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Microbial colonization and community stability in a marine post-smolt RAS inoculated with a commercial starter culture

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A B S T R A C T

The performance of a commercial starter culture was investigated in a new marine post-smolt RAS, analyzing the microbial communities of 100 samples collected monthly over a year from biofilter biofilm carriers, tank wall biofilm, production water and fish skin. Totally 139 operational taxonomic units (OTUs) were defined in the starter culture, of which the classified members of *Rhodobacterales*, *Bacteroidetes*, *Alteromonadales* and *Planctomycetes* were largely the first colonizers of the biofilter carriers. Early colonizing OTUs that dominated biofilter biofilm carriers (> 5% relative abundance) were stably present over time, but the development went slowly from a few OTUs with very high relative abundance to several dominant ones with lower relative abundance. Operating taxonomic units not associated with the starting culture became prominent on the biofilter biofilm carriers only towards the end of the trial period. These were termed environmental OTUs. Comparing the two OTU quantitives in a ratio, where counts were based on all OTUs in the sample, the starter culture OTUs: environmental OTUs were 1.2 and 0.9 at the first and last sampling time for the biofilter biofilm carriers. Correspondingly, for all defined OTUs in the RAS sampling sites together, the ratio changed from 0.8 to 0.6 during experiment. Independent of origin, omniscient OTUs at a sampling site, did also have the highest relative abundances and were normally shared between biofilter biofilm carriers and the production water. New and lost OTUs between sampling times were on average 44 % of the OTUs defined, and this OTUflow was strongest for low abundant environmental OTUs. The maturation of the biofilter with respect to nitrification took long time, and the *Nitrospira* strain in the starter culture was not adapted to marine salinities. Still, we report a controlled colonization of the marine RAS by the starter culture.

1. Introduction

Microbiome studies from recycling aquaculture systems (RAS), based on next-generation 16S rRNA gene sequencing, is a powerful and increasingly used approach to predict processes that influence water quality and fish health. (Bartelme et al., 2019; Gonzalez-Silva et al., 2021; Ma et al., 2020; Martins et al., 2013; Mekuchi et al., 2019; Menanteau-Ledouble et al., 2020; Minich et al., 2020; Minniti et al., 2017; Perry et al., 2020; Ruan et al., 2015; Schmidt et al., 2016; Wang et al., 2021). Most of these studies reports from fresh water or brackish water systems. From commercial post-smolt RAS explorative microbiome studies have investigated facilities that practice up to 22‰ salinity (Rud et al., 2017). For marine RAS (35‰), fecal microbiomes were recently explored in Chinook salmon (Steiner et al., 2021). Yet, thorough microbiome studies from full scale marine RAS that produce salmon are scarce, but needed for a better understanding of the microbial dynamics at high salinities.

The first production cycles in new RAS facilities have the greatest risk for large losses of fish stocks (Roalkvam et al., 2020). Prior to the first fish stocking the biofilter biofilm carriers have gone through a microbial colonization and succession process, and often commercialized microbial starter cultures are used to inoculate the biofilm carriers to ensure rapid establishment of nitrifying bacteria in the RAS biofilter (Brailo et al., 2019; Rurangwa and Verdegem, 2015). The term "a matured biofilter" refers to a biofilter where the microbial nitrification process works satisfactorily, and where unwanted ammonium, ammonia and nitrite do not accumulate in the production water above acceptable concentrations, referred to as good water quality (Norwegian Food Safety Authority, 2016). A steady-state biofilm has neither net growth nor decay of members over time (Rittmann and McCarty, 1980). Such a

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situation is, arguably, achieved only if the water quality and flow rate remain unchanged over time, which is not possible in RAS where production water quality changes continuously, and also often, in a shorter time frame than the microbial adaptability. Still, the major goal for further development of RAS technology is to maintain stable good water quality during a whole salmon life cycle. This goal does not necessarily demand a long-time stable RAS microbiome. Thus, the normal variations in the RAS microbiome without influencing growth and health performance in the fish needs to be understood, and also, what causes the dynamics in the RAS microbiomes (Sauer et al., 2007).

The holistic thinking about the RAS microbiome has been based on microbial K- and R-strategist theories. Principally, the water's carrying capacity will be utilized by slowly growing RAS-bacteria (K-strategists) that restrict the growth potential of invasive fast-growing orpotunists (R-strategists) (Attramadal et al., 2014, 2012; Skjermo et al., 1997). A recent study showed that production water quality was not only affected by the total organic load, but also the distribution between dissolved and particulate material (Fossmark et al., 2020). All microbes present in the intake water of a RAS plant will be introduced continuously in low numbers and become part of the plant's gene pool. If not selected by the RAS environment, these will remain rare or non-detectable in microbiome analysis. For early warning of incoming pathogens and for the understanding of rare OTUs dynamics with respect to pathogens risk evaluation it is of importance that the monitoring regime challenges the rare OTU's identification issues and clarify the dept of sequencing (Zaheer et al., 2018).

To understand microbial community dynamic's, diversity analysis that compares the structures at different sampling sites and times are commonly used (Fossmark et al., 2020; Roalkvam et al., 2019). Alpha diversity address both OTUs evenness and richness, whereas beta diversity allows sampling sites comparisons where the OTUs abundance and presence are included (Gorelick, 2011). These methods are normally supported with statistical variance analysis of differences between compared groups, and, when comparing samples between sites, rarefaction of data to compensate differences in the depth of sequencing (Anderson, 2017; Gloor et al., 2016; Weiss et al., 2015; Roalkvam et al., 2019; Weigel and Erwin, 2016; Willis, 2019). The rarefaction and the weighting of these analysis upon dominating OTUs, omits registering the rare OTUs dynamics. Furthermore, OTU richness, i.e. numbers of OTUs defined in sample, is often addressed by indexes and estimators that corrects for OTUs not observed (Hughes et al., 2001). However, dynamics in individual rare OTU's between sampling times are not addressed with these methodologies. In RAS, microbiomes can be retrieved from three major habitats: water, biofilm and tissue, requiring three different DNA extraction kits that potentially also influence the final sequencing result (Sinha et al., 2017; Costea et al., 2017). When comparing biofilm and water samples, it is further complicated that the conceptual biofilm models, in addition to diversity, use ecological terms as adhesion and extinction of species to describe the colonization process (Woodcock and Sloan, 2017; Garrett et al., 2008; Dzianach et al., 2019). Notably, next generation sequencing does not allow to define microbes as lost from a community, and the presence or absence issues is basically a matter of being above or below the boarder of detection (Zaheer et al., 2018).

A commercial land-based post-smolt RAS that used 32‰ Atlantic coastal water was inoculated with a commercial starter culture enriched from brackish water prior to fish stocking. Previously the starer culture had been used successfully in freshwater RAS, but, the operational capacity at marine salinities (32‰) had not been investigated in advance. We followed the microbial colonization of the new RAS over a period of one year, using 16S rRNA gene amplicon sequencing regularly towards samples from the biofilm, water and skin habitats. We describe in detail the succession of the biofilter biofilm in the RAS. The stability of the inoculum members was compared to other microbes colonizing the biofilter biofilm carriers and the production water, using simple count metrics. When modelling the functional relationship between total reads and total OTUs obtained from the biofilm carriers, we were able to identify abnormal sampling times, and finally, we do the first step in evaluating "OTUflow between sampling times" as a comparable unit between sample sites and sampling times, to better understand rare OTUs dynamics in the RAS.

2. Material and methods

2.1. RAS specifications

Samples were retrieved from a RAS belonging to Erko settefisk AS at Stord, at the west coast of Norway. The RAS had capacity to grow 500 000 post-smolt from 100 to 500 g in 4 months at 14 °C and 32‰ salinity. The experimental time period was February 2016 to February 2017, which covered production cycles (PC) 2–5. The RAS unit comprised 4 production tanks of $1100 m^3$, all filled with coastal water from 70 m depth. The production water recycled through drum filters (60 μm, Hex), the moving bed biofilter (300 m^3), the CO_2 degassing (Aqua group) unit and into the pump sump from where it was pumped into the production tanks again (Fig. 1). A part of the pump sump water passed oxygen cones with liquified ozone, giving a pressurized injection of oxygenated water into the production tanks. The biofilm carriers were made from polyethylene with the size $1 \times 1 cm^2$ (5×5 inner lattice). Prior to operation they were inoculated by a microbial starter culture that was enriched from brackish water and made commercial available (Avecom, 2019). A mixture of liquefied ozone and hypochlorite (Loz AS) was injected into the degassing unit for the purposes of disinfection, reduction of organic particles and oxidation of nitrite chemically. Water buffer capacity was regulated by bicarbonate powder that was added directly into the biofilter chamber, and when needed, NaOH was used to regulate the pH. The dilution water was UV filtered and made up 50–125 litre/min, that caused a water retention times of 1.5 h or a degree of recirculation upon 97.8 %. White LED light was on 24/7 in the production hall.

2.2. Measuring water quality parameter

The chemo-spectroscopic analysis of ammonium, TAN, nitrate and nitrite were performed on daily basis, using an Odeon instrument fitted with a photopod (Ponsel Measure). Water and reagents in the form of tablets were mixed in a suitable test tube (Orchids laboratorie), measuring at lower detection limits of 0.02 mg/L-N ammonium/ammonia/TAN, 0.01 mg/L-N nitrite and 0.06 mg/L-N nitrate. The concentrations of ammonia were calculated as the difference between TAN and ammonium. Parameters of pH, CO_2, salinity, temperature, O_2 and redox potential were measured continuously by probes (Schneider/Oxy Guard probes and Unitronics computer software), and raw data are available as dataset (Drønen, 2020).

2.3. Sampling times, sampling sites, biological material and major operational RAS events

The biological material in the RAS were sampled from the biofilter chamber and the tank enumerated 3. From the tank, samples were collected from the wall biofilm, from the production water and from the fish skin. From the biofilter chamber, the biofilm carriers were collected in triplicates, and so also biofilm from the tank wall. These sampling sites are from now termed biofilter biofilm carriers, tank wall biofilm, production water and fish skin. The sampling times were named after the cycle number (C) and week (W) into the cycle as shown in Table 1. WashI and WashII samples were collected the day before new fish stocking. This table summarize also major RAS events during the experimental time period. Notably, there was a severe occurance of skin wounds in the fish population during production cycle 2, and the antibiotics oxolinic acid (5 mg kg fish body weight$^{-1}\cdot d^{-1}$ in 10 days from week 5) and florfenicol (10 mg kg fish body weight$^{-1}\cdot d^{-1}$ in 10 days

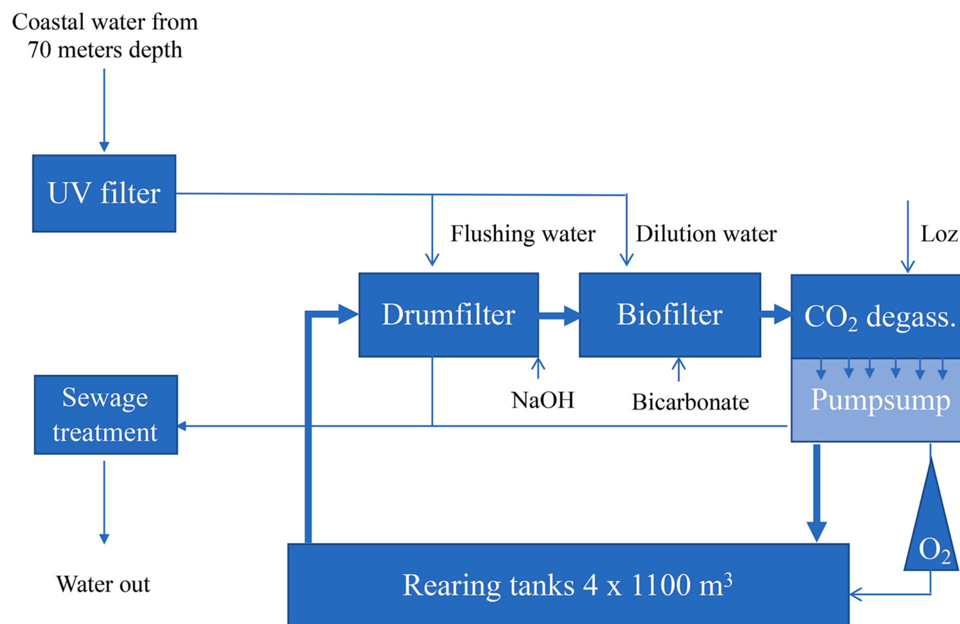

Fig. 1. Simple flow diagram of the marine post-smolt RAS. Dilution water and flushing water for the drum filter (60 μm) were obtained from Atlantic costal water at 70 m depth and UV treated before use. Production water was recirculated in a loop from tank to drum filter to biofilter to CO_2 degassing to tank again. Sites for addition of NaOH, bicrabonate and Loz are shown. Loz: Liquified ozon and hypochlorite.

Table 1
Production cycles, sampling, sampling sites and operational events in the RAS.

Fish stocked (date from-to)	Sampling (Cycle; C Week; W)	Samples sites	Operational events
1.2.16–30.4.16	C2W3*	R**	Antibiotics added
	C2W8	R	Freshwater added
	C2W13	R	
	Wash I	R (No skin)	Biofilter Loz Treatment Re-inoculation
17.5.16–1.8.16	C3W4	R	New PW****
	C3W8	R	
	Wash II	Biofilter	New PW
16.8.16–23.11.16	C4W2	R	
	C4W10	Biofilter, PW	Yellow substance
8.12.16–1.4.17	C5W2	R	Freshwater added
	C5W10	R + E***	

Loz: Oxidative solution of liquefied ozone and hypochlorite; T: treatment and back flushing.
* C2W3; Week 3 in production cycle 2.
** R; Regular sampling sites in the RAS: Biofilter biofilm carriers and the production tank enumerated 3 with the sites: wall biofilm, production water and fish skin.
*** E; Extra ordinary sampling sites for biofilm and water: Biofilms: Wall of the biofilter outer chamber, wall of pump sum, wall depth profiles in all the four production tanks in the RAS, inlet water Bernaud filter. Water: before and after UV treatment.
**** PW; Production water; the water wherein the fish is reared.

from week 7) were administered to the fish through the feed. This was followed by fresh water addition and abrupt drops in salinity and pH. The RAS was extensively washed between the second and the third production cycle (termed Wash I), a pause wherein also the biofilter was "back washed" with Loz and a re-inoculated with 20m^3 biofilm carriers. Another RAS wash (Wash II) was performed after the third cycle, though without biofilter ozonisation. During the fourth production cycle, the water turned strongly yellow. At the end of the experimental period in the fifth production cycle, wall biofilm was sampled extraordinary from the biofilter outer chamber, the pump sump, and depth profiles from the four production water tanks. Biofilm was also sampled from the Bernaud filter in the inlet water this day. At the same sampling time, water samples were collected from the inlet water before and after the UV filter. From the batch with fresh starter culture (Avecom) 5 replicates were sampled for analysis. These samples aimed to distinguish inoculum's associated OTUs (IAO) from environmental OTUs (EO) during microbial community analysis and to calculate the IAO:EO rato.

2.4. *Sampling methodology*

To retain wall biofilm, a toothbrush was taped to a long rod and pulled along the tank wall (10 m), then the brush head was chopped into a 50 ml Falcon tube with 15 ml RNAlater storage buffer. The biofilm carriers were capture by a bean in the biofilter, and carriers' triplets were stored in 10 ml RNAlater in a 50 ml Falcon tube. Prior to −24 °C storage, toothbrush and biofilm carriers were sonicated in a water bath to assist the biofilm loosening. The brush heads and carriers were removed and the organic material pelleted by centrifugation 45 min 5000*g* 7 °C (Heraeus). Water microbes were collected from 240 ml water into a 0.22μm Sterivex filter and stored at −24 °C with RNAlater buffer in the cage. Fish was caught in the tanks by a net and killed with a sharp blunt to the head. Then, 2 × 3 cm skin, 1 mm tick, was cut out with a scalpel in the area lateral to the dorsal fin, and stored in 10 ml RNA later in a 50 ml Falcon tube at −24 °C.

2.5. *DNA extraction, amplicon library analysis and bioinformatics*

The detailed methodical description of the 16S rDNA amplicon library retrieval, the samples successful processing and the detailed usage of each samples data are given in Table 1 of the co-submitted Data in Brief article (Drønen et al., 2021). Shortly, summary of the protocols and methodology is as follows.

DNA was extracted from the samples using kits designed for the sample types: Cell lysis of DNeasy Power Biofilm Kit (Qiagen), DNAeasy PowerWater Kit (Qiagen) and the tissue DNA extraction kit supplied by Roche (High Pure PCR Template Preparation). 16S rRNA gene amplicons were generated in accordance with the two-step PCR amplification protocol recommended by Berry et al. (2011). The first amplification used the universal primers 519f (5′-CAGCMGCCGCGGTAA) (Øvreås

et al., 1997) and 805 r (5′-GACTACHVGGGTATCTAATCC) (Klindworth et al., 2013) in a PCR mixture containing: 12.5 μl HotStar Taq Master Mix (Qiagen), 2 μl of each primer (0.1 mM), 7.6 μl dH_2O and 2 μl DNA-template. The thermal cycle program included 15 min activation of the Taq enzyme at 95 °C, followed by 32 cycles of gene amplification, i. e., 30 s at 94 °C, 30 s at 56 °C and 30 s at 72 °C. The final elongation lasted for 7 min at 72 °C. Positive PCR products of sample triplicates were verified by 1D gel electrophoresis, and pooled triplicates were purified by AMPure XP beads (Agencourt) using a 96 well square storage plate (1.2 ml) adaptable to a magnetic plate. The sample:bead ratio was 0.7 and subsequent supplier's manual was followed (Beckman Coulter). The purified DNA was quantified in the Quantus Fluorometer (Promega Corporation) and used as template (10 ng/mL) for the second PCR, where the primers were tagged for sequencing technical purposes and samples identification (Torrent, 2012). The PCR mixture and thermal program was as previously, only that seven cycles of amplification were used. The purification and agarose steps were repeated as for the initial PCR, to ensure primer and primer-dimer absence. The 96 samples were pooled in equimolar concentration to a final concentration of 0.1 ng/μl. This stock was quantified again prior to the final dilution (40 pM) the very day of sequencing by the Ion Torrent Personal Genome Machine (PGM) technology. The sequence analysis by Ion Torrent was followed by a bioinformatics pipeline thoroughly described by Roalkvam et al., 2019 and in the co-submitted Data in Brief article (Drønen et al., 2021). This comprised USEARCH for sequence merging and read quality filtering (Edgar, 2010), UPARSE for *de novo* 97 % identity OTUs clustering (Edgar, 2013) and Qiime for OTUs taxonomic analysis (Caporaso et al., 2010) using the qiime compatible SILVA128 release (https://www.arb-silva.de/no_cache/download/archive/qiime/) as a reference database. For some of the samples we were not able to obtain a PCR product and these samples were not considered further. For some of the samples, we were unable to obtain a PCR product, and these samples were not evaluated further. Negative PCR controls should have been included through the sequencing step, which was not practiced. Raw data can be downloaded through links provided in the co-submitted Data in Brief article (Drønen et al., 2021), and are available in GenBank with the accession numbers MN890148-MN891672.

2.6. Data handling and statistics

The data OTUs text files were processed by the Microsoft Excel software to sort OTUs data into categories of origin and relative abundance, to register OTUflow, to give samples total OTUs number in replicate samples as mean or pooled, to perform the statistical approaches and to prepare data for Sigma Plot13.0. Means were reported with their standard deviations. Pooled samples were made by including all obtained OTUs from sample replicates, and the relative abundances were averaged. For count metrics of replicate samples and times of identification we used the excel formula = COUNT(range)-COUNT.IF (range; 0). The conjunction probability analysis was performed at http://www.calctool.org/CALC/math/probability/3_events. The amplicon data rarefaction curve was made in R using the 'vegan' package (Oksanen et al., 2015). The functional relationship between OTUs and reads were linked by a 2. order regression line in the Sigma Plot graph. Physiochemical data (DOI:10.17632/zrmjktk992.1) was provided from the Erko settefisk AS in excel sheets and presented by SigmaPlot. Graphs from Sigma Plot, Excel and R (the rarefaction curve) were handled by Photoshop Illustrator CC for final figure presentation.

3. Results

3.1. OTUs distribution statistics in the RAS

The biofilter biofilm carriers in a new marine post-smolt RAS were inoculated with microbes from a commercial starter culture prior to the first fish stocking. The microbial colonization of the RAS and the starter culture itself were analyzed by 16S rRNA gene amplicon analysis, and a total of 100 samples were retrieved from the biofilter biofilm carriers, tank wall biofilm, production water and fish skin sites monthly over a year. To compare data between sampling times and sampling sites we evaluated differences in depth of sequencing among sampling groups, and data for each sampling site were averaged over all sampling times. The average and standard deviation of defined OTUs at the various sampling sites and sampling times is shown in Table 2, whereas total OTUs from pooled samples at various sampling sites and sampling times is shown Fig. 4 in co-submitted Data in Brief article (Drønen et al., 2021). For pooled biofilter biofilm carriers replicate samples this made an average of 147 ± 36 OTUs over all sampling times, whereas averaged replicates made 112 ± 24 OTUs. Samples from the production water, that were in replicates, averaged 146 ± 34 OTUs over all sampling times. The coefficients of variation (CV = standard deviation/mean), were 24 %, 21 % and 23 % for these tree calculations, that are low percentages. The pooled replicate biofilter biofilm samples and the production water generated equalized number of OTUs (∼150 ± 35) between the sampling sites. Thus, the dispersion of OTUs within replicates and the two sampling sites was low. For biofilm samples we therefore pooled replicate samples prior to further analyses and data were maintained unrarefied. In comparison to the biofilter biofilm carriers and the production water, pooled tank wall biofilm samples averaged 114 ± 35 (CV 31 %) and fish skin samples 115 ± 14 (CV 12 %), which were a slightly higher and a slightly lower dispersion respectively.

In the total sample material, 450 operational taxonomic units (OTUs) were defined in the RAS, of which 45 % were classified to genus level. Totally 139 OTUs were defined from the starter culture, while 145 OTUs were defined in the RAS facility the first sampling time (C2W3). Among the latter, 65 RAS OTUs had 100 % similarity to those in the starter culture, and were referred to as inoculum-associated OTUs (IAO). The other 80 OTUs were referred environmental OTUs (EO) as they were assumed to represent environmental microbes. At the end of the sampling period (C5W10), still regarding the whole dataset as an entity from this sampling time, there were defined 92 different IAO and 140 different EO, thus, the IAO:EO ratio changed from 0.8 to 0.6 during 12 months operation. At each sampling time, on average of 211 ± 31 OTUs

Table 2
The average and standard deviation of defined OTUs at the various sampling sites and sampling times.

Sampling time	Mean OTUs number ± SD							
	Biofilter biofilm carriers		Tank wall biofilm		Production water		Fish skin	
	IAO	EO	IAO	EO	IAO	EO	IAO	EO
C2W3	43 ± 0	34 ± 2 (3)	53	56	50	59		
C2W8	55 ± 8	39 ± 4 (5)	41 ± 3	36 ± 1 (3)	53	47		
C2W13	51 ± 5	39 ± 6 (3)	46 ± 5	31 ± 3 (3)	67 ± 14	76 ± 32 (2)	56	39
WashI	64 ± 12	56 ± 18 (3)	55	30	80	123		
C3W4	66 ± 7	66 ± 13 (4)	46	35	47	58	57	51
C3W8	49 ± 5	44 ± 5 (3)	49 ± 4	47 ± 5 (3)	69	113	65	71
WashII	67 ± 6	73 ± 11 (2)						
C4W2	69 ± 11	73 ± 6 (3)	54 ± 1	59 ± 6 (3)	48	80		
C4W10			45	41	58	94	43	23
C5W2			63 ± 8	70 ± 22 (2)	49	62		
C5W10	60 ± 2	61 ± 2 (2)	50	53	63 ± 11	74 ± 13		

IAO; Inoculums associated OTUs, EO; Environmental OTUs.
Brackets; number of replicates.

were defined from the sampling sites all together, of which 89 ± 11 were IAO and 122 ± 23 were EO (IAO: EO ratio 0.7). The OTUs defined varied on average by 14 from sampling time to sampling time, which was 6.6 % of the 211 OTUs defined on average at each sampling time. Notably, 21 out of 139 OTUs defined in the starter culture, were never detected in the RAS facility.

For sampling sites separately, the IAO: EO ratio was 1.2 at the first and 0.9 at the last sampling time for the biofilter biofilm carriers, and equivalently, 0.8 and 0.8 in the production water.

Thus, there were relatively more EO in the production water compared to the biofilter biofilm carriers. On average, the IAO:EO ratio was 0.9 for the biofilter biofilm carriers and 0.7 for the production water at all sampling times, and furthermore 1 and 1.2 for the tank wall biofilm and for fish skin samples respectively, all sampling times on average. Notably, the IAO:EO ratio was lower when counting different OTUs in the RAS from the whole dataset as an entity, than averaging ratio from the separate sampling sites.

3.2. OTUflow in the RAS

OTUs new or lost between sampling times were defined as OTUflow in sample analysis, thus representing the ongoing change in community structure. Added counts of OTUs lost and new from one sampling time to the next was in mean 94 ± 29 or 44 % of the 211 OTUs defined on average at each sampling time regarding all sampling sites together (Fig. 2A). Thus, the average OTUflow was much larger than the variation in total number of defined OTUs between sampling points as reported above (6.6 %). Comparing IAO and EO, on average 21 % of the IAO and 46 % of the EO were flowing between the sampling times. In numbers, this represented 3–28 IAO and 13–48 EO new, and 1–16 IAO

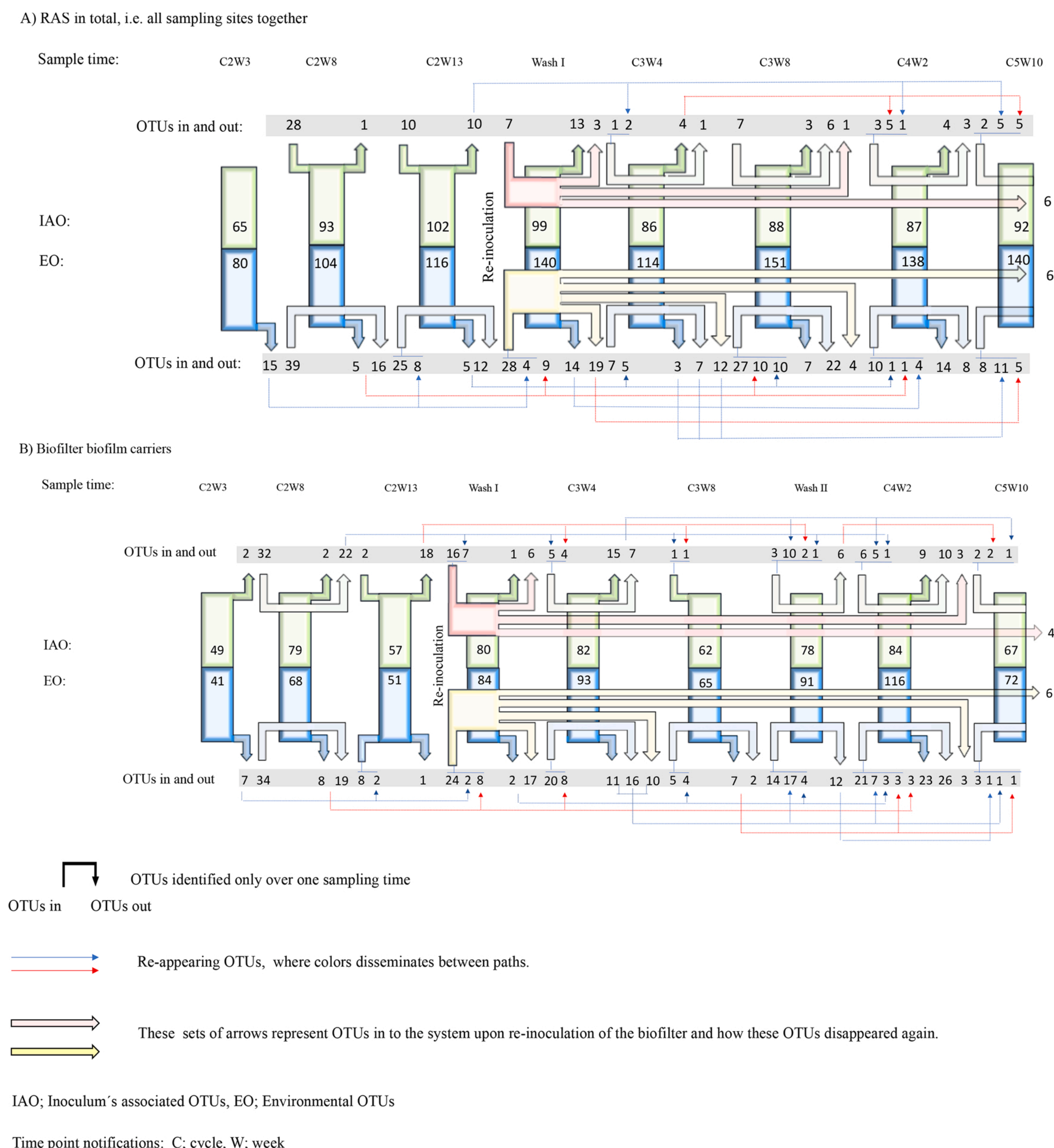

Fig. 2. RAS in total (A) and biofilter biofilm carriers (B) total defined OTUs, OTUflow and re-appearing OTUs as given by 16S rRNA deep sequencing at the different sampling times. RAS in total includes OTUs from all sampling sites, that is, biofilter biofilm carriers, tank wall biofilm, production water and the fish skin. Biofilter biofilm samples at sampling times C4W10 and C5W2 failed in the PCR step and are ommited from comparison. The OTUflow counts are based on the total defined OTUs in samples, i.e. pooled replicates and unrarefied data. On average of sampling times both the production water samples and biofilter biofilm carriers generated ~150 ± 35 OTUs.

and 17–33 EO lost. Notable, OTUs that were identified now and then, the re-appearing OTUs, increased by time, in particular for IAO. In the end (C5W10), >>50 % of OTUs in were re-appearing IAO and ~50 % were re-appearing EO (Fig. 2A).

The OTUflow between sampling times was higher in the biofilter biofilm carriers compared to all sampling sites together, i.e. on average 39 % IAO and 64 % EO. In counts, this corresponded to 2–32 and 6–37 OTUs in and 0–24 and 1–52 OTUs out (Fig. 2B). Most defined OTUs in the biofilter biofilm carriers were also defined in the tank wall biofilm during the experimental period, but here the residence time of OTUs in was usually shorter than in the biofilter. This pattern was observed for both IAO and EO.

The RAS biofilter was re-inoculated between the second and third production cycles, and the new biofilm carriers were pre-colonized by the same starter culture as previously used. After the re-inoculation, but before a new fish stocking (Wash I), 7 new IAO and 41 new EO were identified in the whole RAS facility. Eight months later (C5W10), 12 of these OTUs were still present in the RAS, divided 1:1 by IAO and EO (Fig. 2A). In the biofilter biofilm at sampling time Wash I, 23 IAO and 34 EO were new, of which 10 OTUs were still identified in the biofilter biofilm at the last sampling time (Fig. 2B).

3.3. *OTUs in replicate samples*

3.3.1. *Replicate samples average OTUs and total OTUs as functional relationship to average reads*

Generated OTUs and reads from samples are related quantities where OTUs is a dependent variable of the reads, following there is a functional relationship between the variables. Presumably, samples from the same site that deviates from this relationship reports upon a "non-methodical event". To study sampling times where OTUs defined deviates from the expected, we used the replicate samples from the biofilter biofilm carriers to model a functional relationship between OTUs and reads (Fig. 3). The relationship was modelled both for OTUs as average of replicate samples and as total OTUs from pooled replicates using 2. orders regression curves. Sampling times that deviated strongly from the regression lines were the sampling times C3W8 and Wash I, but also at C2W8.

3.3.2. *Probability of OTUs to be detected in replicate samples and replication cut-off value*

Six triplicate sample set from tank wall biofilm and biofilter biofilm carriers at the time points C2W8, C2W13 and C3W8 were analyzed for OTUs repeated detection in parallel samples, thus, counting their presence and absence in triplicates. Prior to analysis we evaluated the samples DNA yield for possible inconsistence, as this might influence the depth of sequencing. On average 86 ± 10 ng/μl tank wall DNA (CVs 11 %) and 74 ± 35 ng/μl biofilter carrier DNA (CV 50 %) was obtained by the biofilm DNA extraction kit. Only two samples made up the extremes (2.4 ng/μl and 121 ng/μl). This and the low CV values for the sampling material, suggest that DNA yield influenced the analysis little. OTUs were sorted in fractions by their outcome 3 out of 3, 2 out of 3 and 1 out of 3 in triplicate samples, and the mean percentage ($\bar{x}$) distribution of each fraction was as following: $\bar{x}_{3.3wall}$=59.0±5.4 %, $\bar{x}_{2:3wall}$=14.9±4.0 %, $\bar{x}_{1.3wall}$=26.1±3.3 %, $\bar{x}_{3:3biof}$=58.6±4.0 %, $\bar{x}_{2:3biof}$=16.5±3.8 %, $\bar{x}_{1:3biof}$=24.9±6.4 %. Using a 3-events conjunction calculator, the probability that any OTU in the pooled triplicate samples were detected in a single sample at analysis was ~75 %.

The consistency in percent distribution between wall and biofilter samples, was then evaluated towards the relative abundance. A pattern was seen, as 3 out of 3 score were only obtainable for OTUs with relative abundance ≳ 0.015 %, 2 out of 3 score in the range 0.006−0.02 % and 1out of 3 score bellow 0.001 % relative abundance. Thus, 0.015 % relative abundance was set as replications cut-off value in the retrieved dataset. Twice as many EOs (30 % on average) than IAOs (17 % on average) affiliated below this cut-off value (Fig. 4 in Drønen et al., 2021).

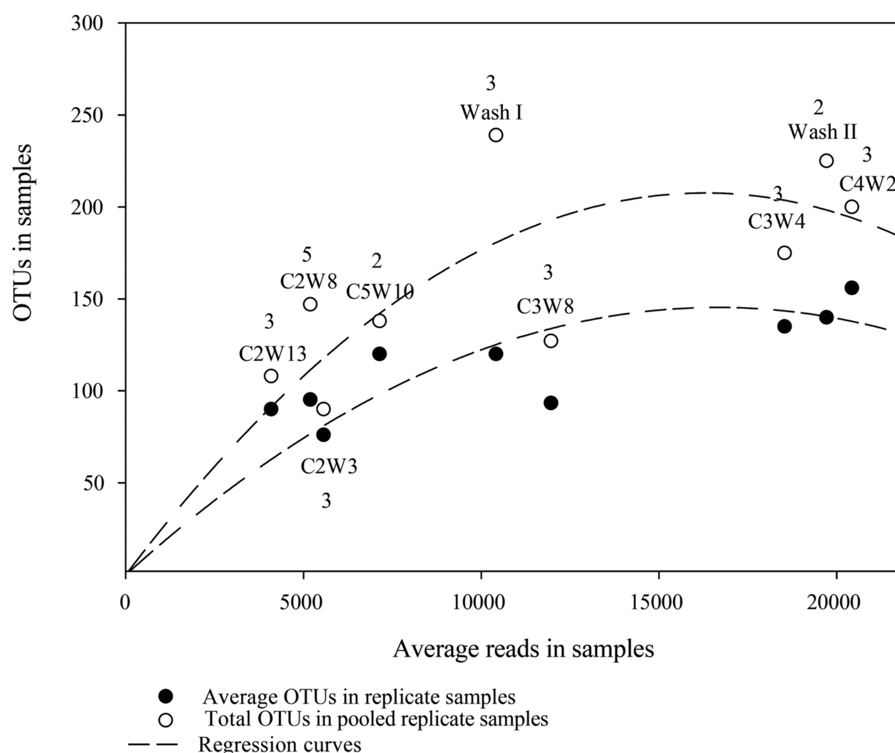

Fig. 3. Modelled functional relationship between defined OTUs and average reads in replicate biofilter biofilm carriers' samples. Filled dots; replicates average OTUs, Open dots; pooled replicates total OTUs. Number of replicates are indicated above the sampling time notification. Dashed curves; (lower) The second order regression lines between average OTUs and average reads, (upper) total OTUs and average reads. Data are unrarefied.

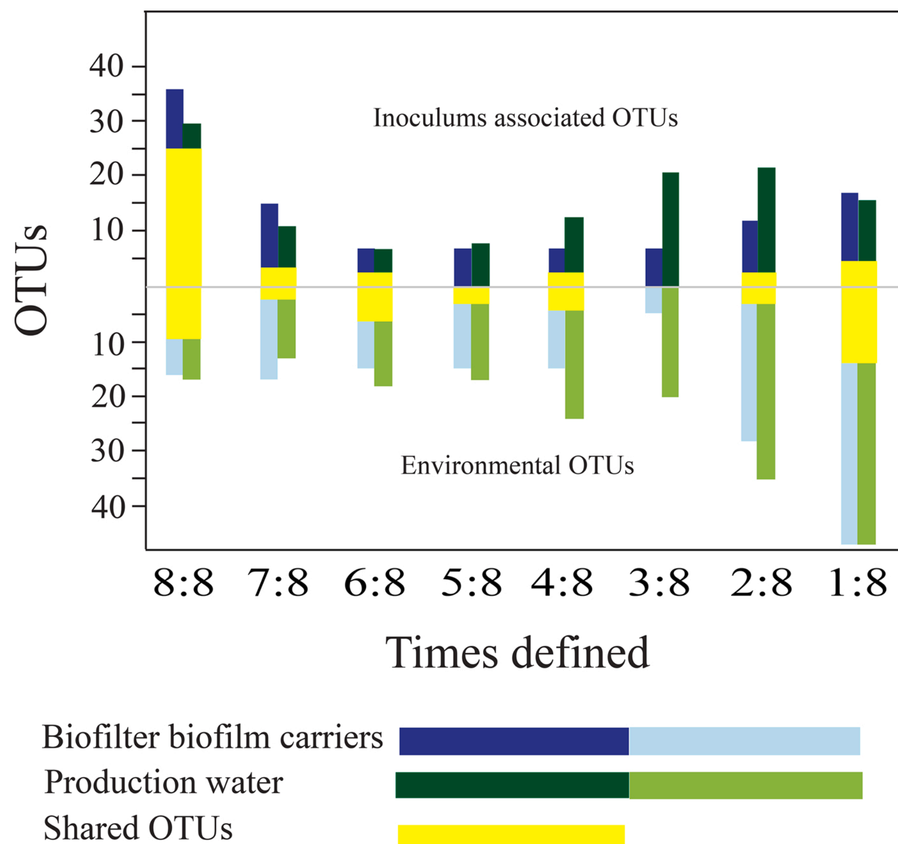

Fig. 4. Comparing OTU stability in biofilter biofilm carriers and production water over 8 sampling times. The comparison is based on the total number of OTUs identified, i.e. pooled replicates and data not rarefied. On average of sampling times both these samles sites generated ~150 ± 35 OTUs.

3.3.3. The correlation between replication outcome and relative abundance

The correlation between replication outcome in samples and relative abundance was confirmed by statistical correlation tools. Totally 8 categories were correlated, i.e., sampling site (tank wall biofilm, biofilter biofilm carriers), outcome in replicates (1–3) and origin of the strains (IAO, EO). The relative abundance of affiliating OTUs was summarized for each of the 8 categories. Summed relative abundance made up one matrix, and were aligned towards matrix 2, contain OTUs percent distribution into 1, 2 or 3 replicate samples. The correlation coefficient for the data set was 0.83, which indicated a positive correlation.

3.4. OTU stability and relative abundance in the biofilter biofilm carriers and in the production water

OTU stability in the production water and the biofilter biofilm carriers was investigated by comparing times of appearance of defined OTUs at the mutual 8 sampling times (Fig. 4). OTUs defined 8 out of 8 times were mainly IAO, and these were also largely shared between the sampling sites. In contrast, the EO were over-represented in the OTUs that were defined only once at the 8 sampling times. The distribution of such “one time” OTUs was relative equal between the biofilter biofilm carriers and the production water, although, only a few OTUs were shared between the two habitats. Interestingly, OTUs observed 2–5 times were more abundant in the production water than at biofilter biofilm carriers (Fig. 4).

The relative abundances of shared and sample-specific OTUs in the production water and biofilter biofilm were further compared (Fig. 5). Inoculums associated OTUs that were identified in 8 out of 8 sampling times were the most abundant OTUs whether shared or not. The shared fraction comprised 35 OTUs that in mean had a factor 3.8 higher relative abundance in the biofilter biofilm than in than water (Fig. 7). Eleven OTUs were also present one or more times with a relative abundance >5%, thus being dominating to our definition. Interestingly, the water mirrored the biofilter biofilm. Although the production water was largely dominated by one OTU, the internal relative abundance pattern was maintained, as a parallel offset, very much identical to the biofilter biofilm when study 8 shared OTUs (Fig. 3 in co-submitted Data in Brief article, Drønen et al., 2021).

In opposite to frequently appearing OTUs, OTUs defined only in one sample had on average very low relative abundance (Fig. 5). These OTUs were in 92 % of cases defined with a relative abundance <0.05 % or in 40 % of cases <0.005 % (Appendix file Drønen et al., 2021). The highest value obtained was 1.1 % relative abundance by a shared EO at the time C4W2 from the biofilm biofilter carriers. The average relative abundance of OTUs defined 2–7 times had high standard deviations, saying that OTUs sporadically made outgrowths also in these fractions, especially among the sample-specific OTUs. Interestingly, on biofilter biofilm carriers this was strongest associated to EO (Fig. 5).

3.5. Dominating and nitrifying bacteria on the biofilter biofilm carriers

A total of 18 OTUs from the biofilter biofilm carriers had > 5% relative abundance at one or more sampling times, and 13 of these were IAO (Fig. 6A). Together, classified OTUs of *Rhodobacteriales* and *Bacteroidetes* constituted > 60 % relative abundance on the biofilter biofilm carriers during the second and third production cycles. Whereas four OTUs dominated in main with >>5% relative abundance the first sampling time, this developed gradually into 6 OTUs with average 7% relative abundance in the end, indicating an increase in diversity over time. Notably, two OTUs of *Rhodobacteriales* (α-*Proteobacteria*) were detected within the >5 % fraction at all sample times (Fig. 6A).

Four classified OTUs of γ-*Proteobacteria* dominated the biofilter biofilm carriers once each at different sampling times (Fig. 6A). Three of

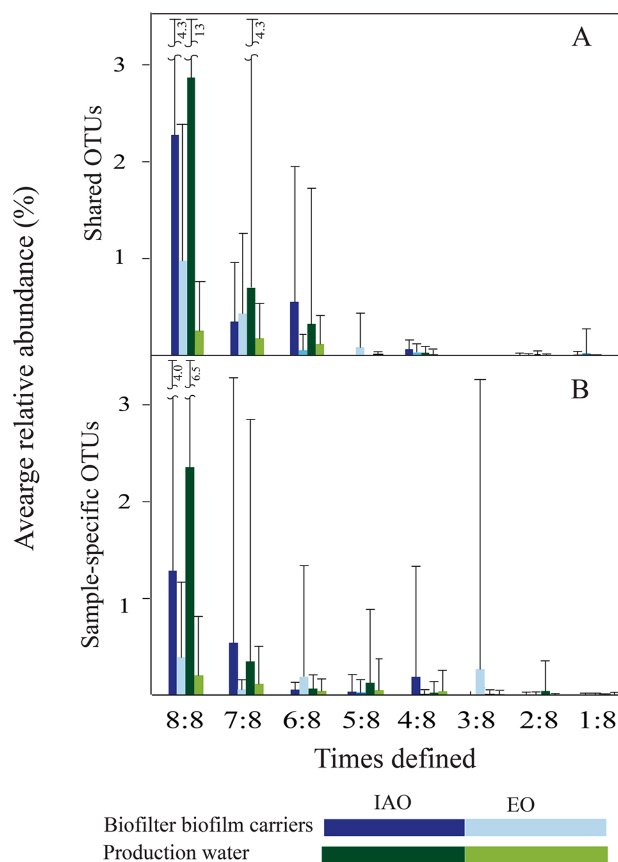

Fig. 5. Average relative abundance of shared (A) and sample-sepcific (B) OTUs from biofilter biofilm carriers and production water versus times of appearance. IAO; inoculums associated OTUs, EO; environmental OTUs. Error bars show the standard deviation. Data is based on the total number of OTUs in pooled replicate samples, and data are not rarefied. The relative abundance data are also presented as boxplot in Fig. 5 in the co-submitted Data in Brief paper, giving more detailed information about medians and outliers.

them affiliated to the order *Alteromonadales*, of which the genus *Colwellia* was dominating on the biofilter biofilm carriers at the first sampling time. The other *Alteromonadales* were two EO defined in the dominating fraction at the last sampling time. This order was also present on the biofilter biofilm carriers with two OTUs in the 1–5 % relative abundance fraction during the second production cycle (Appendix file in Drønen et al., 2021). Thus, OTUs classified as *Alteromonadales* were the dominating *γ-Proteobacteria* on biofilter biofilm carriers during the experimental period, increasing in relative abundance by time. The fourth *γ-Proteobacteria* identified was the genus *Thioalkalispira* within the order *Chromatiales*, which dominated on the biofilter biofilm carriers during the fourth production cycle (Fig. 6A).

Other dominating OTUs on the biofilter biofilm carriers that were not defined at all sampling times were classified as *Planctomycetes* (2 and 3), *Lewinella* (2), *Desulforomonadales* (3 and 5), *Nannocystaceae* (3 and 5), *Thioalkalispira* (4), *Dasania* (5), *Nitrospira* (5) and *Alteromonadales* (5), and they dominated on the biofilm carriers in the production cycles as indicated within the brackets.

The ammonium oxidizing genus *Nitrosomonas* comprised 80–100 % of the nitrifying population in the biofilter biofilm during the second to fourth production cycle (Fig. 6C), and over all sampling times on average the relative abundance was 1.0 % for this genus, with 1.7 % as the highest value at sampling time C5W10 in the fifth production cycle. However, upon the latter sampling time, the nitrite oxidizing genus *Nitrospira* were identified with a relative abundance of 9.6 %, comprising 82 % of the nitrifying population. *Nitrospira* was first identified in the RAS after the re-inoculation step (C3W4) with a relative abundance of 0.02 %, increasing to 0.07 % and 1 % at sampling times C3W8 and C4W2 respectively. The genera *Nitrosomonas* and *Nitrospira* were also associated with the wall biofilm, although detected with lower relative abundance than in the biofilter biofilm.

The dominant population of biofilter biofilm carriers was little affected by the large changes in salinity, pH, CO_2 and N-species in the production water throughout the four production cycles studied. These variations are depicted by trend lines in Fig. 6B. However, there was a fall in the salinity of the inlet water from 25‰ to ~ 21‰ in the fifth production cycle, when *Nitrospira* finally colonized the biofilter biofilm carriers in high abundance.

3.6. Dominating OTUs in the production water

The production water was largely dominated by one OTU, which classified to the genus *Colwellia* and originated from the inoculum. On average this OTU had 50 ± 36 % relative abundance over all sampling times (Fig. 7). However, there were two major drops and three minor drops in relative abundance of *Colwellia* during the experimental period, and during the major drops, i.e., sampling times C3W8 and C5W2, the classified IAO, *Fransicella* (27 % relative abundance) and *Rhodobacteriales* (78 % relative abundance), were the dominating OTUs in the production water respectively. The latter OTU was also frequently dominating on the biofilter biofilm carriers and the second most abundant OTU (8%) after *Francisella* at sampling time C3W8, then together with the classified IAO *Glaciecola* (8%), *Desulfuromonadales* (7%), and *Nannocystaceae* (5%) (Appendix file in the co-submitted Data in Brief article, Drønen et al., 2021). *Fransicella* did also have 19 % relative abundance in the water at sampling time C5W10, whereas, *Psychrobacter* (16 % relative abundance) and an unassigned OTU (7% relative abundance) where the second most common OTUs, after *Colwellia*, at sampling times C2W13 and C4W10 respectively. This unassigned IAO was also frequently dominating on the biofilter biofilm carriers, and when dominating in the water at sampling time C4W10 it was together with two EO, classified as an unknown *γ-Proteobacteria* (7% relative abundance) and *Kiloniella* (6 % relative abundance).

4. Discussion

To shorten time of sea lice exposure in open net pens during salmon production, the technology developed for fresh water RAS was adopted for marine production of post-smolt, mainly by replacing the fresh water with sea water. A commercial starter culture, previously used for fresh water RAS, directed the colonization of the biofilter biofilm carriers, to ensure microbial stability and sound production water in the new plant. The microbial colonization and stability of the RAS facility were monitored from the second to the fifth production cycle by 16S rRNA gene amplicon sequencing, and the retrieved amplicon library included sequences from 5 replicate samples from the starter culture and 100 samples in total from biofilms, water and fish surfaces at different sampling times. Here we demonstrated that a directed and controlled colonization by an enriched consortium was possible, although the key OTUs for nitrite oxidation were not adapted for the highest operative salinities in the RAS.

4.1. RAS colonization by the starter culture

The commercial starter culture supported a number of defined OTUs that dominated on the biofilter biofilm carriers at all sampling times (Figs. 6 and 7). The dominating OTUs were classified as *Rhodobacteriales* and *Bacteroidetes*, that are taxonomic units with members previously described to colonize surfaces from marine coastal waters (Dang and Lovell, 2000). In this context, *Rhodobacteriales* members are regarded as the primary colonizers, following from their efficiency in carbon utilization, intercellular communication, the production of antibacterial components and possibly also the ability to disperse preestablished

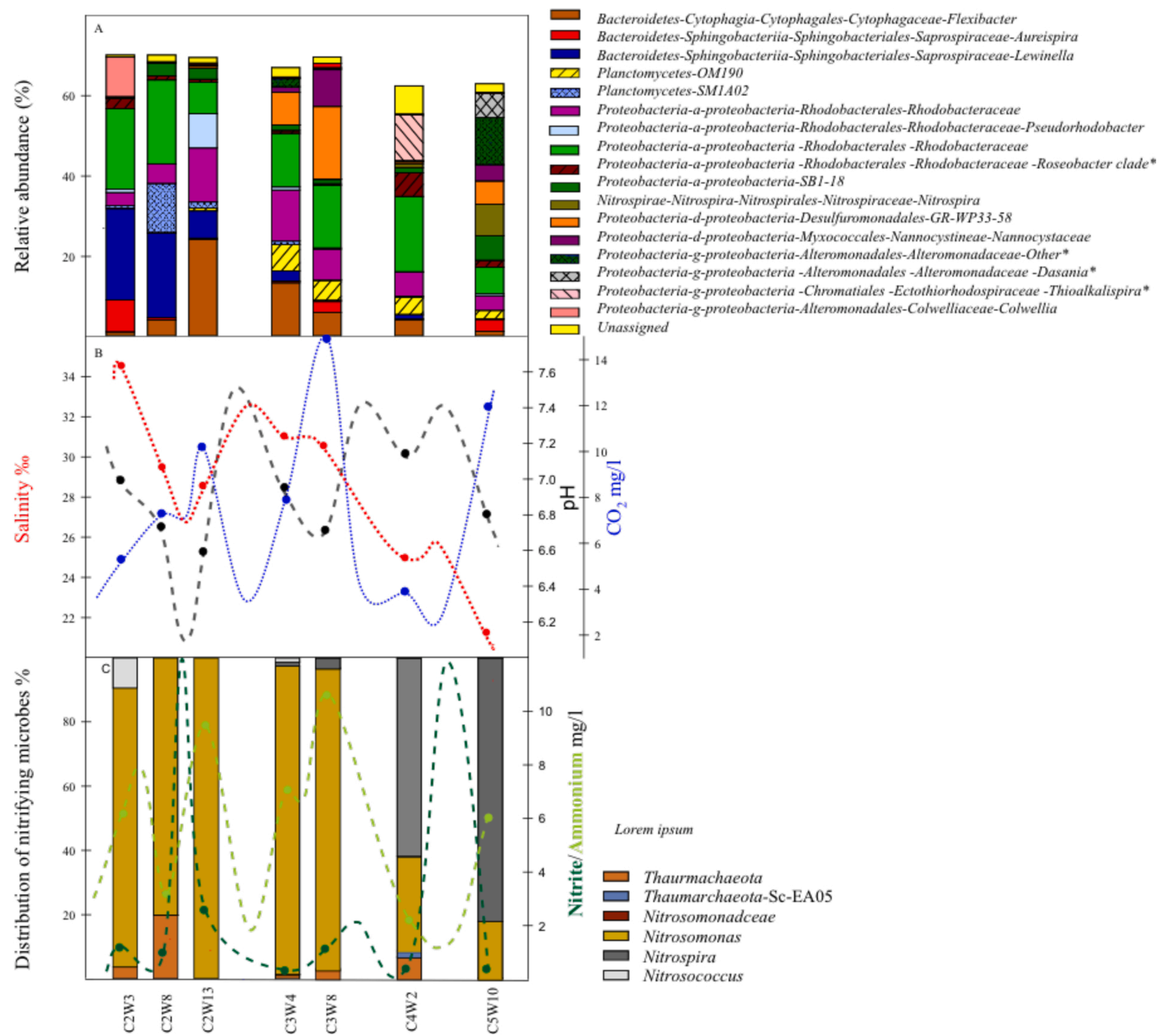

Fig. 6. Dominating and nitrifying bacteria on the biofilter carriers during changing physiochemical conditions. A; OTUs with relative abundance >5 % once or several times during production cycle 2–5. B; Changes in salinity, pH and CO_2. C; Nitrifying microbes and their relative population distribution along with nitrite and ammonium concentrations. Dots notes measured values the sampling day, and dashed line shows the trends between sapling times. *; OTUs with environmental origin (EO).

biofilms formed by others. Members of the *Bacteroidetes* are regarded as the biofilm secondary colonizers, attaching and growing upon extracellular polymeric substances (EPS), i.e. using accumulated polymers and the changed biofilm surface formed by the first colonists (Dang and Lovell, 2000; Zhang and Bishop, 2003). No known salmon pathogens affiliates to the *Rhodobacteriales* or α- *Proteobacteria* in general, and these bacteria are indeed wanted in fish rearing environments. In particular the genus *Roseobacter* has been used for probiotic purposes in aquaculture, and reported as a common member of RAS biofilters biofilm (Frans et al., 2011; Rurangwa and Verdegem, 2015). Members of *Bacteroidetes* are associated with native marine fish species, and alternation between environment and animal gut has been noted (Thomas et al., 2011; Egerton et al., 2018). However, a biofilter-gut relation has still not been investigated nor proven for these bacteria in RAS. Differently from the *Rhodobacteriales*, several fish pathogens affiliates to the *Bacteroidetes*, e.g. the genus *Tenacibaculum* (Austin and Austin, 2007). However, most common fish pathogens affiliates to the γ-*Proteobateria* e.g. *Vibrio*, *Aeromonas*, *Pasteurella, Klebsiella* and *Yersinia*, but also members of the genera *Pseudoalteromonas* and *Shewanella* within the order of *Alteromonadales* (Austin and Austin, 2007). Only a few γ-*Proteobacteria* colonized the RAS biofilter biofilm, except the members within *Alteromonadales*. Dang et al. suggested that the development of *Proteobacteria* were suppressed by *Bacteroidetes* members during primary colonization, and that *Alteromonadales* species succeeded because of high production of external polysaccharides (Raguenes et al., 1996). Thus, over all, the starter cultured microbes formed a colonization of the biofilter carrier surface that lowered the potential establishment of pathogens, this despite the high salinities used during the colonization.

Also OTUs classified as *Planctomycetes* were defined on the biofilter biofilm carriers at the first time of sampling, a taxonomic group whose members has only recently been recognized as early colonists of seawater surfaces in general (Abed et al., 2019; Dang and Lovell, 2016). Genome studies of these slow growing bacteria have shown the presence of adherence properties (Andrei et al., 2019), and they are often observed in RAS and biofilms from industrial sewage facilities (Rurangwa and Verdegem, 2015; Nascimento et al., 2018; Suto et al., 2017). In this context they are believed to perform anaerobic ammonium oxidation (anammox), i.e., oxidation of ammonium to nitrogen by nitrite. Whether they do this process in RAS is unclear. Interestingly, *Planctomycetes* members were also common on the wall biofilm at the production tanks, suggesting also an aerobe function. In recent years the success of *Planctomycetes* species has been seen in the light of their

Fig. 7. Average relative abundance of shared OTUs defined in both biofilter biofilm carriers and production water at 8 sampling times. Standard deviation is shown with error bars. IAO; Inoculums associated OTUs, EO; Environmental OTUs.

production of a broad spectra of rare antibacterial substances, presumed to protect surfaces from overgrowth phototrophic bacteria (Jeske et al., 2016). RAS should thus be a good environment to do enrichment of novel *Planctomycetes* members and mining for new antibiotics.

Almost 15 months passed from the marine RAS startup until successful establishment of the second steps of nitrification by an *Nitrospira* taxon. Consequently, this long time of maturation of the biofilter represented a high-risk period for accumulation of nitrite in the production water and reduced fish welfare (Roalkvam et al., 2020). Why biofilter maturation times seems to be longer in sea water than fresh water is not fully understood, but in our case (paper in preparation) the establishment of *Nitrospira* could be linked to a period with low salinity (<25‰) in combination with activity from a micro-predator from *Myxococcales* within the biofilter biofilm (Keane and Berleman, 2016; Shimkets, 1990). So far, no study has properly addressed the nitrifying potential in RAS at high salinities, but the marine RAS in study solved this biological obstacle by lowering the operational salinity to (<25‰). Large scale salmon production in RAS above this salinity is not common today, and also, a salinity of 12‰ is documented to give the optimal growth conditions for the fish (Ytrestøyl et al., 2020).

4.2. Stability in OTUs with high relative abundance

The more often OTUs were identified over time, the higher were their average relative abundances (Fig. 5). Dominant early colonists with >> 5 % relative abundance decreased over time on the biofilter biofilm carriers and increasingly faced competition from several other dominant OTUs (Fig. 6), which is an observation in line with previous biofilm modeling (Woodcock and Sloan, 2017). A full-fledged maturation of the biofilter was not observed, and the long-time stability of the biofilter microbiome needs further investigation to describe the dynamics beyond the period of predicted mechanisms of colonization. More puzzling was the observed long-time stability of *Colwellia* in the production water. This taxon was not a direct indicator of good water quality, as it also dominated while a severe skin wound outbreak ravaged the RAS in the second production cycle. *Colwellia* strains growing in biofilm has previously been associated with nitrate reduction (Bowman et al., 1998; Schwermer et al., 2008), but are lately also shown to degrade alkanes in oil plume (Mason et al., 2014) and alginate in giant kelp (Lin et al., 2018). As alginate is strongly associated to colonizing biofilm bacteria, it can very well be that *Colwellia* utilize complex carbon compounds in the biofilm matrix. Particle studies in RAS water has shown that organic particles from biofilm are the dominating particles in the water after the biofilter step (Rosche, 2014). If the *Colwellia* population is stabilized by a sustained substrate of organic matter from the biofilter, it could be of great importance for the holistic thinking about RAS and should be investigated further. It was supportive to the seclusion theory that the abundance pattern or the parallel offset of dominant biofilter biofilm bacteria was observed back in the production water (Fig. 3 in co-submitted Data in Brief article, Drønen et al., 2021). Such a mirroring was also seen when comparing omniscient shared OTUs between biofilm and water (Fig. 6).

4.3. OTUflow and instability in OTUs with low relative abundance

A large portion of the OTUs defined in RAS was flowing in and out between sampling times, and we aim here to evaluate the suggested quantitative approach of OTUflow. OTUflow and changes in total OTUs numbers are related units, but OTUflow illuminates much stronger instability in the microbial community than changes in total OTUs numbers, and following, of greater interest with respect to RAS microbiome stability modeling and the evaluation of pathogens detection and dissemination potential. However, the obtained information takes also strongly into evaluation the issues of resolution in data and difficulties in separating biological and methodical observations.

When it comes to resolution, the use of replicate samples enhanced the resolution and more OTUs were defined in pooled samples from sampling sites where the depth of sequencing otherwise was low for individual replicates. More precisely, each of three replicate samples from biofilter biofilm carriers defined 25 % unique OTUs in this study. Furthermore, the unique OTUs were largely associated with low relative abundance, with a "cut-off" for detection in parallel biofilter samples at < 0.015 %. As the cut-off value was the same for IAO and EO, unique sequences in replicates must have been true positives, and thus represent OTUs on the border of the detection limit for our methods.

An upcoming question is then how many replicate samples are enough or how many reads are a minimum to recognize a new pathogen

introduced to the system, as an early warning. To address this and other PCR related issues, we suggest that a mock DNA community sample, i.e., DNA from a microbial community with known composition, is developed for the RAS system in question and is later included in PCR-based methods and down-stream analyses. Another suggestion is that the OTUflow is quantified both for total number of OTUs define and for OTUs above the cut-off value. The ratio between these will be a new index that can be compared towards the mock sample as reference. A third suggestion is that the OTUflow is estimated also vertically, between categories of relative abundance.

The flow of OTUs was higher in percent among the EO than the IAO, both in the biofilter biofilm and over all in the RAS. Thus, the OTUflow brought valuable information upon rare OTUs intrusion to RAS, represented mainly by EO entering the RAS by the dilution water at low abundances, but also the intrusion by re-inoculating with new biofilter carriers. Interesting in this context were the high abundancy EO defined only once in biofilter biofilm and the production water, and the increase in number of re-appearing OTUs with time (Figs. 2 and 4). The frequency of sampling will influence the detection ratio of OTUs on the border of the detection limit, but the increased percentage of re-appearing OTUs suggest that more microbes established in the RAS above the detection limit by time. A master thesis shows that the longer an OTU was present in the production water, the larger the chance that the OTU also were defined in the biofilter biofilm later on (Drønen, 2019). One-time OTUs were thus probably randomly detected without the potential of further establishment, and may explain why they were defined equally between the biofilter biofilm and the production water. That the OTUflow was larger in the biofilter biofilm than in RAS as an entity, suggest that the habitats were different in who they established, and probably also that biofilm was more difficult to colonize than the water. The accumulating EO with low relative abundant was also seen for the tank wall biofilm, but even stronger in the production water. Notably, the accumulation of EO often increased in opposite phase in the biofilter biofilm and the production water (Fig. 4 in Drønen et al., 2021).

The structural changes in the RAS microbial community as effect by RAS events are discussed in a paper in preparation. Here, we clarify closer whether registered changes in OTUflow from time to time can be interpreted as biological or methodical events in the RAS, especially adherence and extinction processes on the biofilter biofilm. Fig. 3 presents the functional relationship between the two quantitative; generated OTUs and reads in the biofilter biofilm carriers replicate samples. The deviation from this relation was pronounced at sampling time C3W8 when the biofilter biofilm was dominated by a bacterium performing bacterial micro-predation by cell lysis, and a clear decrease in OTUflow and total OTUs number was registered at this sampling time (Figs. 2 and 3). Opposite, the high net OTUflow in to the biofilter biofilm upon the wash and re-inoculation events at sampling time WashI did also have deviation from the expected relation as depicted in Fig. 3. However, most notably during the wash and re-inoculation was the adherence of several bacteria closly related to known fish pathogens, which appeared with low abundances in the bioifilter biofilm (paper in preparation). Thus, at time points with either extraordinarily high OTUflow in or out, it could be associated with important RAS events and the sampling times were registered as unique by the modelled functional OTU to read relationship. We believe a further development of the OTUflow approach can help RAS microbes risk modelling and pathogens warning or being a valuable information contributor for machine learning approaches that predict microbial stability and RAS events. Overall, OTUflow can bring in quantitative "backgrounds" information that is not absorbed by diversity indexes based on total OTUs numbers in samples, or by Bray Curtis distance matrix analysis between sampling times.

4.4. Evaluating the inoculum

The microbial consortium in the starter culture originated from brackish water, but demonstrated good colonization properties also in Atlantic coastal water. OTUs defined from the inoculum were prominent in both biofilms and the production water during the year of monitoring, and out of the 139 OTUs identified in the inoculum 118 OTUs were identified once or more in the RAS. Two culture members, *Lewinella* and *Aureispira*, were among the first to colonize the biofilter biofilm carriers, and who's characterized relatives are known to grow by ixotrophy. This growth mechanism predates bacteria selectively, and prevented probably the growth of *Vibrio*'s and *Myxococcales* on the biofilm carriers when abundant (Furusawa et al., 2015). However, *Myxococcales* most likely had an important role in biofilm stabilization and the establishment of the nitrite oxidation process by *Nitrospira*. This process might have been established earlier at lower salinity from the beginning. Furthermore, it prompts the need for biomining new nitrite oxidizing bacteria with better colonization properties and functionality at high salinity. Possibly, it would be an advantage if *Desulfuromonadels*, that reduces sulfur, was replaced with sulfur oxidizing bacteria in the starter culture. It is also possible that the *Myxococcales* members in the starter culture were sources for off flavor compounds in the production water (Lukassen et al., 2019).

4.5. Summary

Here we investigated the microbiome development during four early production cycles in a marine post-smolt RAS in Norway. Prior to operation, the biofilter biofilm carriers were inoculated with a commercial start-culture developed from brackish water. Early colonizing OTUs that dominated biofilter biofilm carriers (> 5% relative abundance) were stably present over time, but the development went slowly from a few OTUs with very high relative abundance to several dominant ones with lower relative abundance. Operating taxonomic units not associated with the starting culture became prominent on the biofilter biofilm carriers only towards the end of the trial period. The second step of the nitrification process did not establish properly before the salinity in the RAS was lowered to <25‰. This was 15 months after the first fish stocking in the RAS. Our study demonstrates the potential of long term biofilter monitoring for evaluation of inoculum performance and for a better understanding of microbial population dynamics in RAS.

Author Contributions

KD did sampling at the location, performed DNA extractions and amplicon library preparations, did the ecological analyses and interpretation of data, and wrote the manuscript. IR did sampling at the location, was involved in the interpretation of the data, and revised the manuscript. HD provided bioinformatics tools for sorting, cleaning, and taxonomical classification of the amplicon reads, and also helped in the analysis and interpretation of the data, and revised the manuscript. HN, AO and, HW revised the manuscript.

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Ethics Statement

The study used fish collected from ordinary production cycles at the fish farm, which are not under the act of animal ethic legislation concerning use of animals in Norway. Therefore, no ethical committee is required. Investigated fish were killed according to the Norwegian law, as described above.

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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References

Abed, R.M.M., Al Fahdi, D., Muthukrishnan, T., 2019. Short-term succession of marine microbial fouling communities and the identification of primary and secondary colonizers. Biofouling 1–15. https://doi.org/10.1080/08927014.2019.1622004.

Anderson, M.J., 2017. Permutational Multivariate Analysis of Variance (PERMANOVA). Wiley StatsRef: Statistics Reference Online. John Wiley & Sons, Ltd. https://doi.org/10.1002/9781118445112.stat07841.

Andrei, A.-Ş., Salcher, M.M., Mehrshad, M., Rychtecký, P., Znachor, P., Ghai, R., 2019. Niche-directed evolution modulates genome architecture in freshwater Planctomycetes. ISME J. 13, 1056–1071. https://doi.org/10.1038/s41396-018-0332-5.

Attramadal, K.J.K., Salvesen, I., Xue, R., Øie, G., Størseth, T.R., Vadstein, O., Olsen, Y., 2012. Recirculation as a possible microbial control strategy in the production of marine larvae. J. Aquac. Eng. Fish. Res. 46, 27–39. https://doi.org/10.1016/j.aquaeng.2011.10.003.

Attramadal, K.J.K., Truong, T.M.H., Bakke, I., Skjermo, J., Olsen, Y., Vadstein, O., 2014. RAS and microbial maturation as tools for K-selection of microbial communities improve survival in cod larvae. Aquaculture 432, 483–490. https://doi.org/10.1016/j.aquaculture.2014.05.052.

Austin, B., Austin, D.A., 2007. Bacterial Fish Pathogens : Disease of Farmed and Wild Fish. Springer.

Avecom, 2019. Avecom - Steering and Optimizing Your Microbial Processes [WWW Document]. URL. www.avecom.be.

Bartelme, R.P., Smith, M.C., Sepulveda-Villet, O.J., Newton, R.J., 2019. Component microenvironments and system biogeography structure microorganism distributions in recirculating aquaculture and aquaponic systems. mSphere 4. https://doi.org/10.1128/msphere.00143-19.

Berry, D., Ben Mahfoudh, K., Wagner, M., Loy, A., 2011. Barcoded primers used in multiplex amplicon pyrosequencing bias amplification. Appl. Environ. Microbiol. 77, 7846–7849. https://doi.org/10.1128/AEM.05220-11.

Bowman, J.P., Gosink, J.J., McCammon, S.A., Lewis, T.E., Nichols, D.S., Nichols, P.D., SKERRATT, J.H., Staley, J.T., McMeekin, T.A., 1998. Colwellia demingiae sp. nov., Colwellia hornerae sp. nov., Colwellia rossensis sp. nov. and Colwellia psychrotropica sp. nov.: psychrophilic Antarctic species with the ability to synthesize docosahexaenoic acid (22: 63). Int. J. Syst. Bacteriol. 48, 1171–1180. https://doi.org/10.1099/00207713-48-4-1171.

Brailo, M., Schreier, H.J., McDonald, R., Maršić-Lučić, J., Gavrilović, A., Pećarević, M., Jug-Dujaković, J., 2019. Bacterial community analysis of marine recirculating aquaculture system bioreactors for complete nitrogen removal established from a commercial inoculum. Aquaculture 503, 198–206. https://doi.org/10.1016/j.aquaculture.2018.12.078.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., et al., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336. https://doi.org/10.1038/nmeth.f.303.

Costea, P.I., Zeller, G., Sunagawa, S., Pelletier, E., Alberti, A., Levenez, F., Tramontano, M., Driessen, M., Hercog, R., Jung, F.E., et al., 2017. Towards standards for human fecal sample processing in metagenomic studies. Nat. Biotechnol. 35, 1069–1076.

Dang, H., Lovell, C.R., 2000. Bacterial primary colonization and early succession on surfaces in marine waters as determined by amplified rRNA gene restriction analysis and sequence analysis of 16S rRNA genes. Appl. Environ. Microbiol. 66, 467–475.

Dang, H., Lovell, C.R., 2016. Microbial surface colonization and biofilm development in marine environments. Microbiol. Mol. Biol. Rev. 80, 91–138. https://doi.org/10.1128/MMBR.00037-15.

Drønen, K., 2019. Microbial Colonization and Community Shifts in a Marine Post-smolt RAS during the First Year of Operation As Analyzed by Deep Sequencing. Master thesis. University of Bergen.

Drønen, Karine, 2020. Physiochemical data from marine post-smolt RAS 2016-2017. Mendeley Data V3. https://doi.org/10.17632/zrmjktk992.3 [dataset].

Drønen, K., Roalkvam, I., Dahle, H., Olsen, A.B., Nilsen, H., Wergeland, H., 2021. Microbiome dataset from a marine recirculating aquaculture system (RAS) for salmon post-smolt production in Norway. Data Br. Submitt. Aquac. Rep.

Dzianach, P.A., Dykes, G.A., Strachan, N.J.C., Forbes, K.J., Pérez-Reche, F.J., 2019. Challenges of biofilm control and utilization: lessons from mathematical modelling. J. R. Soc. Interface. https://doi.org/10.1098/rsif.2019.0042.

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460–2461. https://doi.org/10.1093/bioinformatics/btq461.

Edgar, R.C., 2013. UPARSE: highly accurate OTUs sequences from microbial amplicon reads. Nat. Methods 10, 996–998. https://doi.org/10.1038/nmeth.2604.

Egerton, S., Culloty, S., Whooley, J., Stanton, C., Ross, R.P., 2018. The gut microbiota of marine fish. Front. Microbiol. 9, 873. https://doi.org/10.3389/fmicb.2018.00873.

Fossmark, R.O., Vadstein, O., Rosten, T.W., Bakke, I., Košeto, D., Bugten, A.V., Helberg, G.A., Nesje, J., Jørgensen, N.O.G., Raspati, G., Azrague, K., Østerhus, S.W., Attramadal, K.J.K., 2020. Effects of reduced organic matter loading through membrane filtration on the microbial community dynamics in recirculating aquaculture systems (RAS) with Atlantic salmon parr (Salmo salar). Aquaculture. https://doi.org/10.1016/j.aquaculture.2020.735268.

Frans, I., Michiels, C.W., Bossier, P., Willems, Ka., Lievens, B., Rediers, H., 2011. Vibrio anguillarum as a fish pathogen: virulence factors, diagnosis and prevention. J. Fish Dis. 34, 643–661. https://doi.org/10.1111/j.1365-2761.2011.01279.x.

Furusawa, G., Hartzell, P.L., Navaratnam, V., 2015. Calcium is required for ixotrophy of Aureispira sp. CCB-QB1. Microbiol. (United Kingdom) 161, 1933–1941. https://doi.org/10.1099/mic.0.000158.

Garrett, T.R., Bhakoo, M., Zhang, Z., 2008. Bacterial adhesion and biofilms on surfaces. Prog. Nat. Sci. 18, 1049–1056. https://doi.org/10.1016/j.pnsc.2008.04.001.

Gloor, G.B., Wu, J.R., Pawlowsky-Glahn, V., Egozcue, J.J., 2016. It's all relative: analyzing microbiome data as compositions. Ann. Epidemiol. https://doi.org/10.1016/j.annepidem.2016.03.003.

Gonzalez-Silva, B.M., Jonassen, K.R., Bakke, I., Østgaard, K., Vadstein, O., 2021. Understanding structure/function relationships in nitrifying microbial communities after cross-transfer between freshwater and seawater. Sci. Rep. 11, 2979. https://doi.org/10.1038/s41598-021-82272-7.

Gorelick, R., 2011. Commentary: do we have a consistent terminology for species diversity? The fallacy of true diversity. Oecologia 167, 885–888. https://doi.org/10.1007/s00442-011-2124-8.

Hughes, J.B., Hellmann, J.J., Ricketts, T.H., Bohannan, B.J.M., 2001. Counting the uncountable: statistical approaches to estimating microbial diversity. Appl. Environ. Microbiol. https://doi.org/10.1128/AEM.67.10.4399-4406.2001.

Jeske, O., Surup, F., Ketteniß, M., Rast, P., Förster, B., Jogler, M., Wink, J., Jogler, C., 2016. Developing techniques for the utilization of Planctomycetes as producers of bioactive molecules. Front. Microbiol. 7, 1242. https://doi.org/10.3389/fmicb.2016.01242.

Keane, R., Berleman, J., 2016. The predatory life cycle of Myxococcus xanthus. Microbiol. 162, 1–11. https://doi.org/10.1099/mic.0.000208.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 41, e1. https://doi.org/10.1093/nar/gks808.

Lin, J.D., Lemay, M.A., Parfrey, L.W., 2018. Diverse bacteria utilize alginate within the microbiome of the giant kelp Macrocystis pyrifera. Front. Microbiol. 9, 1914. https://doi.org/10.3389/fmicb.2018.01914.

Lukassen, M.B., de Jonge, N., Bjerregaard, S.M., Podduturi, R., Jørgensen, N.O.G., Petersen, M.A., David, G.S., da Silva, R.J., Nielsen, J.L., 2019. Microbial production of the off-flavor geosmin in tilapia production in Brazilian water reservoirs: importance of bacteria in the intestine and other fish-associated environments. Front. Microbiol. 10, 2447. https://doi.org/10.3389/fmicb.2019.02447.

Ma, Y., Du, X., Liu, Y., Zhang, T., Wang, Y., Zhang, S., 2020. Characterization of the bacterial communities associated with biofilters in two full-scale recirculating aquaculture systems. Chin. J. Oceanol. Limnol. https://doi.org/10.1007/s00343-020-0120-8.

Martins, P., Cleary, D.F.R., Pires, A.C.C., Rodrigues, A.M., Quintino, V., Calado, R., Gomes, N.C.M., 2013. Molecular analysis of bacterial communities and detection of potential pathogens in a recirculating aquaculture system for Scophthalmus maximus and Solea senegalensis. PLoS One. https://doi.org/10.1371/journal.pone.0080847.

Mason, O.U., Han, J., Woyke, T., Jansson, J.K., 2014. Single-cell genomics reveals features of a Colwellia species that was dominant during the deepwater horizon oil spill. Front. Microbiol. 5, 332. https://doi.org/10.3389/fmicb.2014.00332.

Mekuchi, M., Asakura, T., Kikuchi, J., 2019. New aquaculture technology based on host-symbiotic Co-metabolism. In: Gojobori, T., Wada, T., Kobayashi, T., Mineta, K. (Eds.), Marine Metagenomics. Springer, Singapore. https://doi.org/10.1007/978-981-13-8134-8_13.

Menanteau-Ledouble, S., Gonçalves, R.A., El-Matbouli, M., 2020. Feed supplementation with a commercially available probiotic solution does not alter the composition of the microbiome in the biofilters of recirculating aquaculture systems. Pathogens 9, 830. https://doi.org/10.3390/pathogens9100830.

Minich, J.J., Poore, G.D., Jantawongsri, K., Johnston, C., Bowie, K., Bowman, J., Knight, R., Nowak, B., Allen, E.E., 2020. Microbial ecology of atlantic salmon (Salmo salar) hatcheries: impacts of the built environment on fish mucosal microbiota. Appl. Environ. Microbiol. 86 https://doi.org/10.1128/AEM.00411-20.

Minniti, G., Hagen, L.H., Porcellato, D., Jørgensen, S.M., Pope, P.B., Vaaje-Kolstad, G., 2017. The skin-mucus microbial community of farmed Atlantic salmon (Salmo salar). Front. Microbiol. 8, 2043. https://doi.org/10.3389/fmicb.2017.02043.

Nascimento, A.L., Souza, A.J., Andrade, P.A.M., Andreote, F.D., Coscione, A.R., Oliveira, F.C., Regitano, J.B., 2018. Sewage sludge microbial structures and relations to their sources, treatments, and chemical attributes. Front. Microbiol. 9, 1462. https://doi.org/10.3389/fmicb.2018.01462.

Norwegian Food Safety Authority (Mattilsynet), 2016. Aquaculture Operation Regulation. https://www.mattilsynet.no/om_mattilsynet/gjeldende_regelverk/forskrifter/forskrift_om_drift_av_akvakulturanlegg_akvakulturdriftsforskriften.616. www.lovdata.no.

Øvreås, L., Forney, L., Daae, F.L., Torsvik, V., 1997. Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Appl. Environ. Microbiol. 63, 3367–3373.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Wagner, H., 2015. Vegan: community ecology package. R Package Vegan, Vers. 2.2-1.

Perry, W.B., Lindsay, E., Payne, C.J., Brodie, C., Kazlauskaite, R., 2020. The role of the gut microbiome in sustainable teleost aquaculture. Proc. R. Soc. B Biol. Sci. https://doi.org/10.1098/rspb.2020.0184.

Raguenes, G., Pignet, P., Gauthier, G., Peres, A., Christen, R., Rougeaux, H., Barbier, G., Guezennec, J., 1996. Description of a new polymer-secreting bacterium from a deep-sea hydrothermal vent, Alteromonas macleodii subsp. fijiensis, and preliminary characterization of the polymer. Appl. Environ. Microbiol. 62, 67–73. https://doi.org/10.1128/aem.62.1.67-73.1996.

Rittmann, B.E., McCarty, P.L., 1980. Model of steady-state-biofilm kinetics. Biotechnol. Bioeng. 22, 2343–2357. https://doi.org/10.1002/bit.260221110.

Roalkvam, I., Drønen, K., Dahle, H., Wergeland, H.I., 2019. Microbial communities in a flow-through fish farm for lumpfish (Cyclopterus lumpus L.) during healthy rearing conditions. Front. Microbiol. 10.

Roalkvam, I., Drønen, K., Dahle, H., Wergeland, H.I., 2020. A case study of biofilter activation and microbial nitrification in a marine recirculation aquaculture system for rearing Atlantic salmon (Salmo salar L.). Aquac. Res. https://doi.org/10.1111/are.14872.

Rosche, T., 2014. Particle Size Distribution of Suspended Solids in a Commercial Recirculating Aquaculture System. Master thesis. Norwegian University of Life Science.

Ruan, Y.J., Guo, X.S., Ye, Z.Y., Liu, Y., Zhu, S.M., 2015. Bacterial community analysis of different sections of a biofilter in a full-scale marine recirculating aquaculture system. N. Am. J. Aquac. 77, 318–326. https://doi.org/10.1080/15222055.2015.1017128.

Rud, I., Kolarevic, J., Holan, A.B., Berget, I., Calabrese, S., Terjesen, B.F., 2017. Deep-sequencing of the bacterial microbiota in commercial-scale recirculating and semi-closed aquaculture systems for Atlantic salmon post-smolt production. J. Aquac. Eng. Fish. Res. 78, 50–62. https://doi.org/10.1016/j.aquaeng.2016.10.003.

Rurangwa, E., Verdegem, M.C.J., 2015. Microorganisms in recirculating aquaculture systems and their management. Rev. Aquac. 7, 117–130. https://doi.org/10.1111/raq.12057.

Sauer, U., Heinemann, M., Zamboni, N., 2007. Getting Closer to the Whole Picture Downloaded From 316. https://doi.org/10.1126/science.1142502.

Schmidt, V., Amaral-Zettler, L., Davidson, J., Summerfelt, S., Good, C., 2016. Influence of fishmeal-free diets on microbial communities in atlantic salmon (Salmo Salar) recirculation aquaculture systems. Appl. Environ. Microbiol. 82, 4470–4481. https://doi.org/10.1128/AEM.00902-16.

Schwermer, C.U., Lavik, G., Abed, R.M.M., Dunsmore, B., Ferdelman, T.G., Stoodley, P., Gieseke, A., de Beer, D., 2008. Impact of nitrate on the structure and function of bacterial biofilm communities in pipelines used for injection of seawater into oil fields. Appl. Environ. Microbiol. 74, 2841–2851. https://doi.org/10.1128/AEM.02027-07.

Shimkets, L.J., 1990. Social and developmental biology of the myxobacteria. Microbiol. Rev. 54, 473–501.

Sinha, R., Abu-Ali, G., Vogtmann, E., Fodor, A.A., Ren, B., Amir, A., Schwager, E., Crabtree, J., Ma, S., Abnet, C.C., et al., 2017. Assessment of variation in microbial community amplicon sequencing by the Microbiome Quality Control (MBQC) project consortium. Nat. Biotechnol. 35, 1077–1086.

Skjermo, J., Salvesen, I., Øie, G., Olsen, Y., Vadstein, O., 1997. Microbially matured water: a technique for selection of a non-opportunistic bacterial flora in water that may improve performance of marine larvae. Aquac. Int. 5, 13–28. https://doi.org/10.1007/BF02764784.

Steiner, K., Heasman, K., Laroche, O., Pochon, X., Preece, M., Bowman, J.P., Walker, S.P., Symonds, J.E., 2021. The microbiome of Chinook salmon (Oncorhynchus tshawytscha) in a recirculation aquaculture system. Aquaculture 534, 736227. https://doi.org/10.1016/j.aquaculture.2020.736227.

Suto, R., Ishimoto, C., Chikyu, M., Aihara, Y., Matsumoto, T., Uenishi, H., Yasuda, T., Fukumoto, Y., Waki, M., 2017. Anammox biofilm in activated sludge swine wastewater treatment plants. Chemosphere 167, 300–307. https://doi.org/10.1016/J.CHEMOSPHERE.2016.09.121.

Thomas, F., Hehemann, J.H., Rebuffet, E., Czjzek, M., Michel, G., 2011. Environmental and gut Bacteroidetes: the food connection. Front. Microbiol. 2, 93. https://doi.org/10.3389/fmicb.2011.00093.

Torrent, I., 2012. Ion torrent. Torrent, Ion 8–10. https://doi.org/10.7171/jbt.12.

Wang, J., Jaramillo-Torres, A., Li, Y., Kortner, T.M., Gajardo, K., Brevik, Ø.J., Jakobsen, J.V., Krogdahl, Å., 2021. Microbiota in intestinal digesta of Atlantic salmon (Salmo salar), observed from late freshwater stage until one year in seawater, and effects of functional ingredients: a case study from a commercial sized research site in the Arctic region. Anim. Microbiome 3, 14. https://doi.org/10.1186/s42523-021-00075-7.

Weigel, B.L., Erwin, P.M., 2016. Intraspecific variation in microbial symbiont communities of the sun sponge, Hymeniacidon heliophila, from intertidal and subtidal habitats. Appl. Environ. Microbiol. 82, 650–658. https://doi.org/10.1128/AEM.02980-15.

Weiss, S., Xu, Z., Amir, A., Peddada, S., Bittinger, K., Gonzalez, A., Lozupone, C., Zaneveld, J., Vazquez-Baeza, Y., Birmingham, A., Knight, R., 2015. Effects of library size variance, sparsity, and compositionality on the analysis of microbiome data. PeerJ Prepr. https://doi.org/10.7287/peerj.preprints.1157.

Willis, A.D., 2019. Rarefaction, alpha Diversity, and statistics. Front. Microbiol. 10, 2407. https://doi.org/10.3389/fmicb.2019.02407.

Woodcock, S., Sloan, W.T., 2017. Biofilm community succession: a neutral perspective. Microbiology 163, 664–668. https://doi.org/10.1099/mic.0.000472.

Ytrestøyl, T., Takle, H., Kolarevic, J., Calabrese, S., Timmerhaus, G., Rosseland, B.O., Teien, H.C., Nilsen, T.O., Handeland, S.O., Stefansson, S.O., Ebbesson, L.O.E., Terjesen, B.F., 2020. Performance and welfare of Atlantic salmon, Salmo salar L. post-smolts in recirculating aquaculture systems: Importance of salinity and water velocity. J. World Aquac. Soc. 51, 373–392. https://doi.org/10.1111/jwas.12682.

Zaheer, R., Noyes, N., Ortega Polo, R., et al., 2018. Impact of sequencing depth on the characterization of the microbiome and resistome. Sci. Rep. 8, 5890. https://doi.org/10.1038/s41598-018-24280-8.

Zhang, X., Bishop, Paul, 2003. Biodegradability of biofilm extracellular polymeric substances. Chemosphere 50, 63–69. https://doi.org/10.1016/S0045-6535(02)00319-3.