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Production of lumpfish (*Cyclopterus lumpus* L.) in RAS with distinct water treatments: Effects on fish survival, growth, gill health and microbial communities in rearing water and biofilm

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ABSTRACT

Lumpfish (*Cyclopterus lumpus* L.) in Norway is currently produced in traditional flow-through systems (FTS). Hatcheries frequently show signs of bacterial infections, unstable microbial communities in the rearing water and varying mortality. Recirculating aquaculture systems (RAS) is proposed to create stable and healthy microbial environments, with less probabilities for blooming of opportunistic microbes. Studies have also shown that RAS increases the survival of marine fish. The aim with this study was to investigate the effect of various RAS water treatment designs on water and biofilm microbiota, survival, growth and gill health of lumpfish. An experiment with lumpfish was conducted, from 2 months post hatch to the transfer into sea cages. Five different water treatment regimens were compared: 1. RAS with no additional water treatment, 2. RAS with a filtration unit for removal of small particles, 3. RAS with filtration and disinfection with UV-irradiation, 4. RAS with filtration and disinfection with UV-irradiation and ozone and 5. FTS as a reference. The microbiota of the rearing water and tank wall biofilm were sampled and characterized by Illumina sequencing of 16S rDNA amplicons. Lumpfish juveniles reared in the RAS treatments were exposed to a more stable and diverse rearing water microbiota, with a lower share of opportunistic bacteria, a probable reason for the higher survival and better gill health of the fish compared to siblings reared in the FTS. Lumpfish reared in RAS without disinfection were exposed to a more diverse and stable water microbiota, with a lower share of opportunistic and potential harmful bacteria, compared to the lumpfish reared in RAS with disinfection and FTS. This resulted in better gill health. Fish in RAS with filtration, but no disinfection, had a better gill health than the fish in the RAS without filtration, possibly due to the reduction of small particles. The lumpfish were exposed to different microbial communities of both water and biofilm, due to the different treatments of the incoming tank water. In conclusion, our results indicate that implementation of RAS in the production of lumpfish has a potential to increase both survival, growth and gill health of the fish and that RAS with filtration of small particles, but without disinfection, result in the best fish health and performance among the investigated treatments.

1. Introduction

Efficient sea lice control remains one of the most important challenges for the salmon farming industry today. The lumpfish (*Cyclopterus lumpus* L.) is of great use as a strategy for biological control in aquaculture due to its appetite for the sea lice (*Lepeophtherius salmonis* Krøyer). The number of lumpfish used by the salmon farming industry has increased exponentially since 2008, and 31 million lumpfish were produced and put in sea cages in Norway during 2018. The number of cleanerfish hatcheries in Norway, most of them producing lumpfish, has increased from five to 31 in five years (Norwegian Directorate of Fisheries, 2019; Kyst.no., 2019). The first pilot trials for the commercial production of lumpfish started in 2011 (Imsland et al., 2014) and consequently research and development are still at an early stage (Powell et al., 2018). Although lumpfish appear to be fairly robust between hatching and transfer to sea cages, signs of systemic bacterial

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infections are frequently observed in hatcheries (Alarcon et al., 2016). Research has also shown that the microbial communities in the rearing water are highly unstable (Dahle et al., 2017). In addition, the hatcheries have varying survival, ranging from 30 to 90% (producers of lumpfish, Norway, pers. comm., 2019). The most frequent bacterial diseases reported for lumpfish are caused by the pathogens *Tenacibaculum* spp., *Moritella viscosa*, *Aeromonas salmonicida*, *Vibrio anguillarum, Vibrio ordalii, Pseudomonas anguilliseptica* and *Pasteurella* sp. (Alarcon et al., 2016; Hjeltnes et al., 2018; Scholz et al., 2018). Currently, lumpfish are produced in flow-through systems (FTS). Knowledge on optimal husbandry and microbial water quality for rearing of lumpfish in land-based production systems is still in its infancy and research is needed.

Aquaculture is undergoing a rapid technological development and the demand for sustainability has driven the development of new aquaculture systems. There is a growing interest in the use of recirculating aquaculture systems (RAS) motivated by saving energy for cooling or heating, controlling and stabilizing physicochemical water quality and reducing environmental impact (Martins et al., 2010; Dalsgaard et al., 2013). RAS have properties that can contribute to microbial stability, which has been shown to be particularly important and successfully used in the rearing of marine fish larvae (Vadstein et al., 1993; Skjermo et al., 1997; Attramadal et al., 2012a, 2012b; Drenstig and Bergheim, 2013; Attramadal et al., 2014; Attramadal et al., 2016; Vadstein et al., 2018; Vestrum et al., 2018; Duarte et al., 2019). It has been suggested that RAS favour K-selection of bacteria and outcompete r-strategic bacteria (Attramadal et al., 2012a, 2012b; Attramadal et al., 2014; In prep.), according to the r/K-theory (McArthur and Wilson, 1967; Pianka, 1970; Vadstein et al., 1993). According to this theory, r-selection occurs in unstable environments with high availability of resources and little competition, while K-selection occurs in stable and predictable environments where the bacterial density is close to the carrying capacity (CC) of the system, and where the ability to compete for resources is favoured. Experiments have shown that RAS increases the survival of marine larvae and crustaceans compared to FTS due to K-selection of the rearing water (Attramadal et al., 2012a, 2012b, Attramadal et al., 2014).

Disinfection of the intake water reduce the entry and spreading of pathogens into the system (Sharrer et al., 2005; Wietz et al., 2009) and is of paramount importance for the biosecurity of land-based facilities. However, disinfection of rearing water in the RAS treatment loop efficiently reduces competition by killing bacteria without reducing the CC, and therefore favour r-selection and subsequent proliferation of opportunistic bacteria in the rearing water (Sharrer et al., 2005; Attramadal et al., 2012a, 2012b; Attramadal et al., 2014; Attramadal et al., 2016). For well dimensioned and managed RAS where the hydraulic retention time (HRT) of the rearing tanks is longer than the doubling time for the fastest growing planktonic bacteria, which is typical in marine juvenile production, disinfection within the RAS treatment loop is therefore hypothesized to constitute a disadvantage for the health of the fish (Attramadal et al., 2012b). Disinfection in the RAS treatment loop has been shown to change both the number and the activity of bacteria in the system and rearing tanks, as well as the microbial composition (Attramadal et al., 2012b; Interdonato, 2012). Experiments with lobster larvae showed less variable mortality and a tendency towards higher survival in RAS without disinfection compared to RAS with disinfection in front of the rearing tanks (Attramadal et al., In prep.).

While large particles are removed from RAS by mechanical filtration, smaller particles tend to remain in the system and may accumulate over time (Chen et al., 1993; Becke et al., 2018). Within a RAS, suspended solids originate from feces, uneaten feed and biofilm (Noble and Summerfelt, 1996; Summerfelt et al., 1999). The management of solids is one of the most important and challenging technical issues in RAS (Badiola et al., 2012). Particles are known to harm gill structures (Bruton, 1985) and elevate stress levels in fish (Lake and Hinch, 1999; Sutherland et al., 2008), although susceptibility varies among fish species (Becke et al., 2018). Particles in RAS also provide surface area supporting bacterial activity (Pedersen et al., 2017) and affect the CC in rearing tanks by providing organic matter. There is currently limited knowledge about how particles affect lumpfish performance.

The aim of this study was to investigate effects of RAS and various water treatment design configurations of RAS on microbial communities in water and biofilm, microbial environment, survival, growth and gill health of lumpfish. We tested four different set-ups with an increasing amount of water treatment, including: 1. RAS with no additional treatment (RAS), 2. RAS with a filtration unit for removal of small particles (20 μm) (RAS-F), 3. RAS with a unit with mechanical filtration (20 μm) and disinfection with UV-irradiation (RAS-F-UV), 4. RAS with a unit for particle filtration (20 μm) and disinfection with UV-irradiation and ozone (RAS-F-UV-O). In addition, an FTS was included as a reference system. We used these designs to address the following hypotheses: 1) Lumpfish juveniles reared in RAS will be exposed to a more stable microbial environment, dominated by K-strategists, leading to higher survival, growth and better gill health compared to siblings reared in the reference FTS. 2) Disinfection in front of the fish tanks in RAS will create r-selection in the tank water and thereby reduce microbial water quality and reduce fish performance. 3) Removal of small particles by filtration will improve gill health in addition to microbial water quality (through lowering the microbial carrying capacity). Increased knowledge of the microbial communities created by these systems will be useful for improvement of operational design and sustainable lumpfish production in the future.

2. Materials and methods

2.1. Experimental setup

A 146 days long experiment with lumpfish was conducted at Ecomarine Seafarm AS at Dønna, Norway, in cooperation with Let Sea AS. Four different treatments were included directly before the water entered rearing tanks, which were all connected to the same RAS loop: 1) RAS without disinfection or filtration for removal of small particles (RAS), 2) RAS with mechanical filtration (20 μm, mechanical filter) (RAS-F), 3) RAS-F with mechanical filtration and a UV unit (RAS-F-UV), 4) RAS-F-UV with mechanical filtration, UV and an ozone unit (RAS-F-UV-O). In addition, a traditional flow-through system (FTS) was included in the experiment as a reference system (Fig. 1). The RAS had been running for one week with the designated treatments and water in the tanks and the biofilter was mature and stable before the experiment started. Each treatment included three replicate grey fish tanks (800 L with coned bottom of 4% slope and central bottom drain). The intake water (140-m depth) was the same for all treatments and was filtrated (200 μm) and UV treated (Fig. 1). Two different UV reactors were used for the UV treatments; UV from Xylem Water Solutions (Germany) for the RAS-F-UV and Smart UV from Pentair (USA) for the RAS-F-UV-O. An Eclipse 40 Ozone generator at 230 V was used (Del, USA) for the ozone treatment. The water from the RAS tanks was in a pump sump and pumped over a drum filter of 40 μm. The RAS included a submerged fixed bed upflowing biofilter (14.0 × 3.5 × 3.0 m) with 50% filling and strong aeration. Removal of organic matter was done by flushing sediments from the biofilter once a day. Degassing was done in a pump sump with aeration. The light regime was 24:0 with led lights. Hydraulic retention time of the rearing tanks (HRT) was set to 60 min for both RAS and FTS.

2.2. Rearing regime

Lumpfish were hatched and fed cryopreserved live feed for the first 7 days (Planktonic AS, Norway) and thereafter fed commercial dry feed for lumpfish (Otohime, Japan) and reared in an FTS hatchery the first two months, according to commercial production procedures at

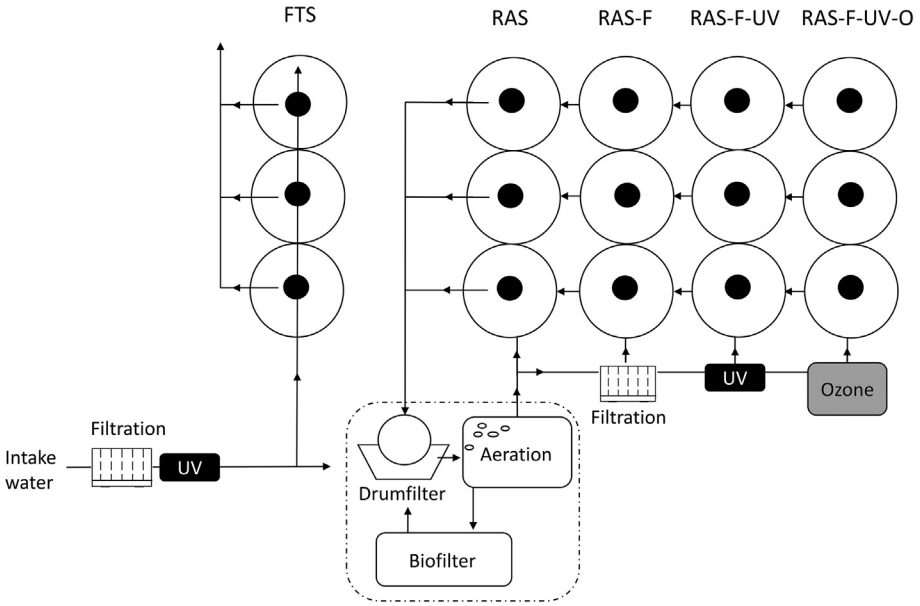

Fig. 1. Schematic set up of the FTS and the RAS designs.

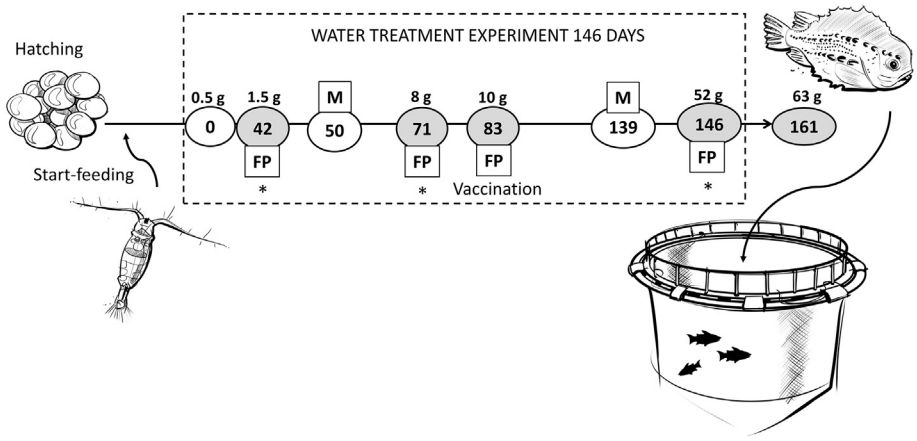

Fig. 2. Timeline for the experiment from hatching (2 months prior to experiment), start-feeding, start (day 0) to end of water treatment experiment (day 146), and transport to sea cages (day 161). Open circles (day 0, 50 and 139) = sampling of microbiota (M), grey circles (day 42, 71, 83 and 146) = analysis fish performance (FP), registration of fish weight and survival. Gill health was analysed at day 146. * = sorting of fish, vaccination at day 83. The total production time was 221 days (7.5 months). The weight differed between treatments and is hence an average of the total production. Sketches by Carl Nørstebø (Eggs Design, Norway).

Ecomarine Seafarm. At 0.52 g, 10.000 lumpfish juveniles were transferred to each tank (6.5 kg/m^3) in the on-growing systems used in the experiment (Fig. 2). The juveniles were fed continuously with an automatic belt feeder using a commercial diet (Clean Lumpfish, Skretting AS, Norway) the first two months (pellet size 0.5–0.8 mm), then the RAS treatments were fed with Lumpfish Grower (Biomar AS, Norway) with increasing pellet size (1.1–2.0 mm) for the rest of the experiment. The fish from the FTS treatment was fed Clean Lumpfish for ten days longer than the RAS treatments, due to smaller fish weight, and then Lumpfish Grower with increasing pellet size. From day 69 the water treatment for RAS was converted to a RAS-F, due to challenges with maintaining the RAS without filtration, because of the need of a heat exchanger, depending on filtration, to lower the temperature. The fish tanks were cleaned once a day by careful siphoning of the walls and bottom of the tanks. The fish were sorted at day 42 and 71 (Fig. 2), due to size differences and to maintain an optimal biomass in the tanks (15–30 kg/m^3). At day 83 the fish (8–11 g) were vaccinated with ALPHA MARINE micro 3.1 vaccine (Pharmaq AS, Norway) with antigens against *Aeromonas salmonicida* genotype VI, *Vibrio anguillarum* serotype O1 and *Vibrio anguillarum* serotype O2a (Fig. 2). The experiment ended at day 146 with sampling and monitoring of fish performance, and fish of 59–68 g were transported to sea cages at day 161 (Fig. 2), with a total production time of 221 days (7.5 months).

2.3. Water quality analyses

The pH, oxygen, salinity, total ammonia nitrogen (TAN), nitrate, nitrite and temperature were measured daily after the biofilter and before entering the tanks, and unionized ammonia was calculated from TAN, pH, salinity and temperature. CO_2 was measured occasionally. Temperature, salinity and oxygen saturation were measured daily in each tank. Temperature, pH, CO_2 and oxygen was monitored by portable electrodes (Oxyguard, Denmark). The nitrogenous waste products were measured with a palintest and a photometer (Palintest, England).

2.4. Fish performance

Survival and growth of larvae were calculated for four different periods, day 0–42, 43–71, 72–83 and 84–146, when the larvae were sorted or vaccinated (Fig. 2). Survival was calculated as the number of alive larvae at different time points according to number of larvae at the beginning of the period. Gills from seven fish of each treatment (totally 35 individuals) were dissected from randomly picked fish at the end of the experiment (day 146). The fish were anesthetized in advance with an overdose with Tricaine Methanesulfonate (MS222, Sigma-Aldrich, USA). Gills were fixated (4% formaldehyde) and sent to Pharmaq Analytics AS (Bergen, Norway) for analyses of gill pathology and health by histology. Formalin-fixed tissue was paraffin-embedded and

processed for histological analysis using standard procedures (Bancroft and Gamble, 2008). Gills were sectioned in the sagittal plane at 2 μm thickness before mounting on poly-L-lysine-coated slides (Superfrost Plus, Thermo scientific, Germany) and stained with haematoxylin and eosin (HE). A gill score was calculated based on the occurrence of various histopathological changes, where a score of 1–10 are considered as mild changes, 11–20 moderate changes, and 21 and up considered as comprehensive changes. The growth was calculated by measuring wet weight of fish at the same timepoints as determination of survival. Specific growth rate (SGR) was calculated according to Eq. (1) (Hopkins, 1992), with W_f being the weight at time t, and W_i at initial time, t = the time in days.

$$SGR = [\ln W_f - \ln W_i]/t * 100 \quad (1)$$

Thermal unit growth coefficient (TGC) were used to calculate the growth rate with consideration to temperature (Thorarensen and Farrell, 2011):

$$TGC = [W_f^{1/3} - W_i^{1/3}/T * t] * 1000 \quad (2)$$

with W_f being the weight at time t, and W_i at initial time. T being the average water temperature (°C) in the system for the relevant period, t = the time in days. An average of SGR and TGC for the four different periods were calculated.

2.5. Microbial community analyses

Bacterial concentration in the rearing water was determined by flow cytometry (BD Bioscience, USA). Tank water was sampled at two different time points, day 50 and 139 (Fig. 2), immediately fixated with glutaric dialdehyde (at a final concentration of 0.5%) and stored in darkness at 4 °C, until analysis. The Samples were diluted 1:10 with 0.1× TE-buffer, and then cells were stained with SYBR®Green I DNA Gel Stain (Life Technologies, Thermo Fisher Scientific Inc., England) for 15 min. Samples were analysed with a BD Accuri™ C6 Flow Cytometer (BD Bioscience, USA) with a flow rate 34.5 ųl/min, threshold at 2000 units, and a sampling time of 3 min. The results were interpreted by using BD Accuri C6 Software. The number of colony forming units (CFU) was determined from growth on Difco Marine agar 2216 (BD, USA) (Salvesen and Vadstein, 2000). 10-fold dilutions were plated for each sample, and each dilution was plated in duplicate. Samples were incubated in darkness at 12 ± 1 °C and inspected after 2 and 14 days. Total CFU were calculated as the average of colonies after 14 days of incubation. The percentage of opportunistic bacteria/r-strategists was calculated as the fraction of fast-growing bacteria (counted on day 2 of incubation) of total CFU (Skjermo et al., 1997; Salvesen and Vadstein, 2000). The percentage of cultivable bacteria (CB) was calculated as the percentage of the total CFU counts of the total cell count with flow cytometry.

For characterization of the microbial communities in the rearing tanks, both biofilm of the tank wall and rearing water were sampled two times from each rearing tank (Fig. 2) during the experiment: 1) after 50 days, 2) after 139 days of the experiment. Three water and biofilm samples were collected from each tank at each sampling time. The water samples were filtrated using a Sterivex™ filter unit (pore size 0.22 ųm, Merch Millipore, USA) and Omnifix® syringes. 150–200 mL water was filtrated for each water sample, until the filter was clogged. Biofilm from the walls of the tanks were sampled by using swabs (Copan Diagnostics, USA). Filter and swab samples were frozen (−20 °C) immediately after sampling, transported to SINTEFs laboratory and stored at −80 °C until further analyzes. DNA was extracted using FastDNA® Spin Kit for Soil (MP Biomedicals, USA) following the protocol. Genomic DNA Clean & Concentrator™-10 (Zymo Research, USA) was used to purify the DNA. To determine the concentration and pureness of DNA, a NanoDrop Spectrophotometer (Thermo Scientific Inc., England) was used. Microbial community composition of the samples collected were characterized by 16S amplicon sequencing at the Centre for Biotechnology (CeBiTec), Bielefeld University, Germany. In brief, 16S rDNA amplicons were generated from DNA-samples by two PCR rounds using the 2 × HiFi HotStart ReadyMix (Kapa Biosystems, USA). To amplify the third and fourth variable regions (V3, V4) of the 16S rRNA gene, the primers Pro341F (5′-CCTACGGGNBGCASCAG-3′) and Pro805R (5′-GACTACNVGGGTATCT AATCC-3′) (Takahashi et al., 2014) covering the domains Bacteria and Archaea were used for the first PCR round. Sequencing adapters and multiplexing indices where added using the Nextera XT Index kit (Illumina, USA). Following each PCR round, amplicons were purified using the QIAquick PCR purification Kit (Qiagen, Germany) and finally the amplicon size and concentration was determined on a BioAnalyzer (Agilent Technologies, USA). Amplicons were pooled, and the normalized DNA libraries (4 pM DNA) were mixed with PhiX (5%) Control v3 (Illumina), denatured at 96 °C for 2 min and each library was run on a MiSeq sequencer (Illumina) lane using the MiSeq Reagent Kit v3 in the 2 × 300 bp paired-end mode. The resulting sequencing data were deposited at the European Nucleotide Archive, under Study PRJEB36184 (accession numbers ERS4260801- ERS4260856). The Illumina sequencing data were processed using the USEARCH pipeline (version 11; https://www.drive5.com/usearch/). The command Fastq_mergepairs was used for merging of paired reads, trimming off primer sequences and filtering out reads shorter than 380 base pairs. Further processing included demultiplexing and quality trimming (the Fastq_filter command with an expected error threshold of 1). Chimera removal and clustering at the 97% similarity level was performed using the UPARSE-OTU algorithm (Edgar, 2013). Taxonomy assignment was performed applying the Sintax script (Edgar, 2016) with a confidence value threshold of 0.8 and the RDP reference data set (version 16). The resulting OTU (Taxonomixal operation units) table was normalized to 20.000 number of reads per sample by determining the fraction of the OTUs for each community profile, and then multiplying with 20 000, and finally rounding off the read numbers to integers. The USEARCH commands Alpha_div and Sintax_summary was used to calculate alpha diversity indices (observed OTU richness and Shannon's diversity) and generate taxa summary tables, respectively. Sequence data was aligned (Wang et al., 2007) to the closest relative in the 16S ribosomal RNA sequences of Bacteria and Archaea in RDP (https://rdp.cme.msu.edu/).

2.6. Statistics

The data are presented as mean ± standard error of the mean (SE). Statistical tests were performed at the 95% confidence level ($p = .05$). Data for larval wet weight were log10 transformed to secure a homogenous variance and tested for differences by one-way ANOVA and *t*-tests in SPSS 16.0 (SPSS Inc., USA). The data for larval survival were Arcsin-transformed before statistical comparison (one-way ANOVA) in SPSS. SPSS was also used for comparisons of the chemical variables. One-way ANOVA or Kruskal-Wallis were used, depending on the homogeneity of variance of the variables. Statistical analyses of the amplicon sequencing data were performed using the program package PAST (Hammer et al., 2001). For ordination of samples we used principal coordinate analysis (PCoA) (Davis, 1986) based on the Bray-Curtis similarities (Bray and Curtis, 1957). To test for differences in community structure between the sample groups, we applied one-way PERMANOVA based on Bray-Curtis similarities (Anderson, 2001). The null hypothesis of no difference in community profiles between groups of samples was rejected for *p* values less than 0.05. The Similarity Percentages (SIMPER) analysis (Clarke, 1993) was used to determine the contribution from the OTUs to the Bray-Curtis dissimilarity among samples.

Table 1
Physicochemical water quality measured in the rearing tanks of each treatment and downstream biofilter during the experiment (mean ± SE).

	RAS	RAS-F	RAS-F-UV	RAS-F-UV-O	FTS	Biofilter
Temperature (°C)	10.3 ± 0.3	10.0 ± 0.3	10.4 ± 0.4	10.3 ± 0.4	7.5 ± 0.4	11.2 ± 0.2
Oxygen saturation (%)	89.9 ± 1.1	95.4 ± 1.10	101.0 ± 1.2	91.5 ± 1.3	89.0 ± 0.7	
Salinity (ppt)						26.4 ± 0.3
pH						7.1 ± 0.0
Total ammonia N (mg TAN L^{-1})						1.0 ± 0.1
Unionized ammonia (mg NH_3-N L^{-1})						1.0 ± 0.1
Nitrite (mg NO_2-N L^{-1})						0.2 ± 0.0
Nitrate (mg NO_3-N L^{-1})						16.2 ± 5.1
CO_2 (mg/L)						13.4 ± 0.7

3. Results

3.1. Chemical water quality

The chemical water quality was generally satisfying, both downstream biofilter and in the fish tanks (Table 1). Notably, the temperature was significantly lower in the FTS (average of 7.5 °C) than in the RAS (average of 10.3 °C) (ANOVA, $p = .001$) (Table 1). During the first period of the experiment (day 1 to 41), oxygen saturation was low in all treatments (63–80%), except for RAS-F-UV. In the second period (day 42 to 70) the oxygen saturation was higher, but still unstable, and the RAS had the lowest saturation. In the third period (day 71 to 82), the oxygen saturation was stable and satisfying for all treatments (Table 1).

3.2. Fish performance

The survival of the lumpfish (Fig. 3) was significantly higher in the RAS treatments than in the FTS during the first and third period (Kruskal-Wallis, $p = .025$; $p = .046$). The average survival during these periods were 79.1 ± 3.8% and 97.9 ± 0.1% for FTS and the RAS treatments, respectively. At the second and last periods there were no significant differences between the survival in the different treatments, even though the RAS and RAS-F had a higher average survival, compared to the other treatments.

The growth, measured as average specific growth rate (SGR), was higher for the RAS treatments than the FTS, although it was not significant (ANOVA, $p = .58$) (Table 2). By compensating for the effect of temperature on growth, thermal growth coefficient (TGC) was calculated. No significant differences in TGC for the experimental period was identified (ANOVA, $p = .99$) (Table 2).

Table 2
Average specific growth rate (SGR) and thermal growth coefficient (TGC) during the experiment ± SE.

	RAS	RAS-F	RAS-F-UV	RAS-F-UV-O	FTS
SGR	3.4 ± 0.1	3.2 ± 0.3	3.3 ± 0.3	3.3 ± 0.3	2.4 ± 0.1
TGC	2.0 ± 0.1	2.1 ± 0.2	2.0 ± 0.1	2.0 ± 0.2	2.1 ± 0.2

The gill health was analysed by histology and showed that the RAS-F had a significantly lower gill score than RAS-F-UV, RAS-F-UV-O and FTS (Kruskal-Wallis, $p = .044$; $p = .006$; $p = .001$), indicating better gill health in the RAS-F system (Fig. 4). The RAS treatment had a significantly lower gill score than FTS (Kruskal-Wallis, $p = .009$) and were close to significant different from RAS-F-UV-O (Kruskal-Wallis, $p = .058$). No significant differences in gill score were found between RAS and RAS-F.

The histopathological analysis did not identify damages in the gills related to any specific agent, but several non-specific changes, like mucous cell metaplasia, degenerative changes of respiratory epithelium, lamellar- and filament epithelial hyperplasia, and focal or diffuse inflammation were observed (Fig. 5). As Fig. 4 indicates, these changes were identified more frequently in dissected gills reared in the RAS treatments that included disinfection and in the FTS.

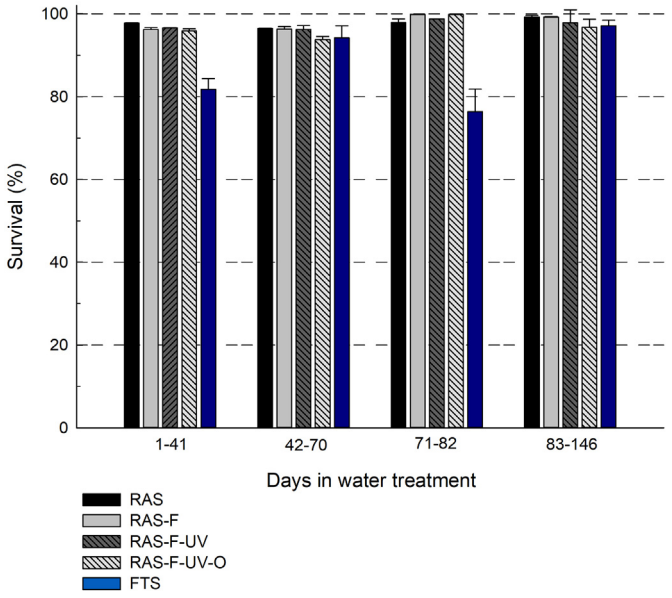

Fig. 3. Survival of fish during the experiment, after each of four different periods in each treatment. Average survival ± SE is given for each treatment. RAS tanks were converted to RAS-F from day 69.

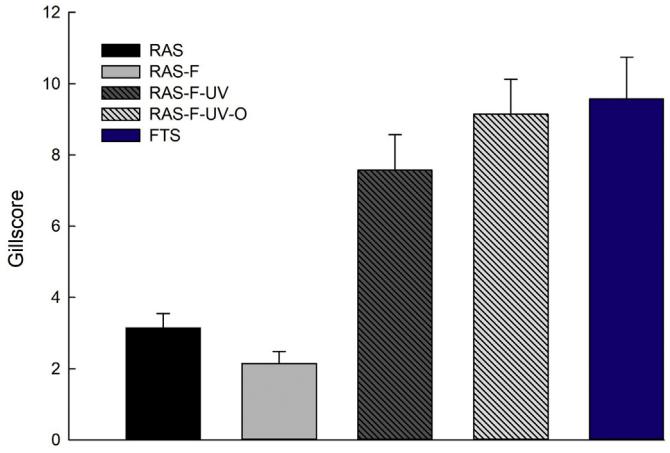

Fig. 4. Gill score for fish from the different treatments (average ± SE). A score of 1–10 are considered as mild changes, 11–20 moderate changes, and 21 and up are considered as comprehensive changes. RAS tanks were converted to RAS-F from day 69.

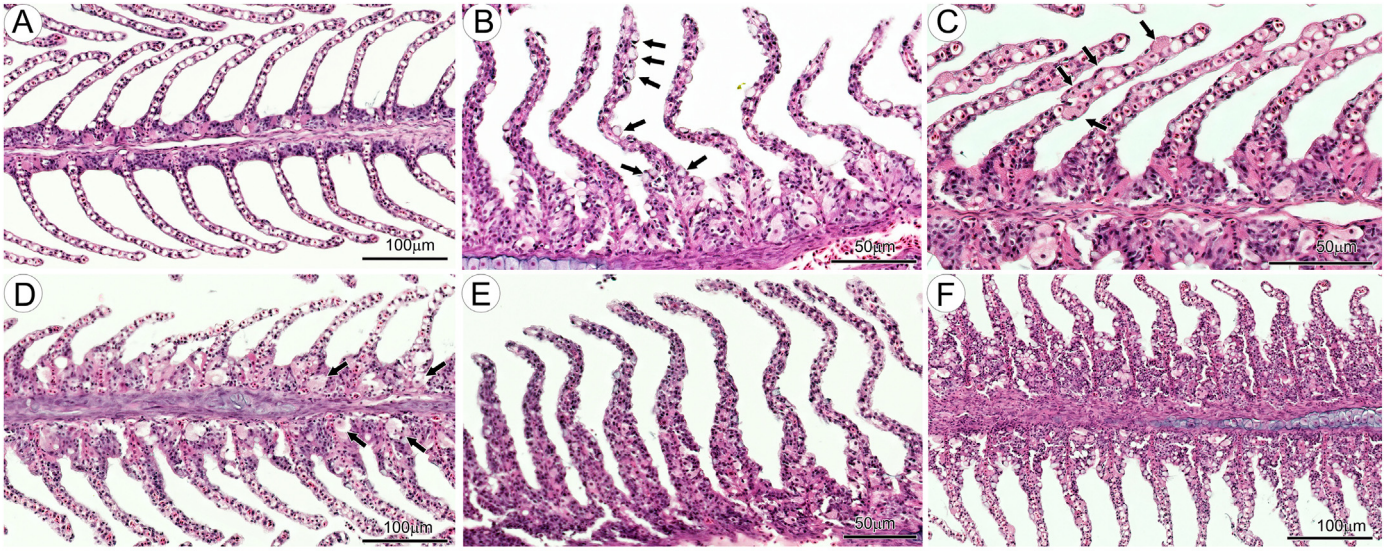

Fig. 5. Examples of the most frequent histopathological changes observed in gills from the experiment. A) Normal gills for comparison, B) Mucous cell metaplasia (examples of mucous cells in the respiratory epithelium along one lamella are indicated by arrows), C) Degeneration of lamellar epithelial cells seen as hypertrophic, eosinophilic cells in the respiratory epithelium (examples indicated by arrows), D) Chloride cell hyperplasia and -hypertrophy (examples indicated by arrows), E) Lamellar epithelial hyperplasia, F) Diffuse inflammation of the filament.

3.3. Microbiota

3.3.1. Effect of water treatment on the water microbiota

The most abundant bacterial classes in rearing water from all systems were Gammaproteobacteria and Alphaproteobacteria (Fig. 6A). The Gammaproteobacteria was the most abundant class at day 50 and was particularly abundant in the FTS and RAS systems with disinfection, with relative abundances as high as 68%, while the Alphaproteobacteria was abundant in all systems at day 139, with relative abundance from 22 to 51%.

A PCoA plot based on Bray–Curtis similarities indicated that the rearing water microbiota differed between the systems (Fig. 7). A PERMANOVA test confirmed that the water microbiota differed significantly between all systems ($p < .5$), except between RAS and RAS-F. The PCoA plot also showed that the water microbiota changed with time for all treatments.

On day 50, The most abundant bacterial family identified in rearing water in RAS-UV and RAS-UV-O was *Thiotrichaceae* (Gammaproteobacteria) (Fig. 6B). In these systems, this family accounted for a high share of the community (up to 53%). In RAS and RAS-F this family comprised only 4% of the reads, and in the FTS the share was even lower, 2%. The same pattern was observed at day 139, at which point the RAS treatments with disinfection had the highest abundance of *Thiotrichaceae*, but the total abundance was lower than what was observed at day 50. At the genus level, the *Thiotrichaceae* was dominated mainly by *Leucothrix,* represented by three OTUs. One of these (OTU_1) was the most abundant OTU in the total data set. SIMPER analysis confirmed that OTU_1 (*Leucothrix*) accounted for most of the differences in the water microbiota between treatments at day 50, with a contribution of 32% to the Bray-Curtis dissimilarity. The *Flavobacteriaceae* was identified in samples from all treatments (Fig. 6B), but the highest abundance was identified in FTS at day 50 (32%). By comparison, the abundance was only 6% in RAS and RAS-F, and around 12% in the RAS systems with disinfection. The *Rhodobacteraceae* was abundant in the RAS and RAS-F treatments, at both sampling dates (12–28%). This family consisted mainly of the genus *Loktanella*, represented by one OTU, which was the second most abundant OTU in the entire data set (OTU_4). *Rhodobacteraceae* was also identified in the RAS treatment with disinfection and in the FTS, but at lower abundances (Fig. 6B). The abundancy of *Rhodobacteraceae* generally increased from day 50 to 139 in all treatments. The FTS showed a surprisingly high variation in the microbial community composition of the water between replicate tanks at day 139 (Fig. 6B). For example, *Oceanospirillaceae* was found in high abundance in only one of the FTS replicate tanks at day 139 (36%) and represented the genus *Oleispeira* (Fig. 6B*)*. Moreover, *Mycoplasma* was highly abundant in another of the FTS tanks (22%). In comparison, the abundances of these taxa were low (less than 1%) in the RAS treatments. *Moritella* (represented by 3 OTUs) was found in all FTS tanks (2–6%) but was in low abundance for the RAS treatments' water samples (less than 0.1%).

Both the observed OTU richness (Fig. 8A) and Shannon's diversity index (Fig. 8B) were significant higher for RAS and RAS-F water compared to the other treatments at sampling day 50. RAS and RAS-F had on average as much as 1360 ± 60 observed OTUs. In comparison, the water of RAS treatments with disinfection (-UV and -UV-O) showed a considerably lower OTU richness (693 ± 109 OTUs), and the FTS showed an even lower richness of only 281 observed OTUs. After 139 days, the bacteria species richness of the rearing water was lower than at day 50 for all RAS treatments, and the FTS had a significantly lower OTU richness compared to RAS and RAS-F (ANOVA, $p = .007$; 0.005).

The Bray-Curtis similarities of the water microbiota was high for comparisons between replicate tanks for all treatments at day 50 (Fig. 9), which indicated stability of the microbial community composition within treatments. This was still the case for three of the RAS treatments on day 139 (RAS, RAS-F, RAS-F-UV), while for the RAS-F-UV-O and FTS, there was a considerably higher variation in the water microbiota between replicate tanks (Fig. 9).

The RAS treatments had a significantly higher concentration of total bacteria in the rearing water, compared to the FTS, at both sampling days (Kruskal-Wallis, $p = .023$) (Fig. 10A). RAS had on average 4.7×10^6 cells mL^{-1} while FTS had 9.4×10^4 cells mL^{-1}. The RAS treatments had a relatively similar total concentration of bacteria, but the fraction of opportunistic bacteria differed considerably between treatments. The RAS treatment showed only 3% of opportunistic bacteria at day 50 (Fig. 10B), which were significantly lower than the water from the RAS-F-UV and RAS-F-UV-O (ANOVA, $p = .030$; 0.014). The RAS-F had 15% opportunistic bacteria at which were significantly lower than RAS-F-UV ($p = .032$). After 139 days there were no significant differences in the fraction of opportunistic bacteria among the

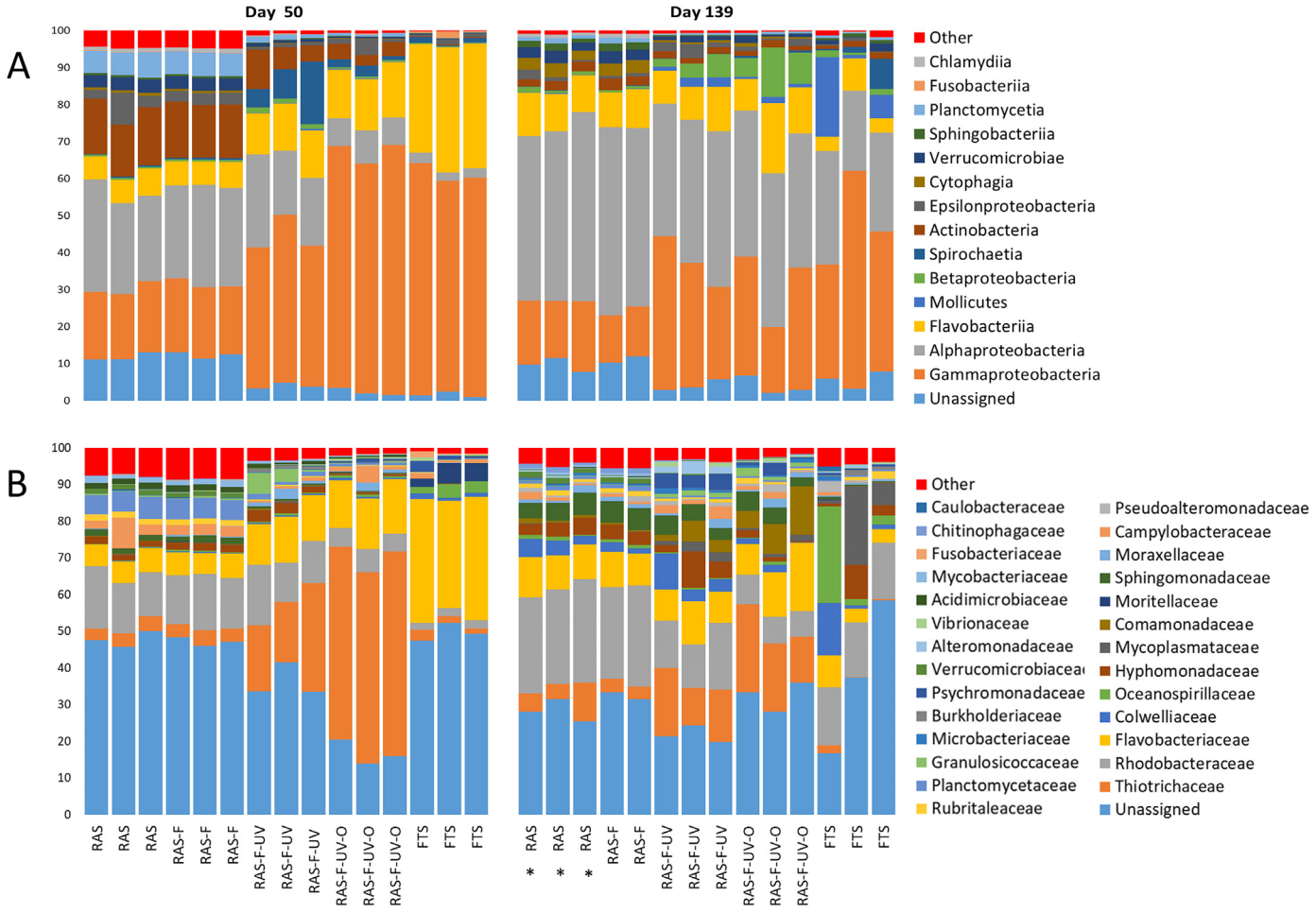

Fig. 6. Relative abundancies of bacterial classes (A) and families (B) in the rearing water of the different treatments, at day 50 and 139. Only classes that are present at abundances > 1% in at least one sample are shown. * = RAS tanks were converted to RAS-F from day 69.

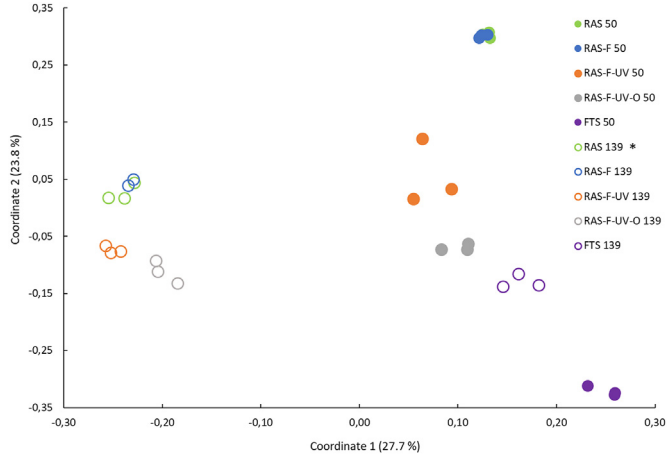

Fig. 7. Principal coordinates (PCoA) plot based on Bray–Curtis similarities for water microbiota from the systems at 50 and 139 days in the water treatments. Filled symbols are day 50, open symbols are day 139. * = RAS tanks were converted to RAS-F from day 69.

treatments (ANOVA, p = .087) (Fig. 10B).

The flow cytometry analysis showed that the bacterial density in FTS was far lower than in the RAS treatments (Fig. 10A). We further examined the fraction of culturable bacteria in the water treatment by relating the flow cytometry measures to the CFU counts. The average cultivability was considerably higher for the FTS than the RAS treatments (Fig. 11), and the difference was found to be significant on day 139 (Kruskal-Wallis, p = .017).

3.3.2. *Effect of water treatment on the biofilm microbiota*

One of the most abundant families identified from biofilm was *Rhodobacteraceae*, identified at the highest abundance in samples from the FTS at day 139, varying from 33 to 43% (Fig. 12). *Flavobacteriaceae* was the second most abundant family, with the highest abundance in FTS (36%) and RAS-F-UV-O (34%) at day 50. The most dominant family from water, *Thiotrichaceae* (Fig. 6B), was also relative abundant in the tank wall biofilm, particularly in RAS-UV-O, where it accounted for up to 30% of the total reads. Another pronounced family was *Hyphomonadaceae*, that was absent at day 50, but present in high abundancy at day 139, 19–23% for RAS and RAS-F, and somewhat lower abundances for the other systems (Fig. 12). As for the water microbiota, the observed OTU richness and Shannon's diversity index were lower for the FTS compared to the RAS treatments at day 50, where the RAS had an average 565 and the FTS 92 observed OTUs. At day 139 the differences in species richness and diversity between the system were not that distinctive (data now shown).

A PCoA-plot based on Bray-Curtis similarities (Fig. 13) indicated that the microbial community composition of the tank wall biofilm differed between sampling times, but the clustering of samples according to treatment system was less profound compared to what found for the water microbiota (Fig. 7). We found no significant differences in tank wall microbiota between systems (PERMANOVA, p > .5). Thus, the tank wall biofilm communities seemed to be less influenced by the different water treatments than the rearing water (Fig. 7). The biofilm

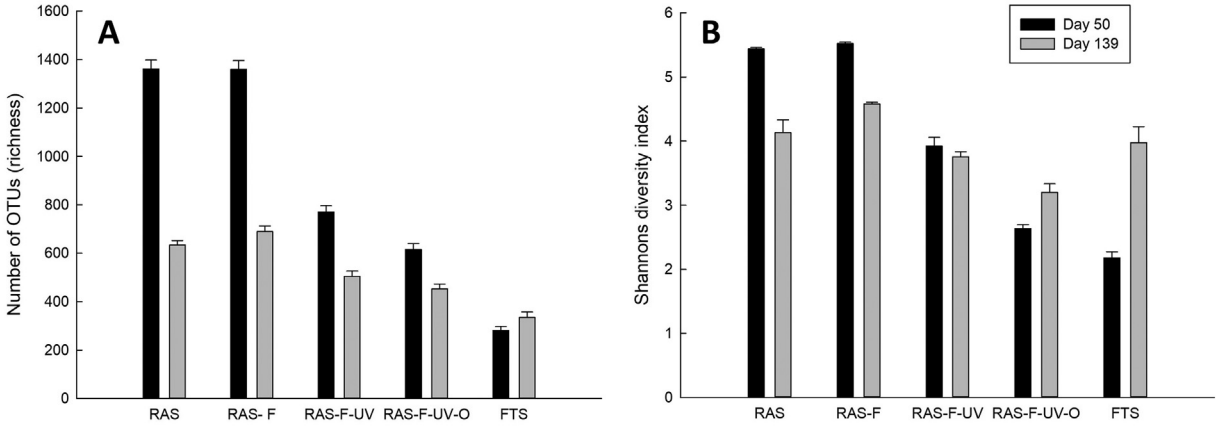

Fig. 8. Means of the observed OTU richness (A) and Shannon's diversity index (B) for the water microbiota at day 50 and 139. Error bars show the standard error. RAS was merged to RAS-F from day 69.

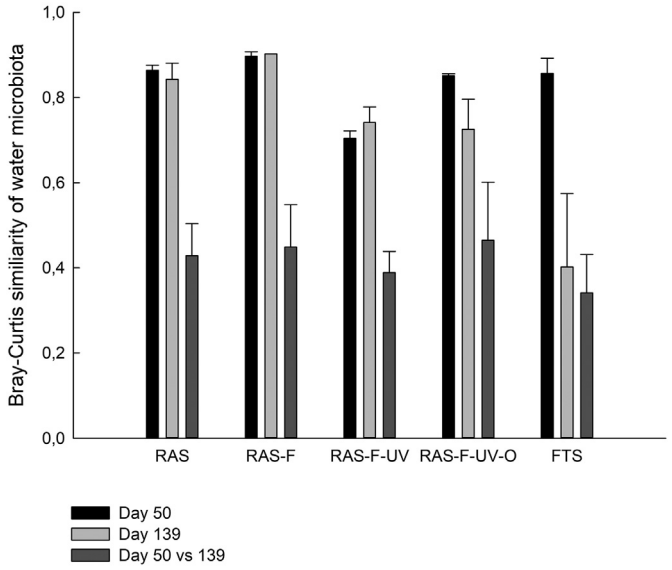

Fig. 9. Average Bray–Curtis similarities for comparisons of water microbiota composition within treatments at day 50 and 139 and for each treatment over time between day 50 and 139. Error bars show the standard error (SE). RAS was merged to RAS-F from day 69.

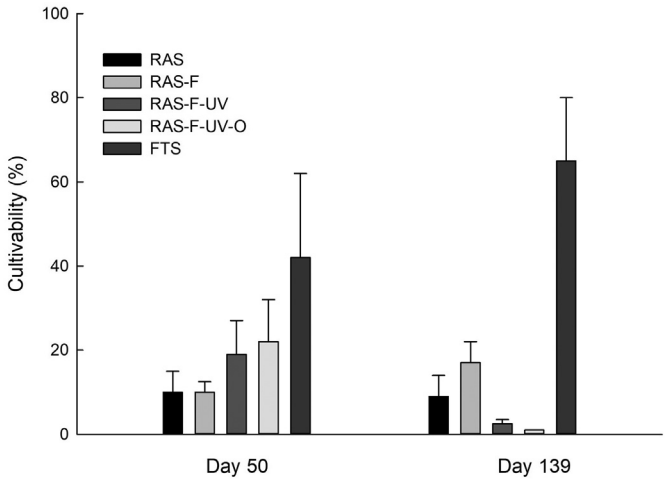

Fig. 11. Average Cultivability (%) at day 50 and 139 ± SE, as the percentage total CFU of the total cell count with flow cytometry.

microbiota changed over time for all treatments (Fig. 13), especially in the RAS treatments.

The tank wall biofilm and the rearing water microbiota differed within systems for all treatments at both sampling dates (Fig. 14AB), even though the differences were not statistically significant (PERMANOVA, $p > .16$). The microbial composition of the tank wall biofilm and rearing water in the FTS were more similar compared to that of the

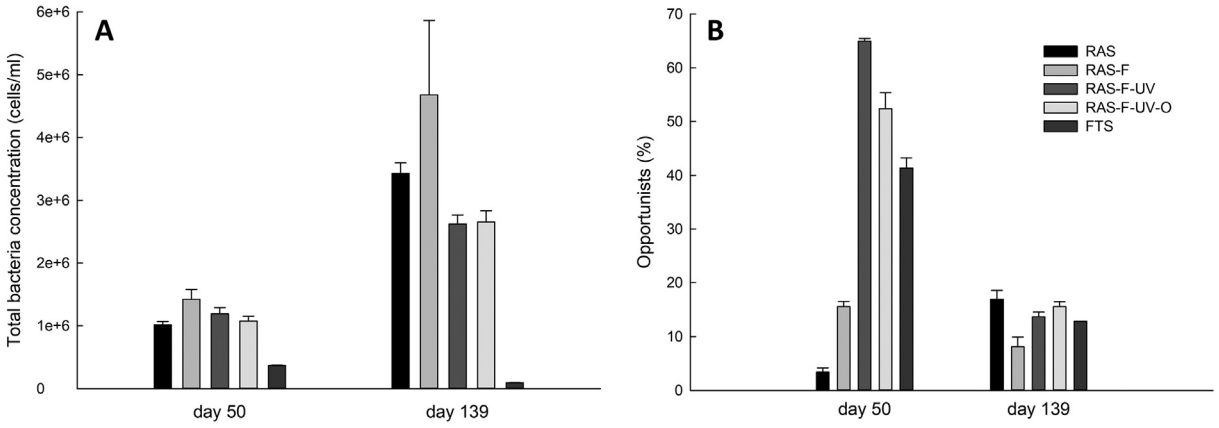

Fig. 10. A) Total number of bacteria (cells/ml) in the rearing water of the different treatments at day 50 and 139, analysed by flow cytometry. B) Opportunists (%), as fraction of fast-growing bacteria of total CFU mL^{-1}. All data presented as average ± SE. RAS tanks were converted to RAS-F from day 69.

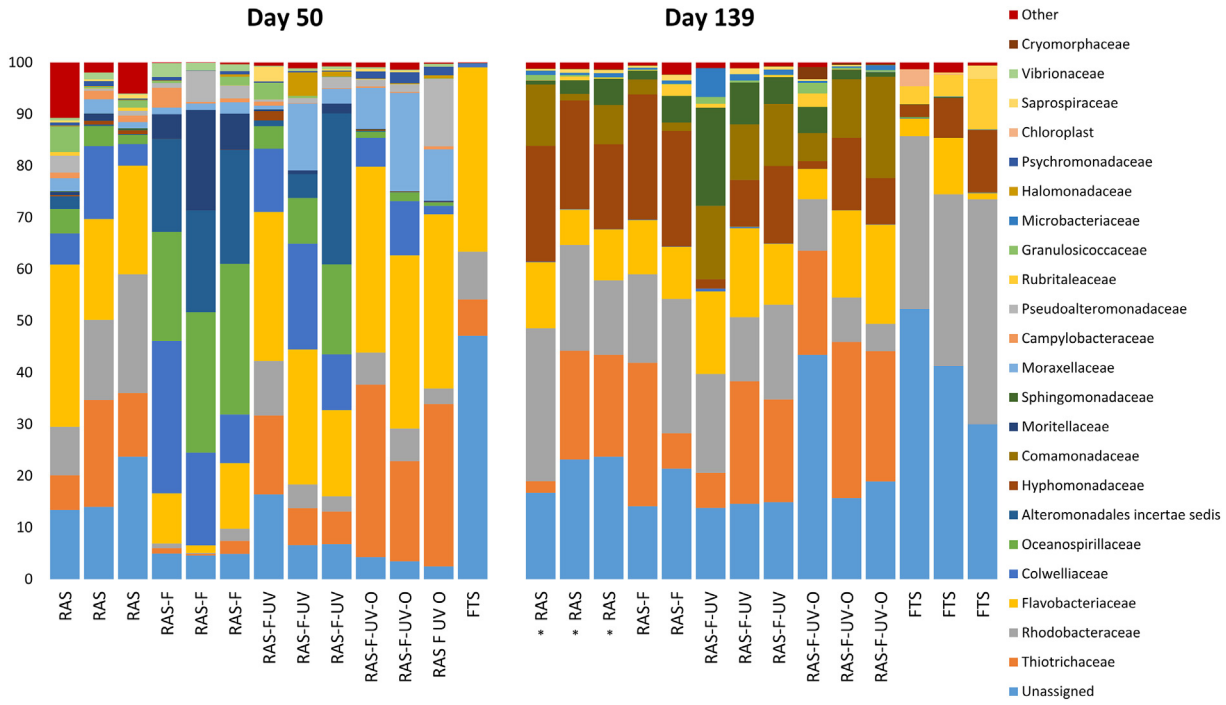

Fig. 12. Relative abundances of bacterial genera in tank wall biofilm samples, at day 50 and 139. Only families observed at an abundancy > 1% in at least one sample are shown. FTS at day 50 included only one sample. * = RAS tanks were converted to RAS-F from day 69.

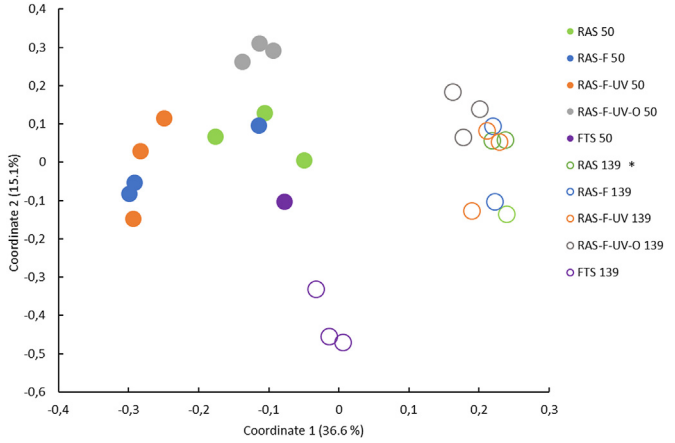

Fig. 13. Principal coordinates (PCoA) plot based on Bray–Curtis similarities for tank wall biofilm microbiota at day 50 (filled symbols) and day 139 (open symbols). * = RAS tanks were converted to RAS-F from day 69.

RAS treatments, on day 50 (Fig. 14A), while the RAS treatments were more similar at day 139 (Fig. 14AB).

4. Discussion

To the best of our knowledge this is the first study to examine the effects of RAS on growth, health, survival and microbial water quality in lumpfish rearing. In addition, it is the first study to compare the effects of different water treatment for individual tanks in the same RAS.

4.1. Chemical water quality

All systems had acceptable chemical water quality during the experiment, which show that the RAS was well dimensioned. However, the oxygen saturation was low in the beginning of the experiment, especially for the RAS treatment (RAS). Juvenile lumpfish is highly sensitive to reduced oxygen saturations and negative effects in terms of growth are already evident for lumpfish reared at 81% oxygen saturation (Jørgensen et al., 2017). The low oxygen saturation could therefore be the reason for the lower wet weight of fish from the RAS treatment after the first period, compared to the other RAS treatments.

4.2. Fish performance

The fish in the RAS treatments showed a significantly higher survival for two of the periods of the experiment, compared to the fish from the FTS. These results are in accordance with previous studies with marine fish larvae, where RAS resulted in higher survival compared to FTS (Attramadal et al., 2012a, 2012b, 2014, 2016), and support the hypothesis that lumpfish juveniles reared in RAS will show a higher survival compared to siblings reared in FTS. For the two periods with higher survival, the RAS treatments, increased survival with 19% in average compared to the FTS. This effect size would constitute a high number of fish in commercial scale, where a high density of fish can be utilized with success (Espmark et al., 2019). In general, the survival was high for all treatments in the experiment (average 76.0–99.9%), including the FTS (76.0–98.0%). Comparably, commercial production of lumpfish in Norway has a lower survival through a production cycle in FTS (Commercial producers of lumpfish in Norway, pers. comm., 2019). The higher survival of fish in FTS in this experiment can be related to the production period. The experiment started two months post hatch, at which point the initial mortality has passed and the fish may be more robust than in the early stages.

Gill health is an important indicator of fish health and welfare in relation to the farming conditions (Marshall and Bellamy, 2010). The extensive interaction between surrounding water and the thin, delicate respiratory epithelium of the gill lamellae during branchial respiration makes the gill tissue an optimal indicator on interaction between the fish and the environment (Mallat, 1985; Strzyzewska et al., 2016). Furthermore, the gills are taking care of processes like gas exchange, acid-base regulation, excretion of nitrogenous waste, ion- and osmoregulation and hormone metabolism as well as being an important immunological tissue (Evans et al., 2005). Thus, optimal function of the

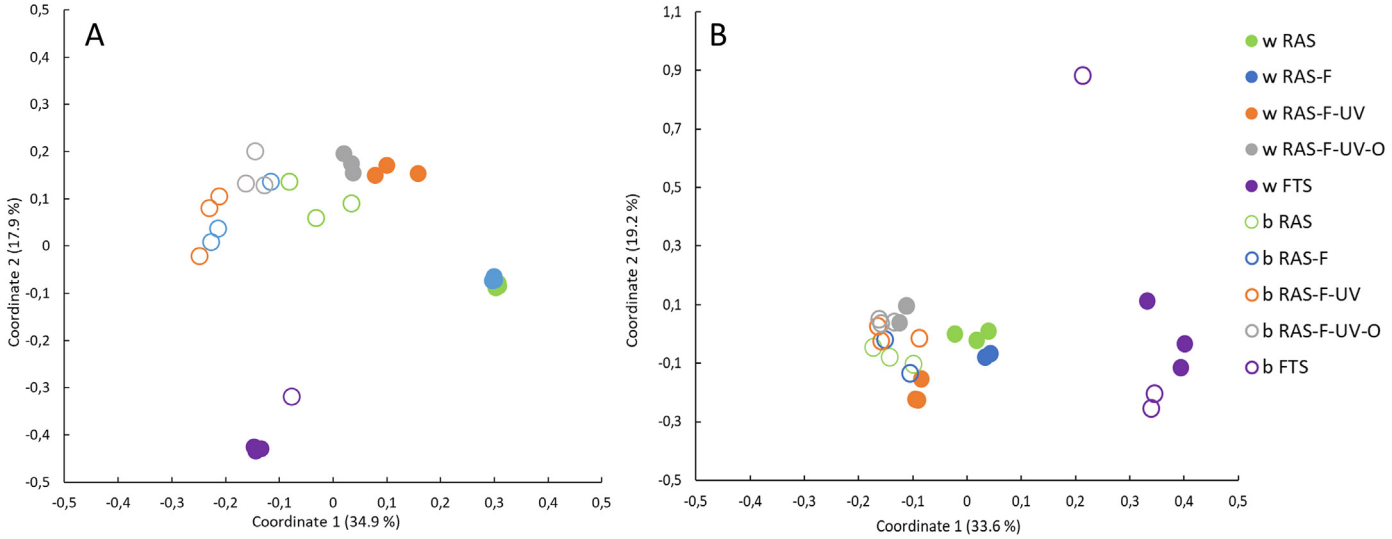

Fig. 14. Principal coordinates (PCoA) plot based on Bray–Curtis similarities for rearing water and tank wall biofilm microbiota from the systems at 50 days (A) and 139 days (B) in the water treatments. Filled symbols = rearing water, open symbols = tank wall biofilm. RAS tanks were converted to RAS-F from day 69.

gill is of outermost importance for fish health and -performance. The fish from the RAS treatments without disinfection (RAS and RAS-F) had a better gill health than those from FTS and the RAS treatments with disinfection (RAS-F-UV and RAS-F-UV-O). The fish of the RAS-F showed the best gill health in this experiment. This implies that the extra mechanical filtration of the incoming tank water of RAS positively affected the lumpfish.

The fish grew better in the RAS treatments than in the FTS, due to the significantly higher temperature, as shown for the Thermal-unit growth coefficient (TGC), which attempts to express growth independent of the temperature (Thorarensen and Farrell, 2011). TGC for all the treatments were rather similar during the experiment. Even though the differences are caused by temperature, this is not entirely irrelevant for system choice, since RAS is a method for maintaining a stable and optimal temperature year around, whereas FTS depends more on the sea temperature, which will vary trough the seasons. At winter, with drop in seawater temperature below 8 °C, *Moritella viscosa* thrives and is a significant problem causing winter ulcer (Einarsdottir et al., 2018; Producers of lumpfish, Norway, pers. comm., 2019). By selecting RAS, the low water temperature during winter can be avoided, and hence possibly the risk of negative interactions with *Moritella viscosa*.

The analysis of fish performance in this experiment indicates that there is a potential to increase both survival, growth and gill health by producing lumpfish in RAS, and that RAS with filtration of small particles, but no disinfection in the RAS treatment loop, seemed to result in the best fish health and performance.

4.3. Microbiota

4.3.1. Effect of water treatment on the water microbiota

Even though the different RAS treatments were connected to the same RAS loop for the entire experiment the microbial community composition of both water and biofilm developed differently due to different treatment of the incoming tank water. These differences were clearly expressed in the rearing tanks with an HRT of only 60 min, where all treatments differed except RAS and RAS-F, at both sampling days. The extra mechanical filtration of the incoming tank water in RAS-F had possibly little influence of the rearing water microbiota or the total concentration of the bacteria. At day 69 the RAS and the RAS-F were merged to RAS-F, and hence the similar water microbiota at day 139 were expected. Since the RAS treatment was changed to RAS-F after 69 days of the experiment, we must note that differences in gill health could have been more pronounced if the different treatment of the incoming water to tanks had been continued during the whole experiment.

Disinfection had a significant influence on the bacterial community composition in this experiment. It has been shown that both UV and ozone change the microbial composition in rearing water and biofilm (Wietz et al., 2009; Interdonato, 2012). Our results indicate that both the UV and the combined UV and ozone treatment changed the microbial community structures. The most abundant family in water was *Thiotrichaceae*, with the highest abundance in the RAS-F-UV and RAS-F-UV-O treatment (21–53%). The *Thiotrichaceae* was represented by three OTUs, all classified as *Leucothrix*. The disinfection apparently selected for the *Leucothrix*. These bacteria can cause fouling of respiratory surfaces or cause internal or systemic bacterial infection in shellfish (Johnston et al., 1971). *Leucothrix mucor* has become a problem in aquaculture (Broch, 2006), especially in the cultivation of lobster at the juvenile stages (Nilson et al., 1975; Dale and Blom, 1987). Since fish in RAS-F-UV and RAS-F-UV-O also had the highest gill score among the RAS treatments, i.e. the most challenged gill health, it might be a correlation between the presence of *Leucothrix* and the poorer gill health. The rearing water in RAS and RAS-F had low abundances of *Thiotrichaceae*, and better gill health. The FTS rearing water had low abundances of *Leucothrix*, but still had the highest gill score. However, FTS was dominated by *Flavobacteriaceae* on day 50. *Flavobacteriaceae* includes important fish pathogens such as *Flavobacterium psychrophilum*, *Flavobacterium columnare* and *Tenacibaculum maritimum. The* samples from FTS contained 18 different OTUs representing *Flavobacterium*. FTS also contained high abundancies of *Mycoplasma* and *Moritella* at the genus level, which were rare in the RAS treatments. Both these genera include pathogenic species (Gudmundsdottir et al., 2006; Suhanova et al., 2011). *Moritella viscosa* has caused several incidents of mortality in the rearing of lumpfish, causing winter ulcers (Gudmundsdottir et al., 2006; Einarsdottir et al., 2018), both in hatcheries and sea cages. *Moritella* was identified in high abundance (< 82%) by Roalkvam et al. (2019) in a normal production of lumpfish in FTS. *Rhodobacteraceae* was abundant in the RAS treatments without disinfection and were increasing from day 50 to 139, with *Loktanella* as the main genus. The RAS treatments with disinfection had a very low abundance of *Loktanella*, and it was rare in the FTS. *Loktanella* include bacterial groups with potential probiotic activity (Makridis et al., 2005; Califano et al., 2017), which can have beneficial effects on fish health (Hjelm et al., 2004; Nayak, 2010). The disinfection of the water going to the RAS-F-UV, RAS-F-UV-O and FTS rearing tanks may have selected against this

potential beneficial bacterial taxon. It must be emphasized that the results from our study of a typical system for marine juvenile production are not directly transferrable to systems for other species, e.g. salmonids, where the HRT of the fish tanks is shorter (Gregersen et al., 2020). With a short HRT (15–20 min) in the fish tanks, disinfection in the RAS loop may keep the level of planktonic bacteria low in the tank water despite high loading of organic matter because the bacteria do not have the time to grow during the short time the water is in the fish tanks (Bakke et al., 2017).

RAS and RAS-F had a significantly more diverse and less variable microbial community composition compared to the other treatments at both sampling days, which might indicate a more mature and K-selected community in the RAS treatments without disinfection, as predicted. This was supported by the higher Bray-Curtis similarities for the RAS and RAS-F for comparisons both between replicate tanks and sampling times, indicating that the microbial community composition in the RAS and the RAS-F were more similar to each other and more stable over time. As hypothesized, RAS without disinfection seemed to promote K-selection.

As expected, the RAS treatments had significantly higher abundancy of total bacteria in the tank water than the FTS at both sampling points, probably due to a higher accumulation of particles in the rearing water, being a substrate for the bacteria in the system. This was measured by both flow cytometry and colony forming units (CFU). RAS had on average 5×10^6 cells mL^{-1} in the rearing water while FTS had 9×10^4 cells mL^{-1}, which is in accordance with previous studies with marine larvae in RAS (Attramadal et al., 2012a, 2012b; Attramadal et al., 2014; Wold et al., 2014). In accordance to the hypotheses, the RAS treatments without disinfection had a lower fraction of opportunistic bacteria compared with the RAS treatments with disinfection and the FTS. In addition, the RAS treatments showed a lower cultivability of the bacteria in the rearing water compared to the other treatments, at both sampling days.

4.3.2. Effect of water treatment on the biofilm microbiota

Lumpfish in aquaculture live in close contact with the biofilm on the tank walls, as they spend much of the time attached with the ventral suction disc to the tank wall and other surfaces (Hvas et al., 2018). Biofilm can represent a reservoir for opportunistic bacterial pathogens and hence the composition can be important for fish health (Wietz et al., 2009). Both the RAS treatments and the FTS had a relatively higher abundance of potential pathogens in the water compared to the biofilms. In biofilm, possible pathogenic and problematic bacteria were identified at highest abundance in the biofilm of the RAS treatments with disinfection, with 19% abundance of *Moritella* from RAS-F-UV and 33% abundance of *Leucothrix* in RAS-F-UV-O. Biofilm microbiota seemed to be less affected by the water treatments, compared to the water microbiota, as the biofilm community varied less between the RAS treatments and especially over time, than the water microbiota. This was expected, since the composition of the layered biofilm is protected against intrusion, like disinfection (Blancheton et al., 2013), and the biofilm is especially protected with surface growth over time (Wietz et al., 2009). In biofilms high competition and K-selection may generally be expected, but frequent cleaning or perturbations may open for more r-selecting conditions.

5. Conclusion

The lumpfish were exposed to different microbial communities of both water and biofilm, due to different treatments of the incoming tank water. Overall, the results support the hypotheses proposed for the experiment. First, lumpfish reared in the RAS treatments were exposed to a more stable microbial community, with a lower share of opportunistic bacteria, which is a probable reason for the higher survival and better gill health of the fish compared to siblings reared in the FTS. Secondly, RAS without disinfection (RAS and RAS-F) had a significantly more diverse and more stable microbial community composition compared to the tanks receiving disinfected RAS water and the FTS. In addition, these treatments had less opportunistic and potential harmful bacteria, which resulted in a better gill health of the fish compared to siblings reared in the RAS with disinfection and FTS. Thirdly, the fish in RAS-F had a better gill health than the fish in the RAS, which was operated without filtration the first 69 days, probably due to the positive effects of reduced particle load. Altogether, our results indicate that there is a potential to increase both survival, growth and gill health by producing lumpfish in RAS, and that RAS with filtration of small particles, but no disinfection, seem to result in the best fish health and performance. By selecting RAS, the industry can improve and increase the production to meet the growing demands from the salmon farming industry. The possibility that the earlier stages of lumpfish would benefit even more of being produced in RAS, from hatching and until delivery to sea cages, should be investigated further.

Ethics statement

The experiments were conducted at a commercial producer of lumpfish, which are not under the act of animal ethic legislation in Norway. Therefore, no ethical committee was required. Sampling of fish for gill health were anesthetized as described in the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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