.

sloppy feeding, e.g. tearing apart prey that does not fit into the copepods mouth whole (Møller, 2005, 2007; Saba et al., 2011). A significant increase in biomass took place between day 5 (*A. tonsa* nauplii stage) and day 7 (*A. tonsa* copepodite stage) (Fig. 1 b). (Attramadal et al., 2014; Brown and Wittwer, 2000; Props et al., 2016; Støttrup et al., 1986). With an increase in *A. tonsa* biomass the natural assumption is an increased production of DOM, for the same reasons as mentioned above. However, the growth potential of the production tank remained steadily at between 0 and 1 doubling. This might be due to inorganic nutrient- and mineral limitations(Berggren et al., 2010; Cotner et al., 2000). The growth potential measured only bacterial growth and did not consider the actual amount of DOM present. Both in *Artemia* and rotifer cultures, a bacterial density of 10^7 cells/ml have been found(Austin and Allen, 1982; Nicolas et al., 1989; Skjermo and Vadstein, 1993). That is 10× higher than what was found in the *A. tonsa* cultures (10^6 cells/ml). The density in *Artemia* and rotifer cultures are usually higher, which could be an explanation for the higher bacterial density. It can also be noted that copepods have a lower ability to predate on bacterial sized particles than Artemia and rotifers (Hansen et al., 1994). As there are large varieties in the way of culturing the different types of live feed (biomass, feed source, temperatures, etc.) a direct comparison between the results obtained here and previous studies cannot be made.

The percentage of fast-growing microbes in the production tank was approximately 70%, indicating a high nutrient availability per cell favoring opportunistic r-strategist. The low percentage of cells with a high RNA-content (30–40%) do however seem to contradict this, as a high RNA-content is linearly correlated with growth rate (Benthin et al., 1991). However, the classification of r-strategists is based on maximum specific growth rate and not realized growth rate. The total cell count for the production tank was a 100× higher than the CFU count, and the latter (with its limitations) was used to calculate the percentage of fast-growing r-strategists (Brown and Wittwer, 2000; Salvesen and Vadstein, 2000). Despite the limitations to this method, we conclude that the production tank was dominated by potentially detrimental r-strategists. However, no genera with known pathogenic species were found in the water of the production tank. In conclusion this suggest that the microbial quality in the production tank was not as poor as the fraction of r-strategists suggest.

As expected, the filtered and UV-treated in-water had the lowest total cell count and CFU count (Laroussi and Leipold, 2004). It was also the only sample type in which total cell count and CFU count were comparable (10^4 ml^{-1}) in magnitude. The high percentage of culturable, fast-growing microbes (50–90%) is a clear indication of a bacterial community dominated by opportunistic r-strategists(Andrews and Harris, 1986; Kirkby et al., 1968; Salvesen and Vadstein, 2000). This was supported by the low cell concentration causing an uncrowded environment, and by the high growth potential (3–5 doublings in cell concentration). The latter indicate high per cell nutrient availability, due to a combination of low cell density and production of nutrient due to killing of cells by UV treatment, as well as good nutrient availability from the deep ocean water in Trondheimsfjorden (Steinberg et al., 2002).

Even though the in-water had a less desirable bacterial composition from a microbial water quality point of view (Attramadal et al., 2014; Vadstein et al., 2018a, 2018b), the in-water only accounted for 1–3% of the bacteria in the production tanks. Most of the cells came from the algae reservoir, which seemed to have a more desirable bacterial composition. While the total cell count and the CFU count of the algae reservoir was on average higher than for the other water samples, the percentage of fast-growing microbes (30%) was less than half of what was found for the production tank and the in-water. The growth potential varied between one halving and one doubling in cell concentration, suggesting a crowded environment with low nutrient availability. A balanced growth potential around zero doublings, as for the algae reservoir, indicates just enough nutrient availability to keep the bacterial concentration steady, explained by the fact that *R. baltica* competes with the bacteria for inorganic nitrogen, phosphorus and minerals (Berggren et al., 2010; Cotner et al., 2000). In conclusion, this indicate that the bacterial community in the algal culture was dominated by K-strategists.

The percentage of cells with a high RNA-content (~50%) was, however, higher than for the other water samples. This could be an adaptation to the environment. The algae *R. baltica* is able to ingest bacteria by phagocytosis (Clay, 2015), and their high density in the algal culture will impose a high mortality rate on the bacteria. To compensate for this, the bacteria need to have a high growth rate. This may create a selection pressure, as only those species able to balance growth and predation will survive on longer term.

A. tonsa had the lowest CFU count of all the sample types, with a magnitude of 10^4 CFU's/ml (a density of 10 copepods per ml). This corresponds to a concentration in the low end for *A. tonsa* living in the wild (Tang, 2005). Compared to other sources of live feed, *A. tonsa* had the same density of host associated bacterial cells as for rotifers (10^3 CFU's/individual) and bacterial cell density was a tenth of that associated with *Artemia* (Olsen et al., 2000; Skjermo and Vadstein, 1993). The low CFU count combined with the high and variable percentage of fast-growing microbes (50–80%), indicate that the copepods were an uncrowded environment consisting of mostly opportunistic r-strategists. This is in accordance with the conclusion for the bacterial community in the rearing tank water.

Both the phenotypic and genotypic beta diversity showed that the in-water, algae reservoir, and *A. tonsa* (only genotypic) samples formed defined clusters with significantly different community composition (Figs. 7 and 8). The only exception was some of the samples from the production tanks, taken the first days, before the bacterial communities had stabilized. In this initial period, the production tank had a microbial community similar to the in-water. This was expected as the in-water was the only source of bacteria at day 0. When feeding with algae was initiated (day 1) the production tank was strongly influenced by the microbial community of the algae reservoir, which contributed with 97–99% of the cell input to the tank. When *A. tonsa* started to feed and egest, the production tank gradually developed its own distinct microbial community (Fig. 7). It is well documented that microbial communities change with input of nutrients, competing bacteria and changes in other growth conditions (Dubinkina et al., 2019; Eng and Borenstein, 2019; Zhang et al., 2016). The changes seen in Fig. 7 is therefore in accordance with theory. This development was only documented by the phenotypic beta diversity, which had a much better time resolution than the data for genotypic diversity (sampled only on day 5 and 7). However, the genotypic diversity analysis confirmed the development of a distinct microbial community on day 5 and 7 (Fig. 8).

No well-known genera containing pathogenic species were found in high relative abundance (>5%) in the production tank and the algae reservoir. The only OTU with >5% relative abundance in the production tanks was *Lentibacter* (23%), a genus associated with algal bloom (Li et al., 2012).

The algae reservoir had the highest species richness of all water samples (1.1–1.2× higher than for the production tanks: Table 1), suggesting K-selection (Andrews and Harris, 1986; Attramadal et al., 2014). As the algae reservoir was operated similar to a chemostat (Smith and Waltman, 1996), this is a reasonable assumption. However, the low evenness found (Table 1), indicating a high abundance of a few bacterial species, is not expected for an environment dominated by K-strategists. The production tank had a high genotypic species richness (close to that observed in the algae reservoir) and low phenotypic species richness (like the in-water). This, combined with the low evenness found, suggests a less defined selection regime and a more variable bacterial community structure in the production tanks.

A. tonsa had the highest genotypic species richness, and an evenness close to what was observed in the production tanks. One of the most abundant genera found was *Nautella* (4.5%), which contains several opportunistic species. This genus have been shown to be present in higher numbers in diseased white shrimp larvae than of the healthy ones (Zheng et al., 2016). However, the percentage of *Nautella* OTU's associated with *A. tonsa* was only a fourth of the percentage reported by Zheng et al. (2016).

The three most abundant genera found in the in-water, *Ralstonia* (34%), *Vibrio* (5%) and *Nautella* (3.4%), all contain pathogenic species (Kim et al., 2015; Ryan and Adley, 2014; Zheng et al., 2016). This combined with the low phenotypic and genotypic species richness (Table 1) support the assumption of r-selection. The high evenness, however, contradicts this (genotypic evenness 2-5× and phenotypic evenness 1.3–1.5× higher than the other sample types). One would expect a lower evenness with r-selection. It is worth mentioning that the phenotypic evenness overall showed higher evenness and less differences than the corresponding genotypic evenness. This leads to the assumption that the phenotypic analysis could be less sensitive than the genotypic diversity analysis.

The analysis does not provide taxonomic identification on the species level; thus, identification of fish pathogens was not possible. Only the in-water showed presence of OTU's at abundance ≥5% within genera that also includes pathogens. As taxonomically close species tend to be selected for under the same conditions, and based on the fact that r- and K-selection only can be used to predict the possibility of presence of opportunistic pathogens (Vadstein et al., 2018a), also hemolytic activity was used to evaluate safety of live feed. Hemolytic activity has already been used to assess the live feed quality of *Artemia* (Olsen et al., 2000). The same was done here, and no hemolytic activity was detected for any of the sample types. This suggest that *A. tonsa* have a good microbial live feed quality. Especially compared to the *Artemia* investigated by Olsen et al., where 10% of the total CFU count had hemolytic activity.

As far as we know, this is the first study that does a thorough quantitative and qualitative evaluation of the microbial quality of copepod cultures intended for feeding of marine larvae. In conclusion the *A. tonsa* cultures developed distinct communities of both free-living and copepod associated bacteria. Even though the communities initially were influenced by dispersion (import) from the in-water and especially the algal culture (97–99%), the selection pressure in the copepods cultures was likely the most important process structuring the microbial community. Despite the high fraction of r-strategist, the absence of

pathogenic genera and hemolytic bacteria makes us conclude that the microbial water quality of the *A. tonsa* production system was good. However, further studies are needed to confirm this, including first feeding experiments with fish larvae.

CRediT authorship contribution statement

Ragnhild Lind Rong: Conceptualization, Methodology, Software, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Maren R. Gagnat:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Funding acquisition. **Yngve Attramadal:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Funding acquisition. **Olav Vadstein:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Ragnhild Lind Rong and Olav Vadstein have no competing interests. Yngve Attramadal and Maren Gagnat are employed at C-FEED. However, Yngve Attramadal and Maren Gagnat had no influence on analysis, results or interpreting results in this article. That was all done by Ragnhild Lind Rong, with supervision from Olav Vadstein.

Acknowledgements

Thanks to Madeleine Gundersen for help with analyzing flow data in R, Amalie Horn Mathisen for help in the lab and the staff at C-FEED for help with the experimental execution and sampling. Financial support to this study was provided by NTNU and C-FEED.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2022.738439.

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