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Deep-sequencing of the bacterial microbiota in commercial-scale recirculating and semi-closed aquaculture systems for Atlantic salmon post-smolt production

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ABSTRACT

New aquaculture production systems are evolving for prolong production of Atlantic salmon smolts or post-smolts before stocking in traditional net pens, such as semi-closed containment systems (S-CCS) in sea (Fig. 1) and recirculating aquaculture systems (RAS) on land. The microbiota in these systems can potentially have great impact on the robustness and health of the fish. These two types of aquaculture systems are likely to have different challenges regarding pathogenic invasion due to the different water management, e.g. different treatment of the intake water and different turnover of the water. In this study, we investigated the bacterial microbiota of both water and biofilms in a commercial RAS and in S-CCS in sea during a three months period of post-smolt production. Deep-sequencing of the bacterial 16S rRNA gene (V4) was used for the first time to obtain in depth compositional analysis of microbial communities in commercial scale facilities. Highly diverse communities were detected, with up to 2000 different Operational Taxonomic Units (OTUs) within samples. Both systems were dominated by Proteobacteria with Rhodobacteraceae as the dominating taxa, followed by Bacteroidetes that was dominated by *Polaribacter* among others. However, the microbiota composition was clearly different between the two aquaculture systems, and between water samples and biofilms. In RAS, it was also shown different microbiota composition with water salinity of 12 vs 22 parts per thousand (ppt). Higher abundance of e.g. Myxococcales and Nitrospiraceae was observed at 12 ppt, which coincided with lower total ammonia nitrogen (TAN) levels. Both taxa were also more abundant in the Moving Bed Bioreactor (MBBR)-biofilms than in water, as well as *Planctomyces* among others. In S-CCS, clear temporal changes of the microbiota was observed during the production, where potential pathogens like *Tenacibaculum*, *Aliivibrio*, Alteromonadaceae and *Polaribacter* were increasing in the spring time, as well as one unassigned taxa and chloroplast DNA likely from algae. The implication of these potential pathogens on fish health is unknown. A common observation for both RAS and S-CCS was higher abundance of the potential pathogens in the water compared to the biofilms. Further studies on the microbiota in closed-containment aquaculture systems are needed to obtain more knowledge about their impact on post-smolt production performance, welfare and health.

1. Introduction

New aquaculture production systems are evolving such as production of salmon post-smolts in floating semi-closed containment systems (S-CCS) in sea (Fig. 1) or on land in recirculating aquaculture systems (RAS) (Iversen et al., 2013; Terjesen et al., 2012, 2013). These new production methods for the post-smolt stage of Atlantic salmon have evolved as a response to challenges in open sea cage production, such as sea-lice infestations, escapees, mortalities and infections from pathogenic microorganisms. The microbiota in these closed-containment systems can still have a large impact on the robustness and health of the fish, and the farming industry experiences challenges with disease outbreaks and limited control of these events. Increased knowledge of the

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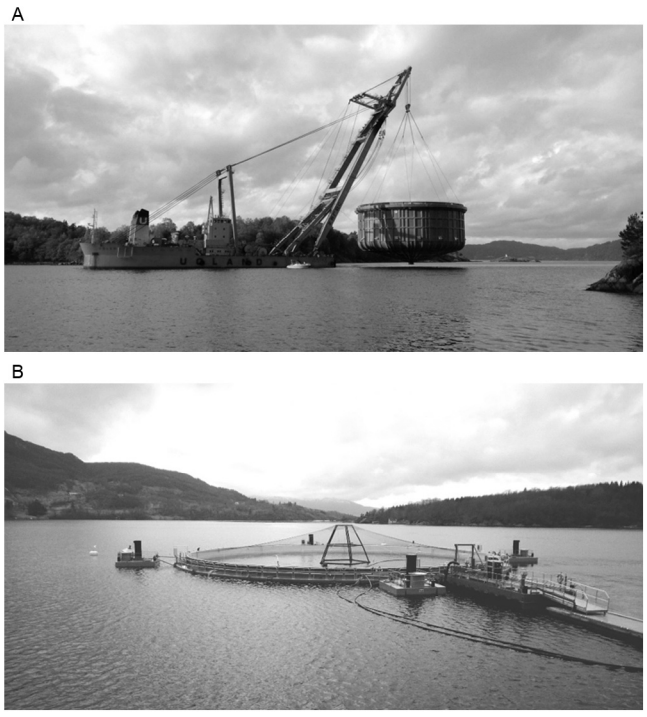

Fig. 1. Commercial-scale semi-closed containment system (S-CCS) of prototype Neptun during transfer (A) and finally floating and operating in Molnes sund, Norway (B). Photos: AquaFarm Equipment AS and Nofima.

microbial communities within the systems in terms of composition, reservoirs, dynamics, functionality and effects of important environmental parameters (e.g. intake water, salinity, feed input, retention time, treatment compartments etc.) would be useful for improved operational design and sustainable farming.

Deep-sequencing for characterization of the microbiota in aquaculture systems is an emerging high-throughput technology that allows for rapid, cost effective, in-depth taxonomic characterization of microbial communities including unprecedented bacteria (Caporaso et al., 2012; Metzker, 2010). This method allows for detection of thousands of bacteria within each sample. It can be used for monitoring the microbiota over time (e.g. days, weeks, months or years) in aquaculture systems and to identify a core microbiota that can be used as reference for future studies, as well as presence of potential pathogens Currently, the technology has been limited to a few studies in RAS (Martins et al., 2013; Ruan et al., 2015). Most studies of microbiota in RAS (reviewed by Blancheton et al., 2013; Rurangwa and Verdegem, 2015; Schreier et al., 2010) are based on either traditional microbiological techniques, targeted molecular methods (e.g. real-time polymerase chain reaction (PCR)) or molecular fingerprinting analysis (e.g. PCR-denaturing gradient gel electrophoresis (DGGE)).

Microbial control within RAS and S-CCS is different due to their different operational design and water management; however, a common challenge is to achieve beneficial microbial communities within the systems, which depends on the supply of bacterial nutrients, organic matter, water exchange and environmental conditions (Attramadal et al., 2012; De Schryver and Vadstein, 2014; Schreier et al., 2010).

Especially in RAS, any disturbance of the natural microbial balance might induce r-selection within the system causing growth of opportunistic pathogens already present as part of the aquatic microbiota (Blancheton et al., 2013). For instance elimination of waste products is critical in RAS, and the biofilter microbiota is an important component in RAS where the ammonia are nitrified by autotrophs into nitrate by ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). The heterotrophic bacteria degrade organic matter, and contribute to maintaining good microbial water quality by occupying niches and preventing proliferation of potential pathogenic species, though they also are competitors for oxygen and space with autotrophic bacteria (Blancheton et al., 2013). Limited outbreaks of pathogens within RAS has been reported (Bornø and Linaker, 2015), even though potential pathogens have been detected in RAS (King et al., 2004; Martins et al., 2013; Michaud et al., 2009).

S-CCS in sea are built as flow-through systems, pumping water from a certain depth to optimize rearing temperature and to avoid surface waters, where the abundance of sea lice is the highest. Due to the high turnover rate of the water and seasonal changes, the microbial community in S-CCS will be exposed to new environmental conditions over time, e.g. changes in temperature and light and an unstable organic load and nutrient profile. Another concern could be the potential of getting upwelling of sediments from the seabed into the system, induced by pumping high volumes of water. From other studies, we know that marine sediments in the sea can harbor pathogenic species (e.g. *Vibrio* spp.) (Blackwell and Oliver, 2008; Shikuma and Hadfield, 2010) and *Moritella viscosa* (Colwell and Morita, 1964; Urakawa et al., 1998). Furthermore, there will always be periodically occurrence of pathogens in the sea due to e.g. bacterial or algal blooms. These pathogens could find their way into the system through the intake water such as the winter-ulcer causing pathogens. *M. viscosa* is regarded as the etiological agent (Lovoll et al., 2009; Lunder et al., 1995), but the etiology of ulcerative outbreaks is likely complex as numerous bacterial species are associated with the diseases, including *Tenacibaculum* spp and *Aliivibrio wodanis* (Bornø and Linaker, 2015; Olsen et al., 2011). Though the route of transmission is likely the intake water or introduced fish, there is limited knowledge about the reservoir of pathogens in aquaculture systems. Microbial biofilms developing in aquaculture tanks and biofilters might represent a reservoir for opportunistic pathogens. They may provide pathogens with survival advantages by providing protection against environmental stressors, such as disinfectants and antibiotics (Sanchez-Vizuete et al., 2015).

The objective of this study was to use state-of-the-art sequencing technology to obtain a preliminary overview of the complex microbiota within land based RAS and floating S-CCS in the sea. The study was done during commercial scale productions of Atlantic salmon post-smolts, since limited knowledge exists on the microbiota in such systems. To the best of our knowledge this is the first finding on microbiota in S-CCS in general. Comparison of microbiota in the water and in biofilms were performed to identify the biofilm associated and the free-living bacteria, and try to identify the main reservoir of potential pathogens in these two aquaculture systems. In addition, relevant system-specific factors were studied. In RAS the effect of salinity on the microbiota was investigated, whereas temporal effects were investigated in S-CCS.

2. Materials and methods

2.1. Description of experimental facilities

2.1.1. Recirculating aquaculture system (RAS)

The experiment was done at the commercial-scale Grieg Seafood facility in Adamselv, Lebesby municipality (Finmark, Norway). Atlantic salmon smolts used in this experiment were hatched at the Grieg Seafood hatchery in Adamselv, and smolts were produced in freshwater RAS (FW-RAS) at the same location, but in a different system. A total of 200,000 smolts with an average starting weight of 89.3 ± 13.8 g (SD) were transferred after smoltification to two separate brackish (BK)-RAS at 8 ppt. The smolts were

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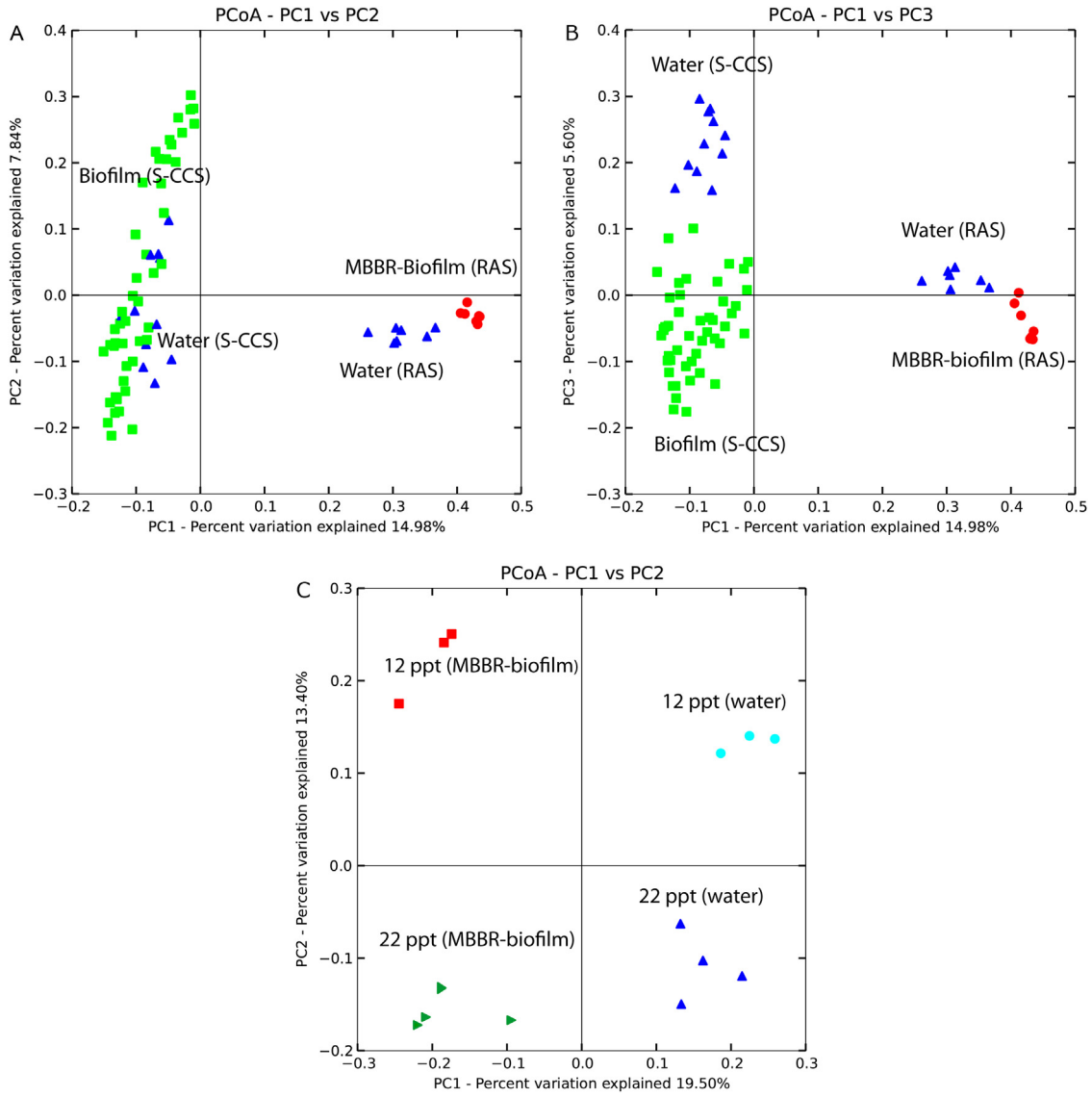

Fig. 2. Microbiota diversity in biofilms and water of RAS and S-CCS presented in PCA plots. (A) Diversity comparison between S-CCS, RAS, biofilms and water with PC1 and PC2, (B) Diversity comparison between S-CCS, RAS, biofilms and water with PC1 and PC3, and (C) Diversity comparison between MBBR-biofilm and water samples from 12 ppt and 22 ppt within RAS (PC1 and PC2).

stocked in two 400 m^3 tanks per system prior to the start of the experiment.

Each RAS consisted of three parallel mechanical drum filters (Hydrotech, Vellinge, Sweeden) with 40 μm pore size for particle removal, followed by a Clearwater Moving bed bioreactor (MBBR) (Inter Aqua Advance, Egå, Denmark; 500 m^3 in volume) for removal of toxic NH_3/NH_4^+ and NO_2, after which the water was oxygenated and returned to the culture tanks. A total of four tanks, two with rearing capacity of 400 m^3 and two with 700 m^3, received recirculating water after treatment in each system. The CO_2 degassing occurred in the bioreactor, in addition to in-tank degassing. At the start of the experiment (July–August 2013), the water salinity in one of the RAS was gradually increased from 8 parts per thousand (ppt) to 12 ppt, and in the other from 8 ppt to 22 ppt over a period of one week. The operational parameters for both BK-RAS are given in Table 1. Total ammonia nitrogen (TAN) was measured daily using Multiparameter bench photometer for aquaculture (HANNA Instruments, Woonsocket, Rhode Island, USA). An additional group of fish was introduced equally into the remaining tanks of both BK-RAS after 43 and 47 days of the experiment, as a part of the planned commercial production at the Adamselv facility. However, this group of fish was, unknown at the time of the experiment, infected by the bacterial pathogen *Yersinia ruckeri*, which was thus introduced to both RAS.

2.1.2. Floating semi-closed containment system (S-CCS)

This study was part of the first test of the commercial-scale floating semi-closed containment system (S-CCS)-prototype Neptun produced by AquaFarm Equipment AS (Haugesund, Norway) (Fig. 1). This system is constructed of glass-fibre reinforced plastic (GRP) and is certified according to NS9415:2009. The sidewalls are coated with Norpol gel and topcoat (Reichold, Durham, NC, USA) and the bottom is coated with a Büfa standard gel and top coat (Büfa, Rastede, Germany). The prototype was located for the trial in the Molnes sund (N 59°43.191 E5°51.528), at the Southwestern coast of Norway, and the system has a circular shape with a circumference of 126 m. The bottom of the tank is angled down toward the center outlet pipe giving a total tank depth of 20 m and a gross volume of 21,000 m^3. Sea water (32 ppt) was pumped into the prototype from a fixed depth of 26 m by four pumps installed

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Table 1
Operational information for the 12 ppt and 22 ppt RAS during production of Atlantic salmon post-smolts. Given values are means ± SD.

System	Total feed load (kg/day)	Total water flow (m3/h)	Make-up water (m3/h)	Make-up water (m3/kg feed)	Degree recirculation (%)
12 ppt RAS	541 ± 292	1999 ± 720	24 ± 16	2 ± 1	98 ± 1
22 ppt RAS	412 ± 268	1768 ± 1004	25 ± 16	1 ± 1	97 ± 2

at four barges just outside the tank at each quadrant. The maximal combined flow rate in this system was 400 m^3/min. Oxygen was added to inflowing water in the intake pipes and was automatically adjusted to maintain a saturation above 80% in the tank. Most of the water (80%) was discharged through 14 side hatches (1 m × 1 m) at 12 m depth and 20% of the water through the bottom center pipe. When the pumps were operating at maximal capacity, the retention time of the water in the tank was 52 min. Additional information about the S-CCS prototype can be found in Summerfelt et al. (2016), farm location H.

A total of 200,000 Atlantic salmon post-smolts weighing approximately 118 gr (VAKI wellboat smolt counter) were transferred from the hatchery Vågafossen settefisk AS to the Neptun prototype. A standard commercial production protocol for post-smolts in sea water (Marine Harvest Norway AS) was followed. Dead fish were removed once a day using a LiftUP system (LiftUP, Eikelandsosen, Norway), counted and noticeable pathologies recorded. Temperature, oxygen (YSI pro20), pH (YSI pro 10) and salinity (TetraCon probe, VWR) were monitored daily at 1 m depth in the prototype. Tank walls, outlet and pumps were cleaned as needed by high-pressure washing and commercial divers.

2.2. Microbiota analysis

2.2.1. Sample collection

The biofilm and water samples for microbiota analysis were collected during three months of post-smolt production in either system. Microbial biofilms were observed attached to the sampling materials, though not quantified. In RAS, approximately 4–5 carrier discs from the Moving Bed Bioreactors (MBBRs) were collected per sample on three occasions during the experiment (2–3 samples per time of sampling, where 1–2 samples from each salinity treatment, in total 7 samples): at the start, after two and three months of the experiment, and frozen at −80 °C for subsequent analysis. The water samples of 100 ml each were collected at the tank outlet level after two and three months of the experiment (3–4 samples per time of sampling, where 1–2 samples from each salinity treatment, in total 7 samples), frozen immediately and was subsequently filtered using 0.2 μm membrane filters (Whatman, Dassel, Germany). Filters were fresh frozen and stored at −80 °C until analysis. In S-CCS, the samples were collected from start of the experiment (Day 0; February 2014) and every week until Day 70 followed by Day 91 (May 2014), except for one water sample missing on Day 63. Sterile dry wipes (32 cm × 39 cm, SodiBox®, Névez, France) were used to collect biofilm of tank walls (3–4 walls per sample date, in total 47 samples). The water samples (one sample per date, in total 12 samples) were collected at 1 m depth from S-CCS by soaking the sterile dry wipes with water. All the samples were frozen and stored at −80 °C until analysis.

2.2.2. DNA extraction

The collected samples were thawed on ice before preparation of microbial pellets. Peptone water was added to the wipes, filters and MBBR-discs before 2 × 1 min of stomaching to release microbial cells of the samples. Firstly, the microbial pellets were collected in falcon tubes by centrifugation for five minutes at 5000 × *g*. The clear supernatants were removed, while the remaining loose pellets underwent additional five minutes of centrifugation at 13,000 × *g* in Eppendorf tubes. The final pellets were subsequently used for DNA extraction. The FastDNA-96™ Fecal DNA Kit with Matrix E (MP Biomedicals, USA) was used for mechanical lysis, following the manufacture's protocol, including the MP-96 Inhibitor Removal Plate.

2.2.3. Deep-sequencing of the microbiota

The microbiota analysis was performed by deep-sequencing following our in-house protocol (Moen et al., 2016), which is in detail presented in supplementary methods of Caporaso et al. (2012). The deep-sequencing involves paired end sequencing (2 × 150 bp) of the variable region 4 (V4) of the bacterial 16S rRNA gene. The V4 contains conserved and hypervariable regions, which makes is suitable for taxonomic classifications. Briefly, polymerase chain reaction (PCR) was performed in triplicates per DNA sample with region-specific primers (targeting conserved regions) that included the Illumina flowcell adapter sequences. The latter permits binding to an Illumina flow cell during the sequencing process. The reverse amplification primer also contained a twelve base barcode sequence unique for each sample, which supports pooling of different samples. Before and after pooling the samples were purified with Ampure (Agencourt Bioscience Corporation) and quantified using the Quant-iT Picogreen ds DNA with picogreen (Invitrogen, Life Technologies). The sample pool was diluted to 4 nM, and sequenced on a MiSeq (Illumina) following the protocol provided by Illumina. The MiSeq run also contained a control library made from PhiX Control v3 that accounted for 10% of reads, which is recommended for increased sequencing yield and quality of low diversity samples (e.g. targeted amplicon libraries). The library quantification and sequencing were performed at Nofima. The MiSeq Control Software (MCS) version used was RTA 1.18.54.

2.2.4. Data processing of sequencing data

Data processing of the sequencing reads was performed using the open-source bioinformatics pipeline Quantitative Insight Into Microbial Ecology (QIIME) v.1.7. and v.1.8 (Caporaso et al., 2010). Briefly, the forward and reverse reads were joined and barcodes failed to assemble were removed. The sequences were demultiplexed into representative sample taqs and quality filtered allowing zero barcode errors and a quality score of 30 (Q30). Reads were assigned to their respective bacterial taxonomy (Operational Taxonomic Unit: OTU) by clustering them against the Greengenes reference sequence collection (gg_13_8) using a 97% similarity threshold. Reads that did not hit a sequence in the reference sequence collection were clustered *de novo*. Chimeric sequences were removed using ChimeraSlayer, and all OTUs that are observed fewer than 2 times were discarded. This resulted in an OTU table containing 52,288 different OTUs, and that was based on a total of 6,180,272 reads. Each of the different OTU identities (OTU id) represents a unique 16S rRNA gene sequence that are assigned to the nearest taxa, where the taxonomic level assigned can vary from phylum, class, order, family, genus and sometimes also species level. This means that several OTU ids can be assigned to the same taxa.

The OTU table was used for alpha diversity (within-sample diversity) and beta diversity analysis (between-sample diversity), using an equal number of sequences across samples, where the OTU table was resampled to an even depth of 27,000 sequences per sample. The alpha diversity was measured using the metrics Chao1

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(estimates species richness by counting numbers of rare OTUs), observed species (counts the number of OTUs), PD_whole_tree (quantitative measure of phylogenetic diversity). The OTU table was further split into one for each system before filtration, where only OTUs that passed the criteria of minimum count of 0.01% of the total sequences per system were kept. This filter was used in order to have a feasible number of OTUs as input for the taxonomic characterization. In total 633 and 607 OTUs passed this filter in RAS and S-CCS, respectively, and these OTU tables were further summarized into taxonomic summary tables for each of different taxonomic levels (i.e. phylum, class, order, family, genus). Taxonomic analysis was mainly performed at the phylum or genus level. Only OTUs at the genus level were used in the statistical analyses, and contained 176 OTUs for RAS and 141 OTUs for S-CCS.

A survey for a list of 15 potential pathogens was performed within the OTU tables at the genus level, which were selected based on the Norwegian Fish Health Report of 2014 (Bornø and Linaker, 2015) and involved *Tenacibaculum*, *Moritella*, *Vibrio*, *Aliivibrio*, *Flavobacterium*, *Yersinia*, *Aeromonas*, *Renibacterium*, *Piscirickettsia*, *Pseudomonas*, *Photobacterium*, *Alteromonas*, *Pseudoalteromonas*, *Psychrobacter* and *Polaribacter*. The bacteria were defined as potential pathogens since their genus or some of their species have been isolated from clinically diseased fish or are known as pathogens or opportunists. Nucleotide search within BLAST database of 16S ribosomal RNA sequences was performed with a 16S rRNA gene sequence corresponding with an OTUid of Enterobacteriaceae detected in RAS, in order to in investigate any similarities with *Yersinia*.

2.2.5. Statistical analysis

For S-CCS differences in alpha diversity (Chao1, observed species, PD_whole_tree) between biofilm and water, as well as temporal changes in the tank was tested using following regression model:

$$y = b_0 + b_1x_1 + b_2x_2 + e$$

In the above model, y is the measured alpha diversity, x1 the sample type (x1 = 1 is biofilm, and x1 = 0 is water) and x2 the number of days after the initiation of the experiment. The same model was applied on the selected OTUs defined as potential pathogens to test for differences between biofilm and water, as well as their temporal changes in S-CCS. For RAS, effect of salinity (12 vs 22 ppt) and sample type (biofilm vs water) on alpha diversity and potential pathogens was tested using two way ANOVA without interaction. Minitab (version 17.2.1) was applied for regression and ANOVA. Results of the alpha diversity analysis is shown with Chao1 metric (Chao, 1984), but similar results were also obtained with the other alpha diversity metrics.

Partial Least Squares Regression (PLSR, Martens and Martens, 2000) was used on the statistical analysis of the OTU tables at the genus level (Unscrambler X 10.3). PLSR is a tool for multivariate regression where one or more responses (Y) are modelled from a set of explanatory variables (X). A PLS model extract linear combinations of original variables that maximize the covariance between X and Y. More specifically linear combinations in X (OTUs) are used as predictors for the Y's (salinity/sample type). Usually a PLS model is interpreted using score plots, loading plots and by looking at regression coefficients. In order to look at stability of models, cross-validation are used to select the number of components to include in the model. Here the Y variables were categorical (salinity, 12/22 and sample type biofilm/water), which means that the regression model can be formulated as a classification problem. In other words, salinity and sample type were predicted from the OTU data, using PLSR with a categorical response. This approach is usually referred to as PLS Discriminant Analysis (PLS-DA, Barker and Rayens, 2003; Indahl et al., 2007; Wold et al., 2001).

PLS-DA with cross-validation and Jack-knifing (Martens and Martens, 2000) was applied to identify OTUs significantly different between biofilm and water within RAS and S-CCS, and between the two salinity levels in the RAS. PLS-DA was applied with biofilm/water as categorical responses in the first model, and 12/22 ppt in the second model. The significant OTUs (variables) with positive loadings on the first component will have higher abundance in the condition corresponding to biofilm or 22 ppt in the first and second model, respectively, whereas those with negative loadings will have higher abundance in the other condition.

PLSR was applied to investigate temporal chances of OTUs in the S-CCS system. In the first model, samples from both biofilm and water were included and the OTU data were corrected with respected to the sample type (mean of each sample type subtracted from raw data). In the second model, only data from biofilm was included. Cross-validation and Jack-knifing were applied to extract significant variables. OTUs identified significantly in the prediction model for the temporal variation was clustered using the function clustergram in bioinformatics toolbox of Matlab (release, 2013b) with standardised euclidean distance and wards linkage between the OTUs. The results are shown as heatmap diagrams.

3. Results

3.1. Microbiota diversity in water and biofilms of commercial-scale RAS and S-CCS

3.1.1. Beta diversity

The difference in microbiota composition between samples from RAS and S-CCS was analysed with beta diversity analysis (unweighted) and presented in Principal Component Analysis (PCA) plots (Fig. 2A and B). A clear separation of samples from S-CCS and RAS was seen along PC1, as well as some separation between water and MBBR-biofilm samples within RAS. PC2 was mainly separating samples within S-CCS (Fig. 2A). Along PC3 a clear separation was observed between water and biofilm samples (Fig. 2B) especially in S-CCS. Since PC1 explains 15%, PC2 7.8% and PC3 5.6% of the variance, this means that the difference between S-CCS and RAS samples were much larger than the difference within S-CCS or between water and biofilm samples. Beta diversity analysis was also performed on RAS samples to investigate the impact of salinity (12 ppt vs 22 ppt) on the microbiota (Fig. 2C). A clear separation between the microbiota from 12 ppt and 22 ppt samples was seen along PC2, while PC1 was separating samples from MBBR-biofilm and water.

3.1.2. Alpha diversity

The diversity of the microbiota within samples were investigated by performing alpha diversity analysis. Almost 2000 observed OTUs per sample were detected in both RAS and S-CCS. The alpha diversity was significantly higher in the biofilm samples compared to the water samples in both RAS and S-CCS (Chao1, $p < 0.001$). Regression analysis revealed temporal changes of the alpha diversity (Chao1, $p < 0.05$) in S-CCS (Fig. 3). No significant difference in alpha diversity between samples from 12 and 22 ppt in RAS was detected (data not shown).

3.2. Taxonomic characterization of microbiota in commercial-scale RAS and S-CCS

3.2.1. Overview of the dominating microbiota

The microbiota in RAS and S-CCS was characterized at phylum and genus level. The dominating OTUs at phylum (>0.1%) and genus (>1%) of RAS and S-CCS are presented in Figs. 4 and 5 , respectively.

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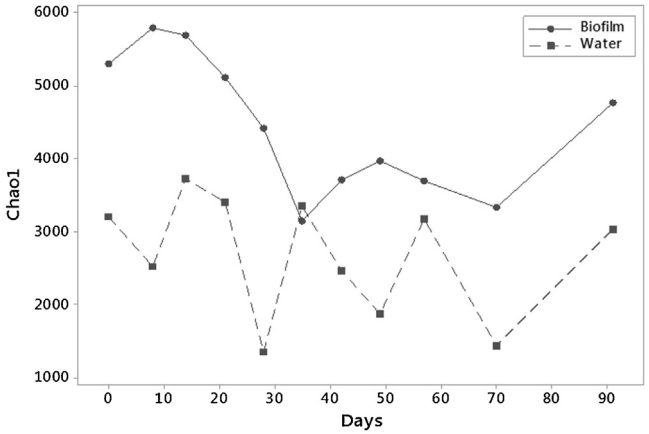

Fig. 3. Alpha diversity (Chao1) in biofilms and water at different time points within S-CCS.

In RAS, Proteobacteria and Bacteroidetes were the main dominating phyla accounting for 89% of the microbiota (Fig. 4A). Relevant for RAS was also the detection of Nitrospirae (0.4%). The dominating phyla in S-CCS are shown in Fig. 5A, with highest dominance of Proteobacteria, Bacteroidetes, Cyanobacteria, an Unassigned OTU and Verrucomicrobia (all >5%). Proteobacteria and Bacteroidetes accounted for 69% of the microbiota in S-CCS. Characterization at the genus level revealed many missing genera, and in this case the taxa at a higher level was reported (Figs. 4 B and 5 B). The corresponding phyla and order was also indicated in the figures. Characterization at the genus level in RAS resulted in only dominance of taxa within Proteobacteria and Bacteroidetes (Fig. 4B), which were dominated by one OTU of Rhodobacteraceae, accounting for 22% of the total microbiota, and Saprospiraceae (7%) and *Polaribacter* (5%), respectively. In S-CCS, the Proteobacteria was highly dominated by the same OTU of Rhodobacteraceae (12%) as within RAS, while *Polaribacter* (9%) was the dominating genera of the Bacteroidetes (Fig. 5B). An Unassigned OTU (6%) at the kingdom level was also dominating in S-CCS. Eight of the OTUs were common between RAS and S-CCS (Figs. 3 B and 4 B).

3.2.2. Differences in microbiota composition between biofilm and water

Differences in microbiota composition between biofilms and water was previously revealed with beta diversity analysis (Fig. 2). Partial Least Squares Discriminant Analysis (PLS-DA) was further applied to identify OTUs with significantly different abundance in biofilm versus water within both RAS and S-CCS, which resulted in total of 66 significant OTUs in RAS and 31 OTUs in S-CCS. The first

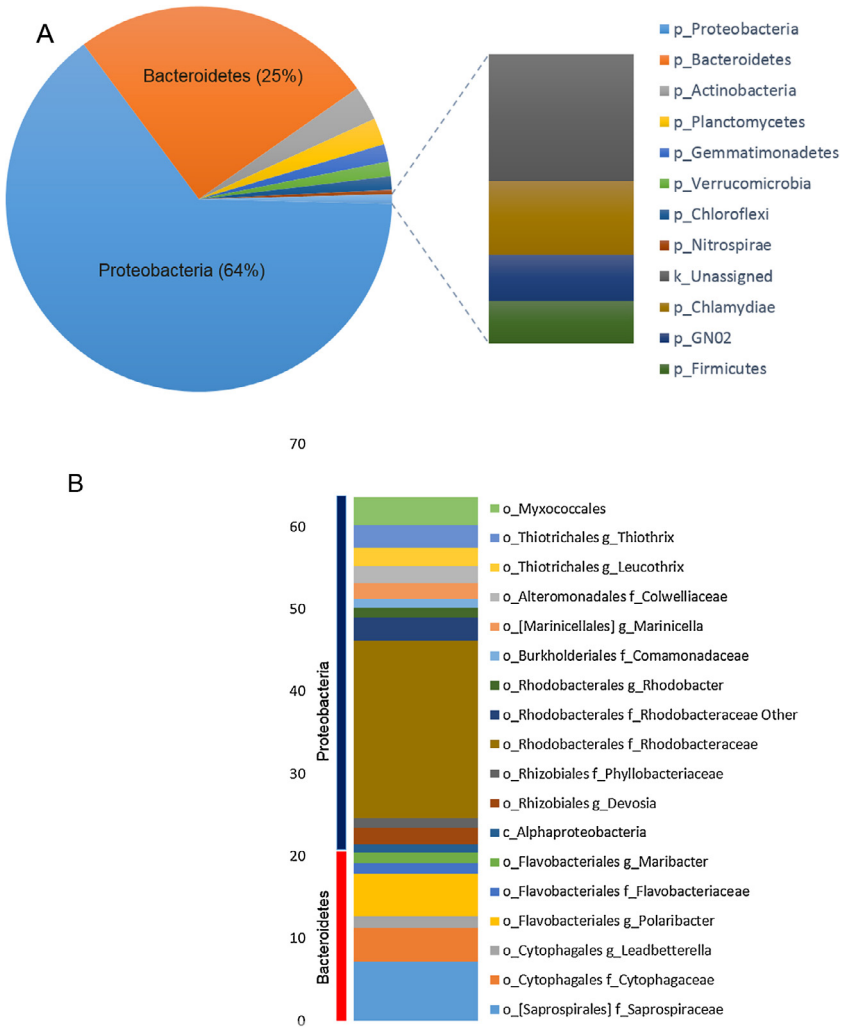

Fig. 4. Taxonomic assignment of dominating OTUs in RAS presented at phylum (A) and genus (B) level shown as percentage of the total microbiota. Data is based on the mean of all samples within RAS. Phyla OTUs > 0.1% and genera OTUs > 1% are shown. At the genus level, the corresponding phyla and order name is included, and when taxa name is missing the higher level of taxa is included. k, kingdom; p, phylum; c, class; o, order; f, family; g, genus.

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Table 2
OTUs significantly different between MBBR-biofilm and water in RAS when applying PLS-DA with full cross-validation and jackknifing[a].

Phylum	Class	Order	Family	Genus	MBBR-biofilm (%)	Water (%)
MBBR-biofilm						
Actinobacteria	Acidimicrobiia	Acidimicrobiales			0.779	0.179
			wb1_P06		0.870	0.138
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae		6.192	1.816
Chloroflexi	Anaerolineae	Caldilineales	Caldilineaceae		1.627	0.075
Planctomycetes	Planctomycetia	Pirellulales	Pirellulaceae		1.593	0.369
		Planctomycetales	Planctomycetaceae	Planctomyces	0.883	0.173
Proteobacteria	Alphaproteobacteria				1.837	0.186
		Kiloniellales	Kiloniellaceae		1.189	0.442
		Rhizobiales			1.752	0.125
			Hyphomicrobiaceae		1.318	0.243
				Devosia	3.236	0.847
			Phyllobacteriaceae	Mesorhizobium	0.619	0.074
		Rhodospirillales			0.545	0.003
	Betaproteobacteria	Burkholderiales	Comamonadaceae		1.426	0.757
	Deltaproteobacteria	Myxococcales			5.887	0.892
	Gammaproteobacteria	Alteromonadales	OM60		1.255	0.326
		Thiotrichales	Piscirickettsiaceae		0.515	0.119
		[Marinicellales]	[Marinicellaceae]	Marinicella	2.956	0.753
Water						
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Leadbetterella	0.638	2.151
	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Maribacter	0.440	2.039
				Polaribacter	1.999	8.380
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae		13.809	29.238
				Phaeobacter	0.310	0.703
	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Glaciecola	0.077	0.737

[a] OTUs where > 0.5% of mean within MBBR-biofilm samples or water samples are included.

Table 3
OTUs significantly different between biofilm and water in S-CCS when applying PLS-DA with full cross-validation and jackknifing[a].

Phylum	Class	Order	Family	Genus	Biofilm (%)	Water (%)
Biofilm						
Unassigned					7.110	4.380
Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Other	0.125	0.042
			Phyllobacteriaceae		1.382	0.573
		Rhodobacterales	Rhodobacteraceae	Other	6.282	2.006
			Rhodobacteraceae		16.089	8.210
				Loktanella	1.193	0.367
				Phaeobacter	1.426	0.454
				Pseudoruegeria	0.404	0.171
	Gammaproteobacteria	Thiohalorhabdales			0.394	0.160
Water						
Acidobacteria	[Chloracidobacteria]	RB41			0.001	0.130
Actinobacteria	Acidimicrobiia	Acidimicrobiales	OCS155		0.022	1.189
Bacteroidetes	Flavobacteriia	Flavobacteriales			0.065	0.179
			Cryomorphaceae		0.080	0.322
				Fluviicola	0.033	0.282
			NS9		0.005	0.119
Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Burkholderia	0.005	0.425
	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter	0.006	0.174
		Oceanospirillales			0.016	1.173
			Halomonadaceae	Candidatus Portiera	0.009	0.546
		Pseudomonadales	Pseudomonadaceae	Pseudomonas	0.002	0.141
		Xanthomonadales	Xanthomonadaceae		0.002	0.275

[a] OTUs where > 0.1% of mean within biofilm samples or water samples are included.

component explained 27% of the variation in RAS and 23% in S-CCS, hence much of the variability in the microbiota was also related to other factors. A representative of the significant OTUs are listed in Table 2 (RAS) and Table 3 (S-CCS), while all significant OTUs are listed in Supplementary material (Table S1 and Table S2).

The OTUs more abundant in the MBBR-biofilm in RAS were in several phyla, e.g. including Proteobacteria (dominated by order Rhizobiales, Myxococcales and Marinicellales), Bacteroidetes (Cytophagales) and Planctomycetes (Table 2). OTUs more abundant in the water in RAS were within Proteobacteria, including the dominating OTU of Rhodobacteraceae, and *Polaribacter* within Bacteroidetes. In S-CCS, the OTUs more abundant in the biofilm were within Proteobacteria, including the dominating OTU of Rhodobacteraceae and OTUs of Rhizobiales, as well as the Unassigned OTU (Table 3). The significant OTUs more abundant in the water than in the biofilm in S-CCS were relative low in abundance (Table 3).

3.2.3. *Salinity effects on the microbiota composition in RAS*

Differences in microbiota composition between salinity treatments, 12 and 22 ppt, in RAS was previously revealed by using beta diversity analysis (Fig. 2C), and PLS-DA was applied and identified 26 OTUs with significantly different abundance within RAS of either 12 or 22 ppt. The first component explained 16% of the variance, hence much of the variability in the microbiota was also related

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Table 4
OTUs significantly different between 12 and 22 ppt in RAS when applying PLS-DA with full cross-validation and jackknifing[a].

Phylum	Class	Order	Family	Genus	12 ppt (%)	22 ppt (%)
12 ppt						
Actinobacteria	Acidimicrobiia	Acidimicrobiales	SC3-41		0.948	0.047
Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae		0.867	0.003
Proteobacteria	Alphaproteobacteria	Rhodospirillales			0.631	0.006
		Rhizobiales	Phyllobacteriaceae		1.697	0.713
		Sphingomonadales			0.515	0.103
	Deltaproteobacteria	Myxococcales			6.221	1.266
22 ppt						
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae		1.947	5.547
				Leadbetterella	0.849	1.803
	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Ulvibacter	0.025	0.568
Proteobacteria	Alphaproteobacteria	Kiloniellales	Kiloniellaceae		0.449	1.091
		Rhizobiales	Phyllobacteriaceae	Other	0.322	1.022
		Rhodobacterales	Rhodobacteraceae	Other	1.193	4.146
				Loktanella	0.273	1.366
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae		0.144	1.322

[a] OTUs where > 0.5% of mean within 12 ppt or 22 ppt are included.

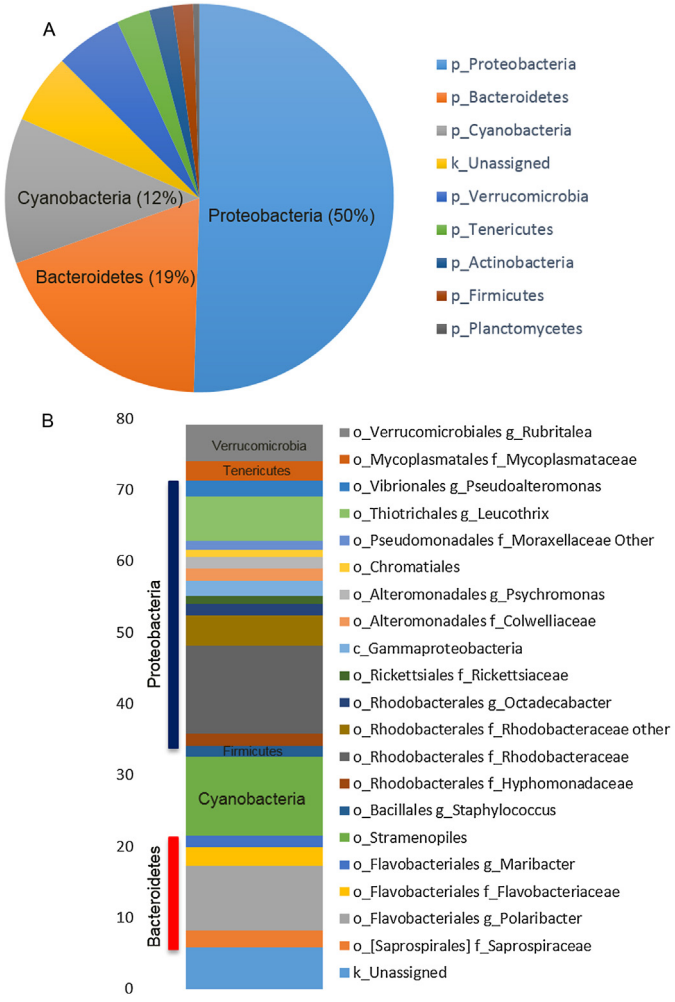

Fig. 5. Taxonomic assignment of dominating OTUs in S-CCS presented at phylum (A) and genus (B) level shown as percentage of the total microbiota. Data is based on the mean of all samples within S-CCS. Phyla OTUs > 0.1% and genera OTUs > 1% are shown. At the genus level, the corresponding phyla and order name is included, and when taxa name is missing the higher level of taxa is included. k, kingdom; p, phylum; c, class; o, order; f, family; g, genus.

to other factors. A representative of the significant OTUs are listed in Table 4, while all significant OTUs are listed in Supplementary material (Table S3). Myxococcales and Phyllobacteriaceae (order Rhizobiales) were the most dominating OTU that were significantly more abundant with 12 ppt. Also Nitrospiraceae was significantly more abundant with 12 ppt, while Cytophagaceae and Rhodobacteraceae (other) were two of the dominating OTUs more abundant with 22 ppt. The salinity might have had an effect on TAN since highest TAN was observed with 22 ppt (Supplementary material, Fig. S1A).

3.2.4. Time-dependent changes of the microbiota in S-CCS

Temporal changes of the microbiota composition in S-CCS was shown with PCA (Fig. 6A and B). A clear time-dependent change in the microbiota composition was observed especially within the biofilms during the 91 days of the experiment (Fig. 6A), whereas the trend was not that clear for the water samples (Fig. 6B). PLSR was applied to identify OTUs significantly changing over time, using both a combined data set of biofilm and water samples (where the first component explained 20% of the variation) and on biofilm samples (where the first component explained 32% of the variation). Hence, the latter model showed that time explained more of the microbiota variation in the biofilms than in water.

OTUs with a significant temporal change were clustered in heatmaps, where 11 significant OTUs common for biofilm and water samples are presented in Fig. 6C, while 33 OTUs significantly changing in the biofilm samples are presented in Fig. 6D. Two main clusters were generated, where Cluster A contained OTUs who's relative abundance decreased over time and Cluster B where the relative abundance increased over time (Fig. 6C and D). Common OTUs for Cluster A in both heatmaps were *Devosia* and Hyphomicrobiaceae (order Rhizobiales), SC3-56 (order Cytophagates) and Sphingomonadales. Common OTUs for Cluster B were Alteromonadaceae and *Aliivibrio*. Interestingly, *Tenacibaculum* was one of the significant OTUs in Cluster B with a common response for both biofilm and water (Fig. 6C). Most of the significant OTUs changing over time in biofilm were in Cluster A (Fig. 6D), including the dominating OTU of Rhodobacteriaceae. OTUs in Cluster B (Fig. 6D) included *Polaribacter*, *Aliivibrio*, Rhodobacterales and several OTUs in the order of Alteromonadales.

3.2.5. Potential pathogens in RAS and S-CCS

In RAS, four of the 15 potential pathogens were identified at the genus level (filtered at 0.01%), and included *Flavobacterium*, *Polaribacter*, *Pseudoalteromonas* and *Photobacterium*. The genus *Yersinia* was not detected in any of the samples from RAS, even though it was revealed that the introduced fish was contaminated by *Yersinia ruckeri*. However, an OTUid of its family Enterobacteriaceae was detected that was not resolved to any genus, and search within

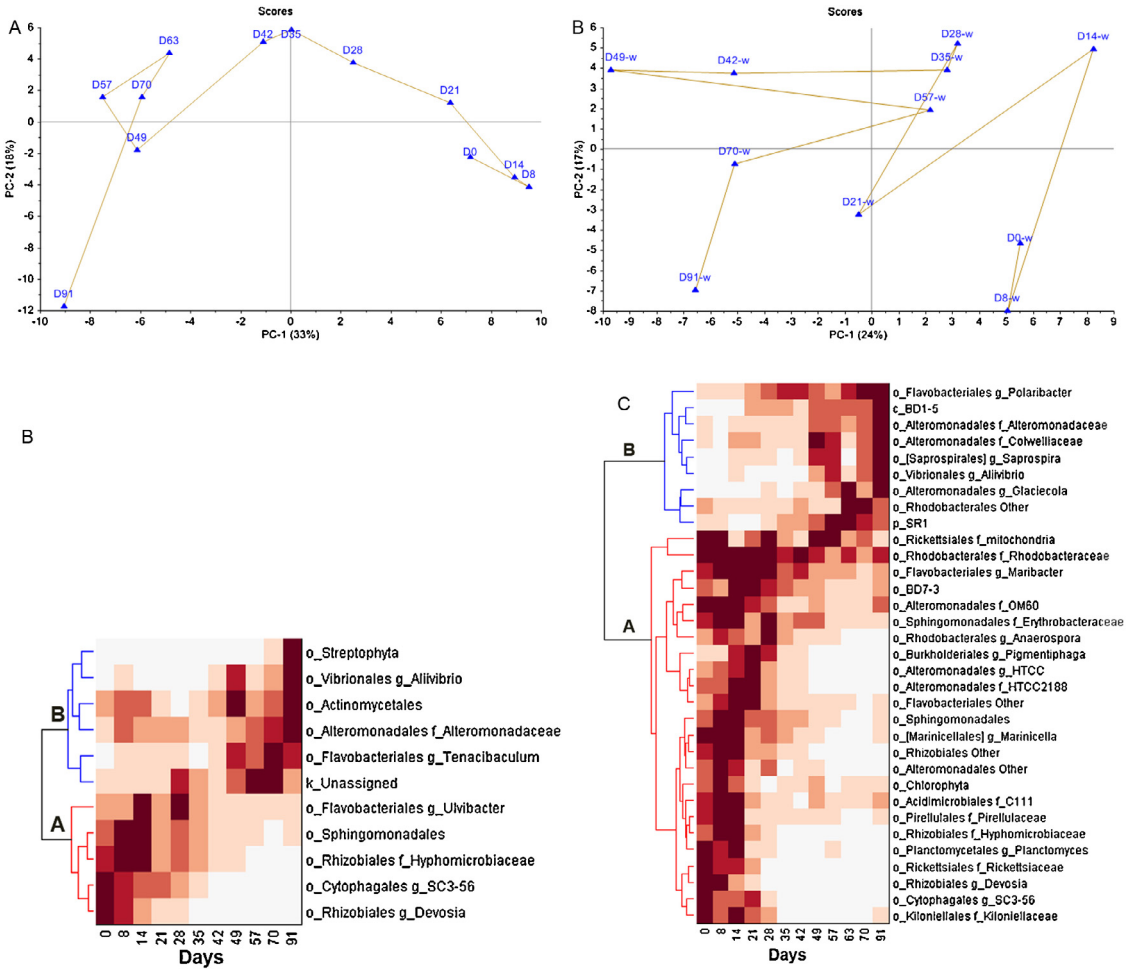

Fig. 6. Time-dependent changes of the microbiota in S-CCS during a period of 91 days. (A) PCA plot of biofilm OTU data over time (D = days), (B) PCA plot of water OTU data over time (D = days), (C) Heatmap of OTUs with significant change over time (days) for combined data of biofilm and water samples, (D) Heatmap of biofilm OTUs with significant change over time (days). k, kingdom; p, phylum; c, class; o, order; f, family; g, genus. Cluster A and B are indicated.

BLAST with the corresponding 16S rRNA gene sequence of this OTUid resulted in 100% hits against different species of *Yersinia* and *Serratia*, and with 99% identity match against one strain of *Yersinia ruckeri*. This OTU was detected mainly in the water after infected fish was introduced to the RAS after 2 months (Supplementary material, Fig. S1D), but was almost depleted one month later in the water of 22 ppt. *Polaribacter* was significantly higher in biofilm than water (also identified with PLS-DA, Table 2), whereas *Photobacterium* had higher abundance in the water samples (Fig. 7A). No significant difference was observed between the two RAS operated at different salinities with any of the four pathogens tested. In S-CCS, nine of 15 potential pathogens were identified at the genus level (filtered at 0.01%), and included *Flavobacterium*, *Polaribacter*, *Tenacibaculum*, *Moritella*, *Psychrobacter*, *Pseudomonas*, *Pseudoalteromonas*, *Aliivibrio* and *Photobacterium*. It has been shown by PLS-DA that *Pseudomonas* was significantly more abundant in the water of S-CCS (Supplementary Table S2), whereas PLSR in section 3.2.4 identified increase of several potential pathogens over time, including *Allivibrio*, *Tenacibaculum*, Alteromonadaceae (family of *Alteromonas*) and *Polaribacter*, and the latter significantly more abundant in the biofilm (Fig. 6C and D). Regression analysis of the nine potential pathogens confirmed these results, as well as decrease of Pseudomonas within the water (Fig. 7B). The four genera best explained by the regression model are shown in Fig. 7B, also showing significantly higher relative abundance of these potential pathogens in the water samples, except for *Polaribacter*.

4. Discussion

4.1. Overall microbiota differences between and within RAS and S-CCS

The microbiota in commercial-scale RAS and S-CCS during post-smolt production of Atlantic salmon was investigated by deep-sequencing to increase our knowledge of the complexity of the microbiota in new emerging closed-containment aquaculture systems for post-smolts. To our current knowledge, this is the first study on microbiota in RAS during post-smolt production of Atlantic salmon, and the first study of microbiota in S-CCS in general.

Deep-sequencing of bacterial 16S rRNA gene amplicons was the chosen method, allowing for in depth compositional analysis of these highly complex microbial communities. Indeed, the complexity was verified by detection of up to more than 50,000 different OTUs in total and almost 2000 unique species per sample in both RAS and S-CCS. As expected, the microbiota composition was different between the two aquaculture systems, likely due to a wide range of external factors, including major differences in technology, water source, water treatment, turnover-rate, fish introduction, seasonal climate variations etc. Thus, the observations in RAS and S-CCS was not meant for direct comparison, and the main scope was to get in-depth overview of the microbiota within each system.

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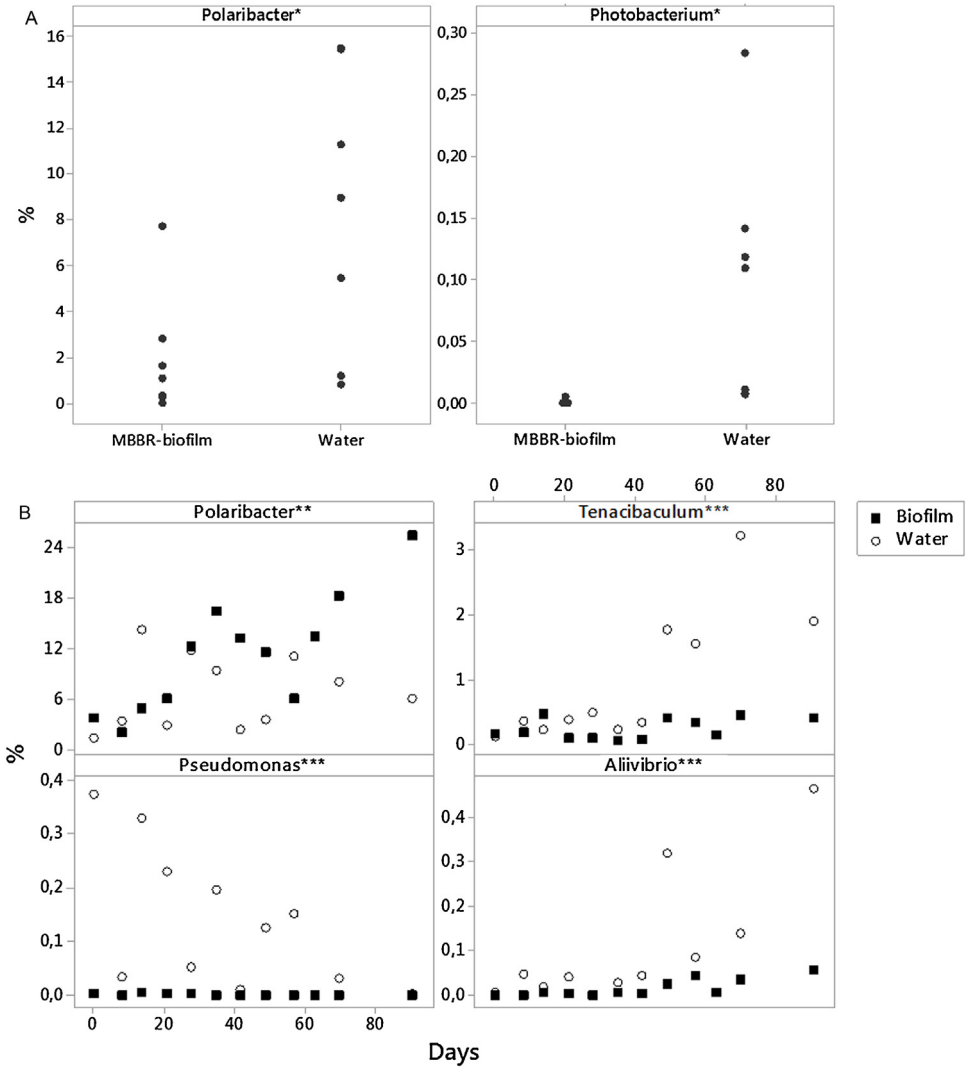

Fig. 7. Potential pathogens (%) in biofilm and water of RAS (A) and S-CCS (B) with significant differences between biofilm and water (*), significant different over time in terms of days (**), both significant differences between biofilm and water and over time (***).

In both RAS and in S-CCS, clear differences in microbiota between water and biofilms were observed in terms of diversity and composition, which reflects that many aquatic microorganisms are capable of colonizing surfaces, leading to formations of biofilms with specialized processes (Costerton et al., 1995; Petrova and Sauer, 2012). Other authors have also reported different microbiota composition attached to RAS biofilter than the free-living in the water phase of aquaculture systems (reviewed in Blancheton et al., 2013). Higher species diversity was detected in the biofilm samples of both RAS and S-CCS compared to the water in the present study, which has also been observed in an industrial recirculating cooling water system (Wang et al., 2013), thus indicating that heterogenic mature biofilms have been established in the systems.

4.2. *RAS and S-CCS were mainly dominated by taxa of Proteobacteria and Bacteroidetes*

Taxonomic assignment of the microbiota revealed more than 1000 OTUs at the genus level, thus the analysis allowed for identification of the more low abundant bacteria in contrast to the more traditional typing methodologies based on 16S rRNA sequencing, e.g. DGGE. A general limitation with 16S rRNA gene taxonomy is that the gene has limited resolution of taxonomy among closely related species, and we experienced especially limitations of taxonomic resolution at the genus level when sequencing the V4 region of the 16S rRNA gene. The next-generation sequencing techniques are generally limited by short read lengths obtained; however, longer sequence reads are currently available (Buermans and den Dunnen, 2014), and will probably expand in the future improving the taxonomic resolution.

The dominating phyla of both RAS and S-CCS were Proteobacteria followed by Bacteroidetes. Both phyla are frequently accounted as dominating in aquatic ecosystems, e.g. in biofilter of marine RAS (Ruan et al., 2015), in water of a RAS for the production of turbot and sole (Martins et al., 2013), in an anaerobic sludge of a wastewater treatment plant (Shu et al., 2015), and in the South Sea of Korea (Suh et al., 2015). The Proteobacteria is a highly diverse phenotypic and phylogenetic lineage (Kersters et al., 2006), and especially one OTU of Rhodobacteraceae was dominating in both RAS and S-CCS in our study. The Rhodobacteraceae are aquatic bacteria that frequently thrive in marine environments and contains of 100 genera (Pujalte et al., 2014), and has been shown to be highly present as heterotrophs in a marine RAS (Michaud et al., 2009). It is likely to assume that this OTU represents bacteria within the Roseobacter clade, a lineage of Rhodobacteriace shown to be among the dominating bacteria in the ocean, comprising up to 20%

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of the microbial community and abundant as both free-living and as attached to particles, cells or surfaces (Buchan et al., 2005; Dang and Lovell, 2016). The Bacteroidetes contains members dominating the marine heterotrophic bacterioplanktons (Kirchman, 2002). They are assumed to be specialized in degradation of complex polymers and have preferences for growth attached to particles, surfaces or algal cells, confirmed by genome sequences of marine Bacteroidetes (Fernandez-Gomez et al., 2013). In both RAS and S-CCS, *Polaribacter* was the most dominating OTU of the Bacteroidetes. This is in agreement with a study of Bacteroidetes across the North Atlantic Ocean, which showed *Polaribacter* to be the most abundant genera of the Bacteroidetes (Gomez-Pereira et al., 2010). *Polaribacter* has recently also been shown to be among the dominating bacteria in RAS compartments and biofilters (Martins et al., 2013; Ruan et al., 2015).

4.3. Microbiota in MBBR-biofilm affected by salinity

Bacteria are known to have different sensitivity to salinity (Herlemann et al., 2011), and here we have shown that salinity also influenced the microbiota in RAS. Changes in salinity in a RAS result also in changes in nitrification capacity (e.g. Nijhof and Bovendeur, 1990); likely a consequence of varying activity of the original nitrifying species present, but also due to qualitative changes to the microbial species composition. Indeed, the levels of TAN in the present study might have been affected by salinity (lowest TAN with 12 ppt), indicating impact on the nitrification efficiency, reported both for trickling biofilters and submerged fixed bed bioreactor (Nijhof and Bovendeur, 1990; Cortes-Lorenzo et al., 2014). This was in agreement with that Nitrospiraceae, likely the nitrite oxidizer in the system, was more abundant with 12 ppt and with highest abundance within the MBBR-biofilm, though almost depleted at 22 ppt in both MBBR-biofilm and the water (Supplementary material, Fig. S1B). Nitrosomonaceae, likely the ammonia oxidizer in the system, was also more abundant in the MBBR-biofilm at 12 ppt (Supplementary material, Fig. S1B), though not significantly different between the two salinities and between MBBR-biofilm and water, because of high presence also in the water at 22 ppt. The present findings, coupled to previous results (e.g. Nijhof and Bovendeur, 1990) has practical implications. Any required change in salinity in this range (12–22 ppt) should be gradual, and the RAS system load be controlled or reduced for a period, as a consequence of reduced nitrification and for allowing the new microbiota to stabilize, including any new nitrifying species. Other investigators have also reported sensitivity to salinity within ammonia and nitrite oxidizing bacteria, where the nitrite oxidizing bacteria were shown to be more sensitive (Bassin et al., 2011; Cortes-Lorenzo et al., 2014; Moussa et al., 2006; Zhao et al., 2014). Myxococcales was among the dominating taxa in RAS, and more abundant with 12 ppt and in the MBBR-biofilm. Though Myxococcales are considered soil bacteria with low tolerance for salt (Reichenbach, 1999), halophilic and halotolerant myxobacteria have been isolated from marine environments, e.g. Jiang et al. (2010). Members of Myxococcales have also been associated with production of off-flavours like geosmin (Dickschat et al., 2005, 2007; Schulz et al., 2004), and also with geosmin production in a RAS (Auffret et al., 2013).

A wide range of different bacteria were shown to be significantly more abundant within the MBBR-biofilm, many likely with specialized capabilities to attach, colonize and survive on surfaces. Movement over surfaces by gliding motility is for instance common within members of the highly abundant Myxococcales and Cytophagaceae (Kaiser, 2003; McBride et al., 2014). *Planctomyces* was also significantly more abundant within the MBBR-biofilm, also previously identified in RAS biofilters (Lahav et al., 2009; Ruan et al., 2015; Tal et al., 2003, 2006), suggesting a role for anammox in the system. Indeed, many species of Planctomycetes grow attached to surfaces via a holdfast anchored at the tip of a long stalk (Ward et al., 2006), and reproduce by budding (Fuerst and Sagulenko, 2011). This is also common for other taxa dominating the MBBR-biofilm, including Rhizobiales, its family Hyphomicrobiaceae and Pirellulaceae (Brown et al., 2012; Oren and Xu, 2014; Ward et al., 2006). The Hyphomicrobiaceae, as well as Pirellulaceae, *Marinicella*, Chloroflexi and Actinobacteria that also were more abundant within the MBBR-biofilms have previously been identified in marine and freshwater nitrification filters (Martins et al., 2013; Michaud et al., 2009; Ruan et al., 2015; Sugita et al., 2005; Tal et al., 2006). The dominating OTU of Rhodobacteraceae was significantly more abundant in the water of RAS, whereas the other OTU of Rhodobacteraceae dominating in RAS was most abundant with 22 ppt, indicating different properties of these two taxonomic clades of Rhodobacteraceae (also commented in 4.2).

4.4. Temporal changes of the microbiota in S-CCS

The temporal changes in the microbiota diversity and composition in S-CCS was mainly observed in the biofilms during the three months study. High turnover of the water in S-CCS, due to the high flow rate of intake of water from the sea, might have caused a more stochastic microbiota in the water than in the biofilms, as well as lower microbiota diversity. The biofilm microbiota detected might also have been more representative due to a mean of four samples compared to one sampling point per time of the water, and larger sampling volume of the water would have been more satisfactory.

The high turnover rate of water in S-CCS (<60 min) might also introduce a microbiota reflecting the diversity in sea, containing high amount of bacteria not yet cultured and taxonomically assigned, also reported by Aravindraja et al. (2013). Indeed, high abundance of an unassigned OTU was detected in S-CCS and not in the RAS, which is characterized by much lower system water turnover rate. High abundance of the phylum Cyanobacteria was also detected in S-CCS; however, at lower taxonomic depth this was resolved to be contamination of Chloroplast DNA in the 16S rRNA gene analysis, also reported by Hanshew et al. (2013). The Chloroplast DNA might indicate detection of algae (phytoplankton), and investigation of the temporal changes within S-CCS identified an increase of the Streptophyta algae especially in May months (also increase of the Cyanobacteria Synechococcaceae was observed in the water, data not shown). This might indicate detection of an algal bloom that is typically developing in the spring months (Buchan et al., 2014). In parallel, several potential pathogens including *Tenacibaculum*, *Polaribacter*, *Aliivibrio* and the family Alteromonadaceae containing potential pathogenic species were increasing in the last period. Indeed, members of the Flavobacteriia are known to dominate communities of algal blooms (see review by Buchan et al., 2014). For instance, Teeling et al. (2012) have identified *Polaribacter* and *Tenacibaculum* within such a community. It should be mentioned that no measurements in the S-CCS could confirm an algal bloom during the experimental period, and our methodology needs to be validated in terms of algal detection accuracy. The increase of *Polaribacter* during the last period might also be due to production of specific bacterial compounds (rhodopsins) that induce growth when associated with light, a feature known within *Polaribacter* (Gomez-Consarnau et al., 2007). *Polaribacter* was only significantly increasing in the biofilm, whereas in RAS it was more abundant in the water than in the MBBR-biofilm. It has been suggested that *Polaribacter* can alternate between two lifestyles, one free-living when nutrients are poor and one attached life form to surfaces and gliding to search for nutrients (Gonzalez et al., 2008).

Rhodobacteraceae OTUs and bacteria within Rhizobiales were more abundant in the biofilm than in the water in S-CCS, but interestingly they significantly decreased during the experimen-

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tal period. The marine Roseobacter clade of Rhodobacteraceae, are known to be frequently surface associated (Dang and Lovell, 2016), and temperature has been reported to be an important environmental factor of Rhodobacteraceae (Campbell et al., 2015), which also changed during the three months study. It could be hypothesized that the temporal changes of the microbiota in S-CCS indeed affected the microbial balance towards a state in favor of opportunistic pathogens.

4.5. Potential pathogens detected in both RAS and S-CCS

Both RAS and S-CCS seemed to be reservoirs for potential pathogens, though only three of the investigated potential pathogens were shared within the two aquaculture systems, including the common marine bacteria *Polaribacter*, *Flavobacterium* and *Pseudoalteromonas*. The water seemed to be the main reservoir for the potential pathogens investigated. One exception was *Polaribacter* that was significantly more abundant in the water in RAS and with biofilm in S-CCS, perhaps due to different nutrient availability affecting the lifestyle as discussed above. In addition, Piscirickettsiaceae was significantly more abundant in the MBBR-biofilm in RAS, but we did not detect *Piscirickettsia salmonis* known to cause piscirickettsiosis in salmonid fish (Rozas and Enriquez, 2014). Salinity was not shown to affect any potential pathogens in RAS. However, the *Yersinia ruckeri* that was introduced into RAS, which we might have detected as Enterobacteriaceae, was less abundant in the water of 22 ppt than 12 ppt one month after the introduction (Supplementary material, Fig. S1C and D).

Even though an increase of several potential pathogens, i.e. *Tenacibaculum*, *Polaribacter*, *Aliivibrio* and the family Alteromonadaceae was detected in S-CCS, no symptomatic fish was observed during the experimental period. Nevertheless, future development of S-CCS should consider to include intake water treatment, such as mechanical and UV-filtration to improve biosecurity. Several of the above-mentioned bacteria are considered to be environmental opportunists and commonly found in the sea and in the fish, but they can invade already weakened fish, though their role, as opportunistic pathogens are unclear. Especially *Polaribacter* was highly abundant in both RAS and S-CCS, and as mentioned commonly abundant in marine ecosystem and with not known pathogenicity. However, isolates have been identified by Multilocus sequence analysis (MLSA) from outbreaks of tenacibaculosis in Norway, showing high similarities with *Tenacibaculum* and virulent lineages of *Polaribacter* was suggested (Habib et al., 2014). To summarize, the employed methodology is limited to identification of potential pathogens since their species were not identified, and combination with other methods are needed to correctly identify known pathogens, opportunists or potential pathogenic species, e.g. real time PCR or metagenomics (Miller et al., 2013).

5. Conclusions

This study demonstrates that deep-sequencing of the microbiota in RAS and S-CCS during commercial-scale Atlantic salmon post-smolt production allowed for detection of highly diverse microbial communities that differed in composition between aquaculture systems, niches, salinity, over time and type of potential pathogens. Such information is needed in order to achieve good control of microbial management in relation to the environmental factors, and due lower time and cost of the analysis than traditional methods, the methodology is suggested used for microbial monitoring.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquaeng.2016.10.003.

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