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Presence and habitats of bacterial fish pathogen relatives in a marine salmon post-smolt RAS

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ABSTRACT

Recycling aquaculture system (RAS) requires well-functioning microbial communities to support fish welfare and to minimize the risk of spreading diseases. In this study of a marine post-smolt RAS we searched for groups of bacteria containing known salmon pathogens in a 16S RAS library that covered samples from four different sites over a year. In the first of the four production cycles included, there was a severe outbreak of fish skin ulcers associated with over 50 % loss of the fish population. To study the presence of potentially pathogenic bacteria from various habitats in the RAS, we used a refined 16 S rRNA gene library approach. The short library sequences of 250 bp were aligned towards 16 S sequences of recognized fish pathogenic bacteria 750-1500 bp long. The latter sequences were widely defined and gathered from GenBank into a fish pathogens database (FPD) prior to the alignment. Pathogen-relatives detected were *Moritella viscosa*, *Psychrobacter immobilis* and *Aeromonas salmonicida*, all with 100 % similarity; *Tenacibaculum dicentrarchi*, *Pseudoalteromonas piscicida*, *Mycobacterium simiae*, *Aliivibrio wodanis* and *Fransicella philomiragia* that had 97-99 % similarity and *Shewanell puterfaciens*, *Flavobacterium johnsoniae*, *Piscirickettsia salmonis*, and *Bacillus mycoides* that had 90-95 % sequences similarity. The pathogen-relatives habituated differently in the RAS as their relative abundances changed between the sites and over time in response to changing operational conditions. The biofilter biofilm was a unique site due to its remarkable low abundance of the pathogen-relatives, whereas three pathogen-relatives with high similarity to classical salmon winter ulcers strains were highly present in the fish skin ulcers. All pathogen-relatives identified were also detected in the production water, three of them at high relative abundance. Thus, we recommend the fish skin and the production water as the best sampling sites for pathogens early warning and specific screening approaches.

1. Introduction

Next generation 16S rRNA gene amplicon sequencing is increasingly used to monitor microbial communities in recycling aquaculture systems (RAS) to better understand connection between microbial activity, water quality, and fish health (Martins et al., 2013; Ruan et al., 2015; Schmidt et al., 2016; Minniti et al., 2017; Bartelme et al., 2019; Ma et al., 2020; Menanteau-Ledouble et al., 2020; Mekuchi et al., 2019; Minich et al., 2020; Perry et al., 2020; Drønen et al.,2021; Gonzalez-Silva et al., 2021; Wang et al., 2021). Martins et al. (2013) were the first to suggest 16S deep sequencing as a tool for fish disease monitoring in aquaculture systems, but concluded that the approach should be used with caution as phylogenetic resolution was low. The 16S rRNA gene is highly conserved and even at the highest level of phylogenetic resolution, the gene is not

alone a reliable marker for taxonomic assignments on the species or strain level. Additional approaches often used for species identification involves culturing or multilocus sequence analysis (MLSA), e.g., towards members of the genera *Vibrio*, *Aeromonas* and *Tenacibaculum*, (Habib et al., 2014; Glaeser and Kämpfer, 2015; Steinum et al., 2016). Fish health studies using16S amplicon libraries have mainly focused on the relationship between microbial diversity and fish growth, or the relationship between microbiota and expressed immunogens (Minniti et al., 2017; Minich et al., 2020; Perry et al., 2020; Steiner et al., 2021; Wang et al., 2021).

Pathogens identification and diagnostics makes use of targeted PCR primers for specific marker genes of each target organism, allowing only a limiting number of organisms to be studied (Panangala et al., 2007; González et al., 2004). When studying the presence of pathogenic

bacteria by a Next generation sequencing (NGS), 16S library sequences are generally short (~250 bp), but only bacteria in the same genera to the pathogens are addressed. Valuable information is still obtained for pathogens risk assessment and refugia's detection in the environment, as there is a correlation between the percent similarity in the marker gene (16S) and similarity broadly in the genome (Lan et al., 2016). The 16S library sequences can be used to identify similarity to other 16S sequences, including 16S sequences of recognized fish pathogens. A comprehencive database of full length 16S sequences from numerous recognized fish pathogens can thus be helpful to identify pathogens in the library and inform upon sequence similarity. For the 16S marker gene one assumes that sequences showing over 95 % identity represent the same genus whereas sequences above 97 % identity represent the same species (Schloss and Handelsman, 2005). The term closely related bacteria is however not associated with a certain percentage of similarity, but describes often distances in phylogenetic trees that are based upon sequence similarity (Washburne et al., 2018). Thus, in practice, "closely" or "distantly" related bacteria refer to high and low similarities between e.g. 16S sequences. Here we suggest to use the term pathogen-relatives followed by the information about the percent similarity between their compared marker sequences. An adequate question to address will be, what is the likelihood for pathogenic bacteria to occur in a RAS environment harboring groups of bacteria, their relatives, identified based on degree of percent similarity.

The potential of intrusion of unwanted fish pathogens that may cause disease outbreaks in RAS is of major interest for risk evaluation. An issue to be addressed is the likelihood of pathogens to be present in deeper water layers, as the inlet water in marine RAS normally is taken from depths deeper than 60 meters below sea level. The groups of bacteria known to be relatively more common at such depths than others are members of the orders *Rhodobacteriales* and *Flavobacteriales* (Cui et al., 2019; Suzuki, 2015). *Flavobacteriales* members like *Tenacibaculum* are thus likely to enter marine RAS through the inlet water, both free living and as particle associated. Pathogens entering a RAS through the inlet water will be exposed for UV irradiation, and bacterial inactivation efficiency strongly depends on UV dosage, particle association and robustness against UV irradiation (Huyben et al., 2018; Teitge et al., 2020). During an UV treatment that inactivates ~99 % of the bacteria, 5000 bacteria/ml will survive and blend into the production water, if the initial concentration is ~5×10^5 bacteria/ml in marine RAS inlet sea water (Whitman et al., 1998). Thus, any bacterium present in the RAS inlet water will be available in the RAS gene pool, but only the selection pressure allows members of the gene pool to enrich in the system.

Among known fish pathogens, there are some bacterial species more likely to cause diseases in marine post-smolt RAS than others. An ubiquitous problem are bacterial skin ulcers, colonized by the same pathogens as seen in the ulcers of fish kept in open sea net pens (Møllerstuen, 2020). The most common species are *Tenacibaculum* sp., *Moritella viscosa*, *Aliivibrio wodanis*, *Vibrio splendidus* and *Vibrio tapetis* (Grove et al., 2008; Olsen et al., 2011; Bornø and Linaker, 2014; Soltveit, 2020). *Moritella viscosa* is considered a primary pathogen in fish skin ulcers, and is documented to be present in 88 % of all winter ulcers, whereas *Tenacibaculum* sp. is associated with ulcers, alone or coexisting with *M. viscosa* (Grove et al., 2008; Olsen et al., 2011). *Tenacibaculum*, *Aliivibrio wodanis* and the *Vibrio* species are believed to be able to colonize already existing ulcers, but not to perform the initial opening of the skin (Olsen et al., 2011; Bornø and Linaker, 2014; Soltveit, 2020).

In the present study we examine the most adequate habitats for pathogen's identification and monitoring in a marine post-smolt RAS system. The sampling sites included were production water, biofilter biofilm carriers, tank wall biofilm, fish skin and fish skin ulcers. From these sites, samples were retrieved monthly for a period of one year, covering four production cycles. Relatives to hitherto recognized fish pathogenic and detrimental bacteria was identified by aligning 16S rRNA gene amplicon sequences towards full-length 16S rRNA gene sequences of recognized fish pathogens. We registered changing relative abundances of the identified pathogen-relatives over time, and during changing operational management. Statistical correlations between the identified pathogen-relatives were analysed. The obtained information can be of importance in risk evaluation of bacteria potential able to cause disease of fish in RAS. Especially, monitoring of microbial communities in RAS can provide early signs of emerging pathogenic bacteria and further studies are therefore needed.

2. Material and methods

2.1. The marine RAS facility and sampling sites

Atlantic coastal water from 70 meters depth was UV irradiated and used as inlet water in a marine post-smolt RAS located at Stord, south of Bergen, Norway. The RAS had capacity to grow 500 000 post-smolt from 100 g to 500 g in 4 months at 14 °C and 32‰ salinity. The RAS contained 4 production tanks (each1000m^3), drumfilters with 60 μm cut-off, a moving bed biofilter (300m^3), CO_2 stripping unit and oxygenation of water. Liquified ozone and hypochlorite (LOZ AS, Trondheim) was injected in the CO_2 degasser for particle reduction and sodium bicarbonate and sodium hydroxide were added to regulate the pH of the production water. The water exchange rate of the RAS was 100-250 l/min, giving a water dilution rate of 6-15 % and a retention time of ~1.5 hours in the production tanks. The degree of recirculation was ~98 %.

A detailed description of the RAS farm was given by Drønen et al., 2021., including how the physiochemical parameters pH, temperature, salinity, redox, oxygen, and N-species were measured. The physiochemical raw data were deposited in the Mendeley data repository (datafileDrønen, 2021).

The commercial starter culture for biofilter inoculation was enriched from brackish water, and the colonization and succession upon the biofilter carriers were reported previously by Drønen et al., 2021, covering the same trial period as in present study. Prior to the first fish release the starter culture was enriched with ammonium and fish feed in increasingly larger batch volumes of carriers until full scale volume enrichment in the flowing RAS. This process lasted for several weeks, and the utilization of ammonium into nitrite was measurable. Based upon experience from fresh and brackish water systems, the second step of nitrification established when fish were released into the RAS system, but the first production cycle terminated after a few weeks due to high nitrite and H_2S formation. From here, our experimental trial period started, covering the second production cycle until the fifth, a period of one year duration. The detailed overview of sampling times, sampling sites and replicates of the biological material are presented in Fig. 1, and a total of 125 samples were collected for microbial analysis. Samples were retrieved monthly from biofilter biofilm carriers, tank wall biofilm, production water and fish skin. Extra sampling was performed from the production water and the biofilter biofilm carriers when the RAS was washed between two cycles, and also at the final sampling time, including water sampled before and after the inlet water UV-filter, wall biofilm of the pump sump and the biofilter outer chamber wall biofilm, and finally, tank wall biofilm profiles from all production tanks (4×4 samples from respectively 0.2, 1-, 2- and 4-meters depth). Five parallel samples were also analyzed from the microbial starter culture used to inoculate the biofilter carriers prior to the first fish stocking. During a severe outbreak of fish skin ulcers in the first included production cycle, samples from damaged fish skin/tissue were also collected. Not all samples were successfully processed through the 16S deep sequencing analysis as noted in Fig. 1.

Two strategies were used to mitigate the reported ulcer outbreak. First by administrating the antibiotics oxolinic acid (5 mg·kg fish body weight^{-1}·d^{-1} in 10 days from week 5) and florfenicol (10 mg·kg fish body weight−1·d−1 in 10 days from week 7) to the fish through the feed. As outbreak prevailed despite this treatment, fresh water was added to the production water from week 8 to week 10 of the cycle, causing an abrupt drop in salinity. This was efficient for fish survival,
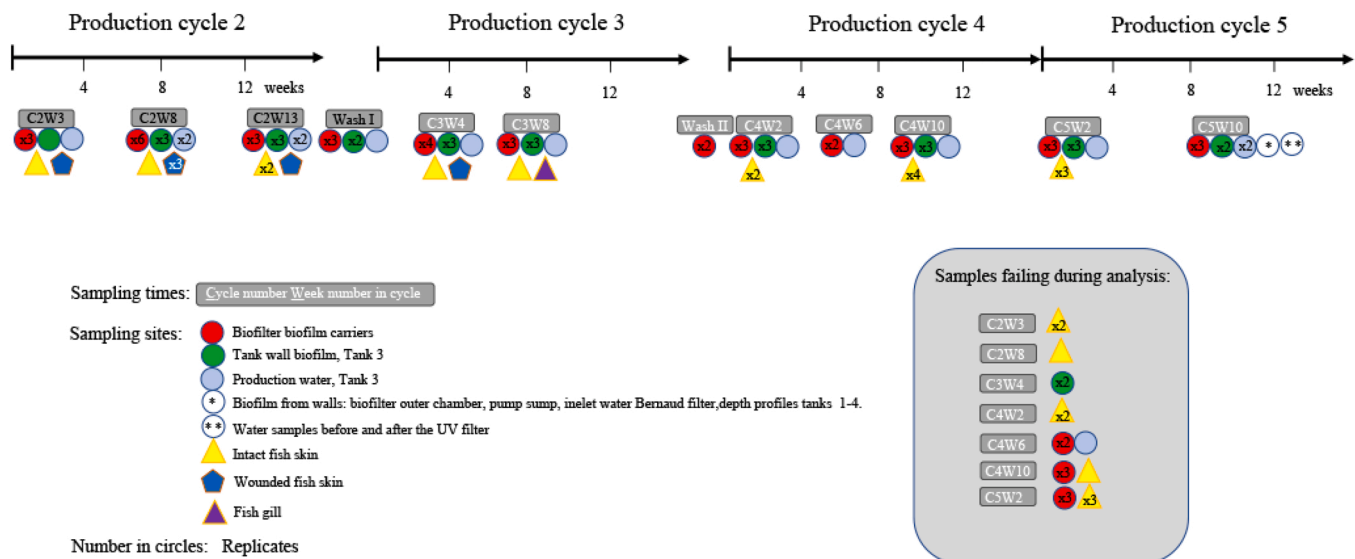
Fig. 1. Biological material retrieved for the 16S rRNA gene amplicon library used to identify pathogen-relatives. Symbols and numbers denote sampling times, sampling sites and replicate samples.

and when fish feeding was fully re-established, there was an abrupt change also in 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 (ozone and hypochlorite) and re-inoculated with 20m^3 biofilm carriers (6-7 % of total carriers volume). Another RAS wash (Wash II) was performed after the third cycle, though without biofilter "back washing". During the fourth production cycle, the water turned strongly yellow, without reported effect upon fish performance.

2.2. DNA extraction, sequencing, and bioinformatics

The detailed methodical description of the 16S rRNA gene amplicon library preparation and the bioinformatic pipeline were given by Drønen et al., 2021 and (Drønen et al., 2022a). Shortly summarized: Biofilm carriers in the biofilter were collected by a sterile ladle, wall biofilm was collected by a sterile toothbrush, water (240 ml) was filtered through a 0.2um Sterivex filter (Millipore) and fish skin samples were by sliced by scalpel 2x3cm and 0.1 mm at the lateral line. All samples were stored in RNA later at -20°C immediately after sampling. From these samples, DNA was extracted using kits designed for the sample types: DNeasy Power Biofilm Kit (Qiagen), DNAeasy PowerWater Kit (Qiagen) and the tissue DNA extraction protocol from High Pure PCR Template Preparation kit (Roche).

The 16S rRNA gene amplicons were generated in accordance with the two-step PCR as recommended by Berry et al. (2011), using HotStar Taq Master Mix (Qiagen) and the universal primers 519f (5'-CAGCMGCCGCGGTAA) (Ovreås et al., 1997) and 805r (5'-GAC-TACHVGGGTATCTAATCC) (Klindworth et al., 2013) for the initial 16S rRNA gene amplification. 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 sec at 94 °C, 30 s at 56 °C and 30 s at 72 °C, and a final elongation at 72 °C for 7 min. Pooled triplicate PCR products were purified by AMPure XP beads (Agencourt) using the sample:bead ratio of 0.7. 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 comprised individual tags and adapters for Ion Torrent sequencing chemistry (Torrent, 2012). The PCR mixture and thermal program was as before, but only 7 cycles of amplification were used. The purified amplicons were pooled in equimolar concentration to a final concentration of 40pM and sequenced by the Ion Torrent Personal Genome Machine (PGM) technology. Positive and negative controls were processed through PCR analysis, i.e. DNA known to yield a PCR product by the primers and samples added dH$_2$O without DNA. The PCR controls were not included in the Ion Torrent sequencing afterwards.

The bioinformatics pipeline was thoroughly described by Roalkvam et al., 2019 and Drønen et al., 2021. The pipeline comprised USEARCH for sequence merging and read quality filtering (Edgar, 2010), UPARSE for *de novo* 97 % identity OTUs (operational taxonomic units Edgar, 2013) clustering and Qiime for OTUs taxonomic analysis using the Qiime compatible SILVA128 release (https://www.arb-silva.de/no_-cache/download/archive/qiime/) as a reference database (Caporaso et al., 2010). The performance of the analysis was evaluated against newer approaches to noise reduction, and considered equally good by experienced analysis personnel (Drønen et al., 2021). A total of 105 samples formed satisfactory products in analysis, and 449 OTUs were defined in the RAS, from where 45 % were identified to genus level. OTU's raw data was published in Data in Brief (Drønen et al., 2022a), and sequence information was made available in GenBank with the accession numbers MN890148-MN891672. The raw data set comprised 1524 OTU sequences that were used for alignment with the FPD database, although only 449 out of these sequences were representing the final defined OTUs for the dataset. In this study, no rarefaction of data was necessary in the downstream analysis, as the variability in the lower detection limit in the analysis is a part of the risk evaluation.

2.3. Pathogen database construction and databases alignment

A 16S RNA full length sequence (750-1500 bp) database comprising detrimental bacteria and known fish pathogens were stored as fasta file and referred to as the fish pathogen database (FPD). Selected sequences to the database were mainly based on fish pathogens species and other bacterial species associated with fish diseases, as listed in the book "Bacterial fish pathogens" (Austin and Austin, 2012). Emerging pathogens recognized after 2012 were also included in best to our knowledge (Table in co-submitted Data in Brief article (Drønen et al., 2022b). Sequences for the database were retrieved from the GenBank database in the fasta formate, e.g,

https://www.ncbi.nlm.nih.gov/nuccore/? term=Tenacibaculum+maritimum+16 S+rRNA) or from the GenBank based database Silva 128 (https://www.arb-silva.de/documentation/release-128/). The software supported by GenBank and Silva allowed filtering upon 16S and sequence length. Multiple sequences were included for each fish pathogen specie when available, as one specie

often comprise several strains with multiple 16S rRNA genes. A total of 295 different species were included and their 6750 corresponding sequences were gathered in a fasta file as reported in the co-submitted Data in Brief article (Drønen et al., 2022b).

The marine RAS 16 rRNA amplicon library sequences (250 bp, V3-V4) were aligned with the FPD full length 16S rRNA sequences in Blast 2.9.0. using the Python platform. The sequence identity cut-off was set to ≥90 %. The matching sequences were register by their percent similarity and termed as pathogen-relatives. The matching 16S rRAS gene library sequences frequently showed several percent similarities to the FPD database sequences, and for our purpose, only the highest percent similarities were reported for each genus. The pathogen-relatives were linked to the information about sampling times and sampling sites by merging the query data with the QUIIME pipeline output file otu_table_taxSilva_withtax.xlsx, using an in-house Python script. The Geneious data software was used for phylogenetic tree building and NCBI Blastn search, using the Geneious' standard parameters (Kearse et al., 2012). The approach of pathogen-relatives identification is not to be used in pathogens diagnostic due to the low specificity.

3. Results and discussion

A marine post-smolt RAS for Atlantic salmon was regularly monitored using 16S rRNA rRNA gene amplicon sequencing during the first year of operation to enlighten the role of the microbial communities in good water quality and fish welfare. Pathogen-relatives in the RAS plant were identified by comparing obtained RAS amplicon sequences (250 bp), with full length 16S rRNA gene sequences in a database (the FPD database) of pathogenic bacteria (FPD, (Drønen et al., 2022b). Based on present knowledge one can assume that sequences with 100 % similarity to pathogenic bacteria cannot be excluded as pathogens, although not confirmed. Bacteria with less than 100 % similarity to any sequence in the database can be considered non-virulent, provided that all strains known to be virulent were included in the FPD database with their 16S information. However, if the strain becomes documented as a disease agent by convention, the 16S sequence should be included into the 16S rRNA gene FPD database.

3.1. Database query to identify pathogen-relatives

Thirteen OTU sequences out of a total of 1524 OTU sequences defined in the RAS amplicon's raw data had a hit with 90-100 % similarity in the FPD database (Table 1). All hits represented pathogens relevant in the context of salmon fish health issues. Three OTU sequences matched FPD sequences with 100 % similarity, and thus could not to be excluded as pathogens. These FPD sequences affiliated taxonomically to *Moritella viscosa*, *Psychrobacter immobilis* and *Aeromonas hydrophila, Aeromonas salmonicida* (Table 1). The latter OTU was not identified taxonomically by the bioinformatic pipeline used for RAS 16 S library formation, but the affiliation of this sequence to the *Aeromonas* genus was confirmed by a query in the NCBI GenBank database. Four

Table 1

Matching 16S sequences between the OTU and FPD databases. The requirement for match was ≥90 % similarity and the OTU sequences were 250 bp long.

OTU sequence	OTU genus	Best match FPD %	Matching FPD sequence	Taxonomic identity FPD sequences	Reference
4	*Moritella*	100	Y17574*	*Moritella viscosa*	Benediktsdóttir et al., 2000 Y17574
1104	*Tenacibaculum*	98.8	KT000261 KT0002611 KX984053 KY041669 OENC01000048	*Tenacibaculum dicentrarchi*	Piñeiro-Vidal et al., 2012 FN545354
1346	*Aliivibrio*	97.6	AJ132227 Y17575 AJ132227 AF329728 AY628647	*Aliivibrio wodanis*	Urbanczyk et al., 2007 AJ132227
703	*γ-Proteobacateria*	100	AB626121** KC884667***	*Aeromonas hydrophila Aeromonas salmonicida*	Seshadri et al., 2006 NR074841 (Griffin et al., 1953)
18	*Psychrobacter*	100	QGGM01000028 KF701041 HQ698589 AJ309942 AB680954	*Psychrobacter immobilis*	Juni and Heym, 1986 HQ698589
1416	*Pseudoalteromonas*	98.0	X82215	*Pseudoalteromonas piscicida*	Buck et al., 1963 X82215
45	*Mycobacterium*	98.0	X52931	*Mycobacterium simiae*	Chapman, 1977 AB547406
210	*Fransicella*	96.8	EU50315	*Fransicella philomiragia*	Hollis et al., 1989 AJ698862
1407	*Shewanella*	94.8	U91552	*Shewanell puteraciens*	Thorell et al., 2019 FJ971881
1556	*Flavobacterium*	93.4	EU531547	*Flavobacterium johnsoniae*	(Bernardet et al., 1996) AM230489
1139	*Piscirickettsia*	90	AY498633	*Piscirickettsia salmonis*	(Fryer et al., 1992) txid1227812
804	*Bacillus*	90.4	AB021192 KF625192	*Bacillus mycoides*	Liu et al., 2018 txid1405

OTUs 18, 703, and 210 were detected in the commercial starter culture.

*Matching all *M. viscosa* sequences

**Matching 32 out of 44 *A. hydrophila* sequences

***Matching 11 out of 15 *A. salmonicida* sequences

OTU sequences showed 97-99 % similarity as best match to the FPD sequences, thus potentially affiliating to the same species as the pathogens. These FPD sequences affiliated to *Tenacibaculum dicentrarchi*, *Pseudoalteromonas piscicida*, *Mycobacterium simiae* and *Fransicella philomiragia,* and were thus also potentially undescribed pathogens. The other matching FPD sequences, *Shewanell puterfaciens*, *Flavobacterium johnsoniae*, *Piscirickettsia salmonis*, and *Bacillus mycoides* did only have 90-95 % sequences similarity to the 16 S rRNA gene library sequences, and thus could not be unequivocally assigned to the same genera as the matching pathogens.

However, the use of percent similarity for taxonomic resolution has many pitfalls. Phylogeny built upon short 16S rRNA gene sequences will be influenced by the sub-regions of the gene used (Johnson et al., 2019). A new thresholds (98.65 %) of interspecific similarity of the16S rRNA gene has been suggested based upon polyphasic taxonomy of bacteria which integrates all available phenotypic and genotypic data into a consensus classification (Kim et al., 2014). Also, standing nomenclature strains of *Mycobacterium* with 16S rRNA gene sequences of length >1320 bp did show similarity values below 97 % upon alignment (Beye et al., 2018). Still, the 16S rRNA gene similarity is a powerful framework in pathogens evolutionary theory, where a high sequence identity indicates a young evolutionary history (Case et al., 2007; Woese and Fox, 1977). Even why emerging pathogens harm their host and how virulence develops during a given set of conditions can be understood by their evolutionary history (Bonneaud and Longdon, 2020). In our context it is important to know that the genome-similarity increases with the inter or intra species percent similarities, making the basis to believe there is a likelihood of pathogen survival as a pathogen-relative has been observed. Thus, to improve the pathogens risk evaluation in RAS, more studies comparing pathogens to related environmental strains are requested.

3.2. Pathogen-relatives in the commercial starter culture

The biofilter carriers were colonized from a commercial bacterial starter culture before the RAS plant received fish for the first time (Drønen et al., 2021). In general, OTUs detected in the RAS with similarity to sequences in the starter culture were termed inoculum associated OTUs (IAO) in our data handling. Three of the 16S RAS library sequences reported in Table 1 were present in the commercial inoculum. Two of these three pathogen-relatives, *Psychrobacter* and *Aeromonas*, had 100 % similarity to the FPD sequences, whereas the third IAO identified both in the RAS and in the starter culture, *Francisella*, had 96.8 % similarity to *Fransicella philomiragia*. This could indicate that the commercial inoculum could represent a source of pathogenic bacteria, introducing them to RAS at an early stage of production.

3.3. Pathogen-relatives with high abundance in the RAS production water

Francisella had a higher relative abundance in the production water than in the other sampling sites and was permanently present and even dominant in the production water with over 5 % relative abundance at two occasions (Fig. 2). The matching FPD sequence affiliated to *Fransicella philomiragia* as the best hit, demonstrating 96 % similarity (Table 1). *Francisella philomiragia* is regarded as a non-pathogenic environmental representative within the *Francisella* genus (Mailman and Schmidt, 2005), though taxonomic inconsistency exist (Mikalsen et al., 2007) and strains within this lineage have recently been identified as serious pathogens for fish, causing francisellosis in cultured and wild fish globally (Colquhoun and Duodu, 2011). Extensive studies of Norwegian coastal marine waters and sediments detected *F. philomiragia*-like strains in 30 % of the water samples, and also somewhat in sediments in areas with francisellosis (Duodu et al., 2012).

Sugar is suggested as the main substrate for non-pathogenic *Francisella* strains during planktonic living, whereas pathogenic intracellular *Francisella* strains seems to have lost the ability to grow on glucose, and

also requires external cysteine or certain transition metals for growth (Gupte and Gupte, 2006; Duodu and Colquhoun, 2010; Ziveri et al., 2017; Ozanic et al., 2021). By this, the ability to grow in culturing media is normally lost in a few days for pathogenic strains. However, a recent study from tilapia farming in the tropical climate zone indicated that pathogenic *F. noatunensis* subsp. *orientalis* re-infected after a longer environmental stay (Delphino et al., 2019). Based on the current knowledge, the most likely environmental reservoir for pathogenic *Francisella* species in RAS would be in protozoa, although long time survival in such environments is associated with loss of virulence (Sjöstedt, 2007; Broman et al., 2011; Ozanic et al., 2021).

The OTU identified taxonomically as *Psychrobacter* (100 % similarity to *Psychrobacter Immobilis*) was the second most common genus in the RAS production water and dominant above 5 % relative abundance at one sampling time (Figs. 2 and 3). However, this OTU was also detected with very high relative abundance in fish skin at the same time. Substrates known to sustain growth of *Psychrobacter* are organic acids and amino acids, but also uric acid from fish, which may explain the bacterium as part of the natural fish skin microbiota (Bowman, 2020). Fish seclude skin mucus continuously and potential growth substrates associated with fish mucus and uric acid are then likely to support growth of the bacterium also in the production water of a RAS.

Furthermore, an OTU taxonomically identified as the genus *Mycobacterium* (98 % similarity to *M. simiae*, Table 1 and Fig. 2) was obtained from the production water, comprising the third highest relative abundance (0.5-5 %) in the library. This OTU had also a strong association to biofilms, and the growth substrates may have sustained the growth at both sites. *Mycobacterium* seems to utilize a wide range of carbon sources in their environmental staying (Hartmans et al., 2006). It is interesting in the context of substrates accumulation in RAS that cultured representatives of the genus utilize gluthatione that is typically present at high cytoplasma levels (0.10-10 mM) in all eukaryotic cells (Copley and Dhillon, 2002; Dasgupta et al., 2010). For unknown reasons, epithelia cells in salmon are observed leaking their content into mucus (Minniti et al., 2019). The highest relative abundance of the genus in the RAS, both in biofilm and production water, was obtained during the fourth production cycle, when the production water was colored strongly yellow. This might have increased the mucus production or caused that more eukaryote epithelial cells being released from the gut. In aquaculture, infection and granulomas formation in salmon has been associated to the *Mycobacterium* strains *salmoniphilum*, *marinum fortuitum* and *chelonae* (Yanong et al., 2010; Zerihun et al., 2011; Aro et al., 2014). However, the similarity of these strains to the pathogen-relative detected in the RAS plant was only 96 %, but as mentioned earlier, it has generally been difficult to use the percent 16 S rRNA gene similarity for species definition in *Mycobacterium* (Beye et al., 2018). Thus, we strongly suggests that identification of *Mycobacterium* in RAS should also be investigated by additional methods.

The high relative abundance (over 1 %) observed for *Francisella*, *Psychrobacter* and *Mycobacterium* members in the production water demonstrated that these genera were selected in the production water at certain sampling times. Thus, their selective growth parameters in the production water should be mapped and evaluated for supporting growth of pathogens in the RAS system.

3.4. Pathogen-relatives with low abundance in the RAS production water

The *Aliivibrio* genus was ubiquitous in the production water, maximizing in abundance (0.5-1 %) during the third production cycle. This cycle tended to select for heterotrophs in general due to a suggested microbial lysis in the biofilter biofilm (Drønen et al., 2021). The sequences affiliating to this OTU in the RAS library had 98.5 % sequence identity to *A. salmonicida* and 98.1 % to *A. wodanis* as examined in a NCBI GenBank query.

The *Shewanella* genus was also identified on five occasions in the
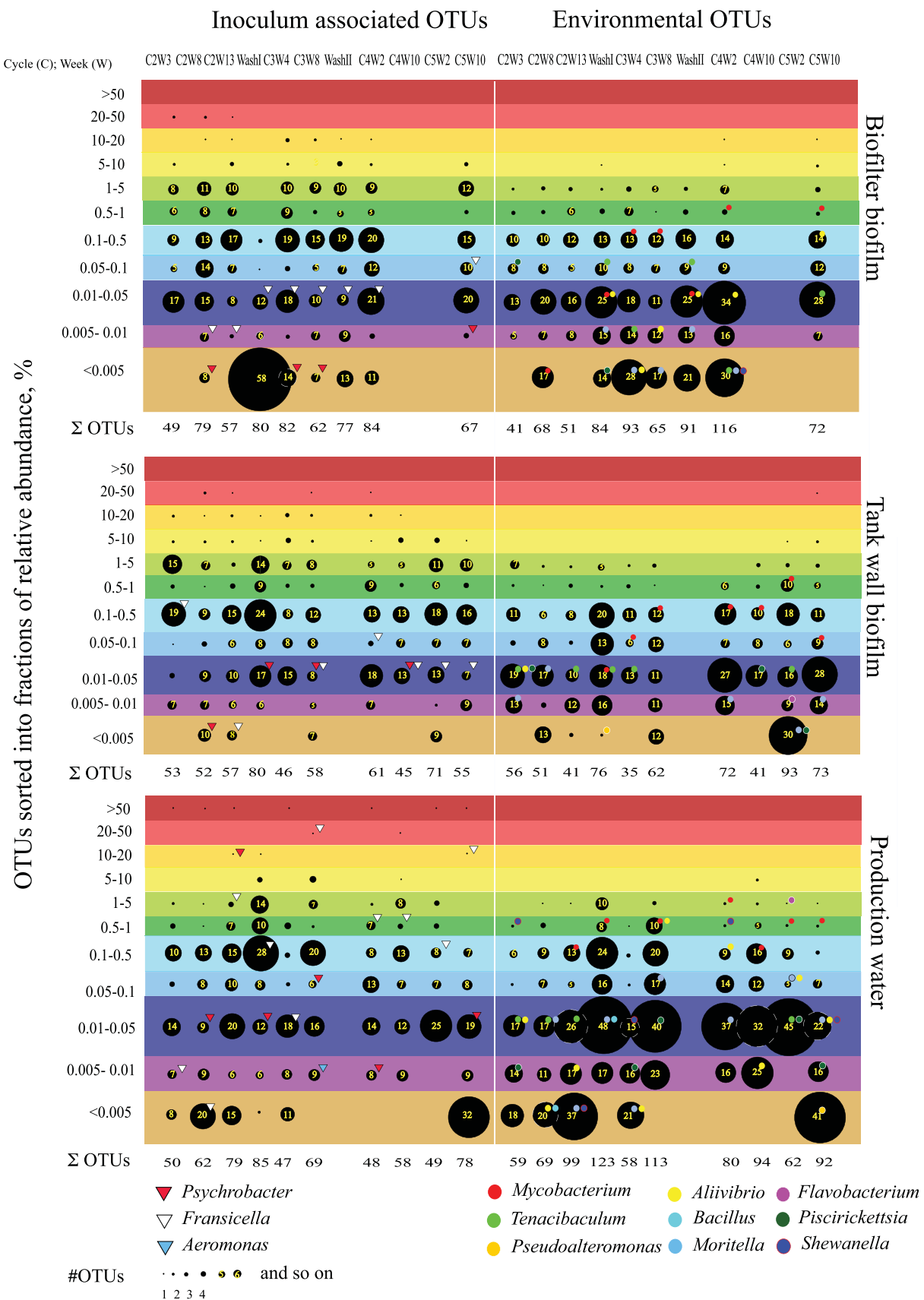
Fig. 2. Relative abundance of pathogen-relatives in the biofilter biofilm carriers, tank wall biofilm and production water at different sampling times. Each pathogen-relative is shown with colored dots or triangles, where dots represent OTUs originating from the commercial starter culture and triangles OTUs with anticipated environmental origin. The number of defined OTUs sorted into a fraction of relative abundance (#OTUs), are shown in the bubbles. Notable, when replicates where analyzed the data report the number of OTUs from pooled replicates. Data were not rarefied for this downstream count metrics.
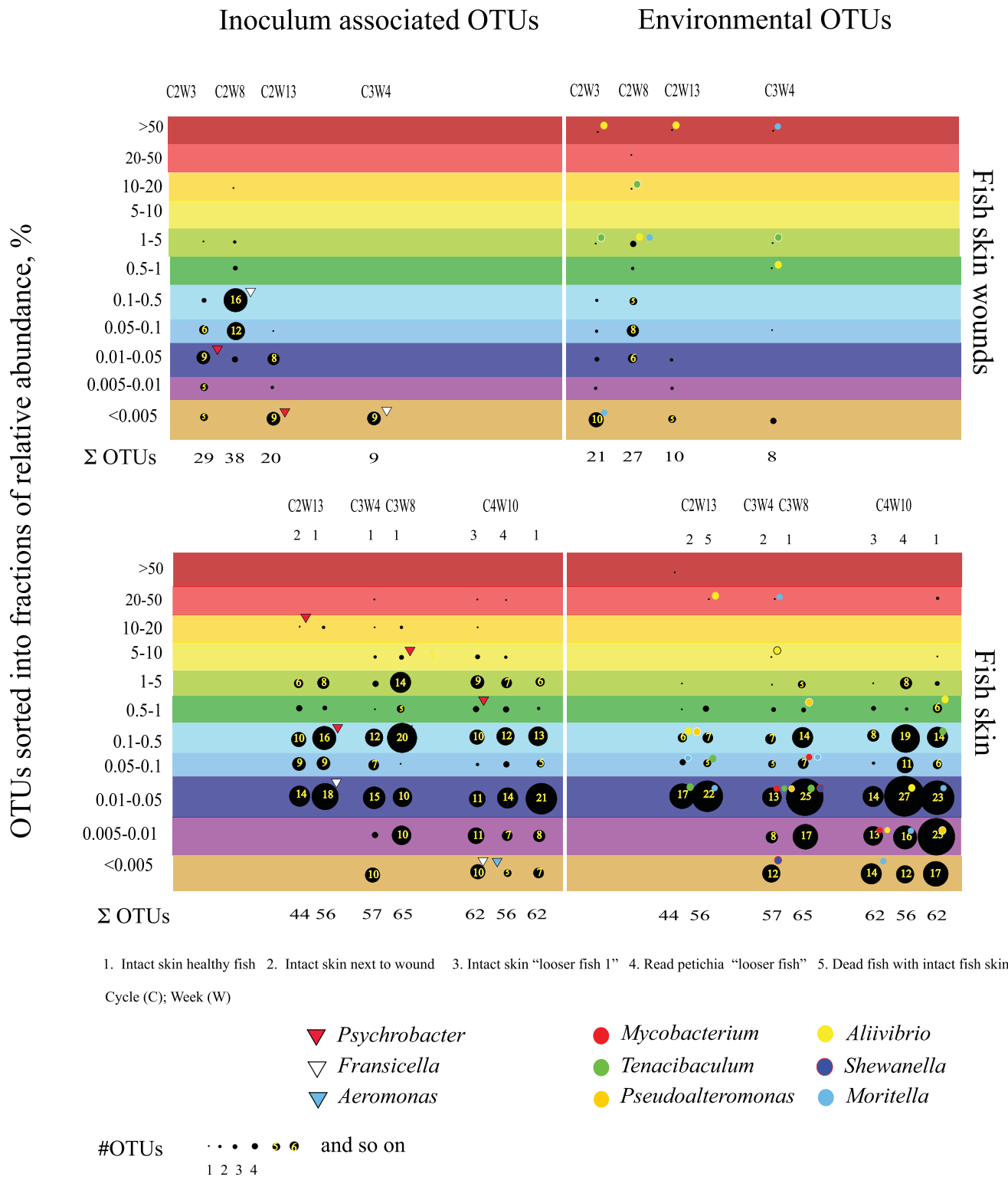
Fig. 3. Relative abundance of pathogen-relatives in fish skin and fish skin ulcers at different sampling times. Each pathogen-relative is shown with colored dots or triangles, where dots represent OTUs originating from the commercial starter culture and triangles OTUs with anticipated environmental origin. The number of defined OTUs sorted into a fraction of relative abundance (#OTUs), are shown in the bubbles. The total number of OTUs in a studied fraction of a sample are denoted ΣOTUs. Notable, when replicates where analyzed the data report the number of OTUs from pooled replicates. Data were not rarefied for this downstream count metrics.

production water, which was more than at the other habitats in the RAS. *Shewanella* bacteria are normally present in the skin flora of marine fish and are strongly implicated in fish spoilage (Adams and Moss, 2008). The *Shewanella* sequence from the RAS, revealed 100 % identity to a

Shewanella clade involving the Polyunsaturated fatty acids (PUFA) producing specie *S. japonica*, *S. pacifica*, *S. olleyana* and *S. electrodiphila*, as revealed by a NCBI GenBank query. None of these species are associated with fish diseases, but the ability to grow on alginate/gelatin

seems to be a common property (Ivanova et al., 2004, 2001). This may explain the association of *Shewanella* with the production water in the marine RAS, as secluded biofilm material will be abundantly present in the water (Drønen et al., 2021, Rosche, 2014).

The genus *Aeromonas* was identified only once in the RAS and then at very low relative abundance in the production water (Fig. 2). The OTU sequence showed 100 % percent similarity to *A. salmonicida* and *A. hydrophila*, but also to most 16 S sequences of this genus as confirmed in a NCBI GenBank query. *Aeromonas salmonicida* and *A. hydrophila* are well known pathogens in aquaculture, where *A. salmonicida* strains is associated most with mesophilic growth, whereas *A. hydrophila* strains more alternate between psychrophilic and mesophilic lifestyles (Rouf and Rigney, 1971). The RAS rearing temperature was in mean 13.8 ±1.3 °C during the experimental period, thus, selecting strongest for a psychrophilic lifestyle. A previous study shows that the *Aeromonas* genus was a natural part of the fish skin microbiome for freshwater salmon, while another study from a marine RAS research station, like our study, did not detect *Aeromonas* in fish skin or water over 1 % relative abundance (Minniti et al., 2017; Uren Webster et al., 2018). Thus, *Aeromonas* strains are unlikely to be selected in marine post-smolt RAS, as the temperature and salinity are not optimal.

Bacillus, *Pseudoalteromonas*, and *Flavobacterium* were detected only once in the water. Only the *Pseudoalteromonas* taxa was detected in one of the other habitats, demonstrating a modest association to fish skin, which could explain the low relative abundance in marine RAS (Fig. 2). *Flavobacterium* members are usually associated with the fresh water habitat (Verma et al., 2011).

The pathogen-relatives with low relative abundance (<1 %) in the production water was not so strongly selected by the production water quality at any sampling time as those with high relative abundance. Thus, the selective parameters in the production water are less interesting in a marine RAS risk evaluation for the genera *Aliivibrio*, *Shewanella*, *Aeromonas*, *Bacillus*, *Pseudoalteromonas* and *Flavobacterium*.

3.5. Pathogen-relatives associated with biofilm on tank wall and biofilter biofilm carriers

The *Mycobacterium* genus was unique in its association to RAS biofilm among the identified pathogen-relatives, demonstrating good abilities to colonize both the tank wall biofilm and the biofilm carriers (Fig. 2). *Mycobacterium* is anticipated to have specific attachment domains to biofilm, like many of the other members of the *Actinobacteriia* order (Blickwede et al., 2004; Hashimoto et al., 2005; Zhang et al., 2018). The establishment in the biofilms occurred during the cleaning and re-inoculation step between production cycle 2 and 3, and maximized in the production cycle 4 and 5 with relative abundances in the range 0.5-1 % (Fig. 2). *Mycobacterium* as a fish health issue in RAS is reported for *Trachinotus carolinus* production in Florida (Yanong et al., 2010). So far *Mycobacterium* has not been considered a problem in salmon post-smolt RAS, but the selective advantage of this bacterium suggests that opportunistic or pathogenic *Mycobacteria* may be difficult to eliminate if first established.

The *Aliivibrio* genus was identified upon biofilm carriers at 6 out of 9 sampling times (Fig. 2) and at 1 out of 10 sampling times upon the tank wall biofilm (Fig. 2), however, at low relative abundance, i.e, once above 0.05 %. Notable, the genus was first detected upon the biofilter biofilm carriers after flushing with concentrated ozone and hypochlorite ("back washed"). This biofilm disturbance and the introduction of new biofilm carriers allowed a re-colonization of the surfaces were also the *Tenacibaculum* and *Moritella* genera attached modest to the biofilter biofilm carriers (Fig. 2). Out of these two, *Tenacibaculum* reached the highest relative abundance upon the biofilter carriers, with relative abundance 0.05-0.1 % at the highest.

None of the pathogen-relatives that were found to be dominant in fish skin ulcers were strongly associated with biofilter biofilm carriers or tank wall biofilm, even when there were a severe outbreak of fish skin

ulceration that killed over 50 % of the fish population. This can be explained both by the lack of a selective growth substrate for the ulcer associated pathogens in the production water (only a low relative amount also in the water), but also that established biofilm was difficult to colonize without the biofilm surface firstly being broken up or removed.

3.6. Pathogen-relatives in fish skin ulcers

The microbiomes of fish skin ulcers included five pathogen-relatives of the genera *Tenacibaculum*, *Moritella*, *Aliivibrio*, *Psychrobacter* and *Fransicella* (Table 1). The *Tenacibaculum*, *Moritella* and *Aliivibrio* genera were dominant (18-99 % relative abundance) at one or two occasions each in the ulcer samples (Table 2). Members of these genera are commonly known for their fish ulcer associations and found in mixed cultures of winter ulcers on Atlantic salmon (Olsen et al., 2011). The *Moritella* sequences demonstated 100 % sequence similarity to most *Moritella* species, i.e., *M. viscosa, M. marina, M. dasanensis* and *M. yoyanosii*, where *M. viscosa* had the highest sequence similarity based on both the e-value and query coverage as shown by a NCBI Blastn query. Similarly, the *Tenacibaculum* sequences had 98.8 % sequence identity to *T. dicentrachi*. Only *M. viscosa* members are regarded capable to open the skin integument, and thereby being the etiological agent of the winter ulcers disease, whereas *Tenacibaculum* and *Aliivibrio* enter epidermis through an already existing opening of the integument and are by that termed secondary pathogens(MacKinnon et al., 2019, Karlsen et al., 2014). Winter ulcer formation in Norwegian waters is reported when water temperatures is below 8 °C, although fish skin ulcers by *Moritella viscosa* is reported above 10 °C from Canada (MacKinnon et al., 2019). Isolated strains of *M. viscosa* from Europe are also reported in literature to grow in the range 4-20 °C with optimums below 10 °C (Table 3). Thus, the temperature of approximately 14 °C in the RAS production water was far above the temperature reported for winter ulcers formation in Norwegian waters (Tables 2 and 3). This indicates that *Moritella* could adapt in their functional temperature range, which is also explainable with why *Moritella* dominated the examined ulcer at sampling time C3W4 and not at the early sampling time C2W3. At both these sampling times the salinity was over 30‰ that is also a requirement for the growth by this bacterium as reported upon salinity growth range from the literature (Table 3). The different temperature and salinity windows of *Moritella*, *Aliivibrio* and *Tenacibaculum* genera may then partially explain their variable dominance in the ulcers.

When the salinity of the production water was below 30‰ at the sampling times C2W8 and C2W13, *Tenacibaculum* or *Aliivibrio* were dominant in the ulcers respectively (Table 2). The antibiotics oxolinic acid and florfenicol were administered successively to the fish before sampling at time C2W8, but ulcer formation escalated in the fish population despite medication, causing more than 50 % mortality (Fig. 4). Although the antibiotic administrations were not efficient towards the ulcer formations, they were not unlikely to influence the competition relation between the *Tenacibaculum* and the *Aliivibrio* strains. Published data are scarce upon accurate sensitivity concentrations for these bacteria towards the used antibiotics, but indicative results for a few

Table 2
Relative abundance of the *Aliivibrio*, *Tenacibaculum* and *Moritella* genera in the ulcer samples from RAS and the actual RAS salinity, temperature, redox and pH values at sampling.

Sampling time	C2W3	C2W8	C2W13	C3W4
Dominating taxa	*Aliivibrio*	*Tenacibaculum*	*Aliivibrio*	*Moritella*
Relative abundance, %	94.5	18.2	99.6	97.7
Salinity, ‰	34.6	30.7	29.1	31.4
pH	7.0	6.8	6.5	6.7
Temperature, °C	14.4	15.0	14.6	13.0
Redox potential, mV	265	210	145	231
CO_2, mg/l	6	7	14	11

Table 3
Classical winter ulcers pathogens salinity and temperature range.

| Ulcer pathogen	Temperature, °C	Salinity, ‰	References
Moritella viscosa	4-25 (<10, Europe) (>10, Canada)	10-40 (>30)	(Tunsjø et al., 2007) Benediktsdóttir et al., 1998 MacKinnon et al., 2020)
Tenacibaculum maritimum	15-34 (30)	10-34	Suzuki et al., 2001
ovolyticum	4-25	24-34	Suzuki et al., 2001
finnmarkense	2-20	15-34	Småge et al., 2016
dicentrarchi	4-30 (23)	10-34 (24)	Piñeiro-Vidal et al., 2012
Aliivibrio wodanis	4-25 (10)	10-40	Lunder et al., 2000 Urbanczyk et al., 2007

Bracked; optimum value. When only the percent sea water was given we anticipated that 100 % seawater equaled 34‰.

examined strains suggested *Aliivibrio* more sensitive to oxolinic acid than *Tenacibaculum* (Lunder et al., 2000; Takle et al., 2015; Irgang et al., 2017; Raverty et al., 2017). Other factors making the sensitivity discussion difficult were the chance that the fish stopped eating between the two administrations or that biofilm formation protected the first colonizer from the second antibiotic exposure. Even more interestingly, a recent study suggests *Tenacibaculum* as the causative agent for post-smolt skin ulcer formation, but maintains the association to a preformed skin lesions (Klakegg et al., 2019). Previously, *Aliviibrio wodanis* is shown to be an efficient colonizer of imperfect tissue, and it́s early colonization would reduce the virulence and high mortality caused by sole *Moritella* infections (Karlsen et al., 2014). The observation of a severe ulcer outbreak without the participation of *M. viscosa* as a primary pathogen strongly indicates that an alternative biological skin opening mechanism may operate in RAS.

3.7 Pathogen relatives on intact fish skin

The pathogen-relatives from intact fish skin microbiomes affiliated taxonomically to the genera *Aliivibrio, Moritella, Tenacibaculum, Mycobacterium, Pseudoalteromonas* and *Psychrobacter*. Compared to fish ulcers, the relative abundances of *Aliivibrio, Moritella* and *Tenacibaculum* were in general lower in intact skin samples (Fig. 3). This was especially valid for the *Aliivibrio* genus. The *Aliivibrio: Moritella: Tenacibaculum* (A: M: T) ratio was 9: 3: 2 in the ulcer examined at sampling time C2W13, whereas intact skin close to the ulcer had a A:M:T ratio of 90: 1: 3. In the following production cycle, the *Moritella* genus was dominant in an ulcer with an A: M: T ratio of 1: 10: 2, while intact healthy skin close by the ulcer had an A: M: T ratio of 30: 750: 1. Thus, the A: M: T ratios in the sample from healthy skin next to an ulcer reflected in this case the situation in the fish skin ulcer quite well. Interestingly, healthy skin samples retrieved during no-ulcer formation situations had the A:M:T ratios 0:10:1 (C3W8) and 100:1:10 (C4W10). In the latter case the relative abundance of *Aliivibrio* was far higher than in the production water, whereas the situation was opposite at C3W8. The highest relative abundance observed for *Aliivibrio* was more than 20 % on intact fish skin, 0.5-1 % relative abundance in the production water and 0.1-0.5 % upon biofilter biofilm carriers (Figs. 2 and 3). Given the higher potential of enrichment of *Aliivibrio* upon fish skin we will suggest this sampling site for the aim of early warning and detailed identification. However, this is not an absolute conclusion, as accession from fish tissue for successful downstream PCR analysis is more difficult than from water.

The genus *Psychrobacter* was also strongly associated with healthy fish skin in the post-smolt RAS facility. Members of this genus is suggested to have an opportunistic lifestyle, which can affect the fish negatively and cause reduced growth, but an infective *Psychrobacter* sp. strain was recently identified in fish kidneys in a consortium with *Moritella* and *Aliivibrio* (McCarthy et al., 2013). Despite a strong association of *Psychrobacter* with fish skin, it is considered to have low fish post-mortem spoilage potential as cultivated members do not produce
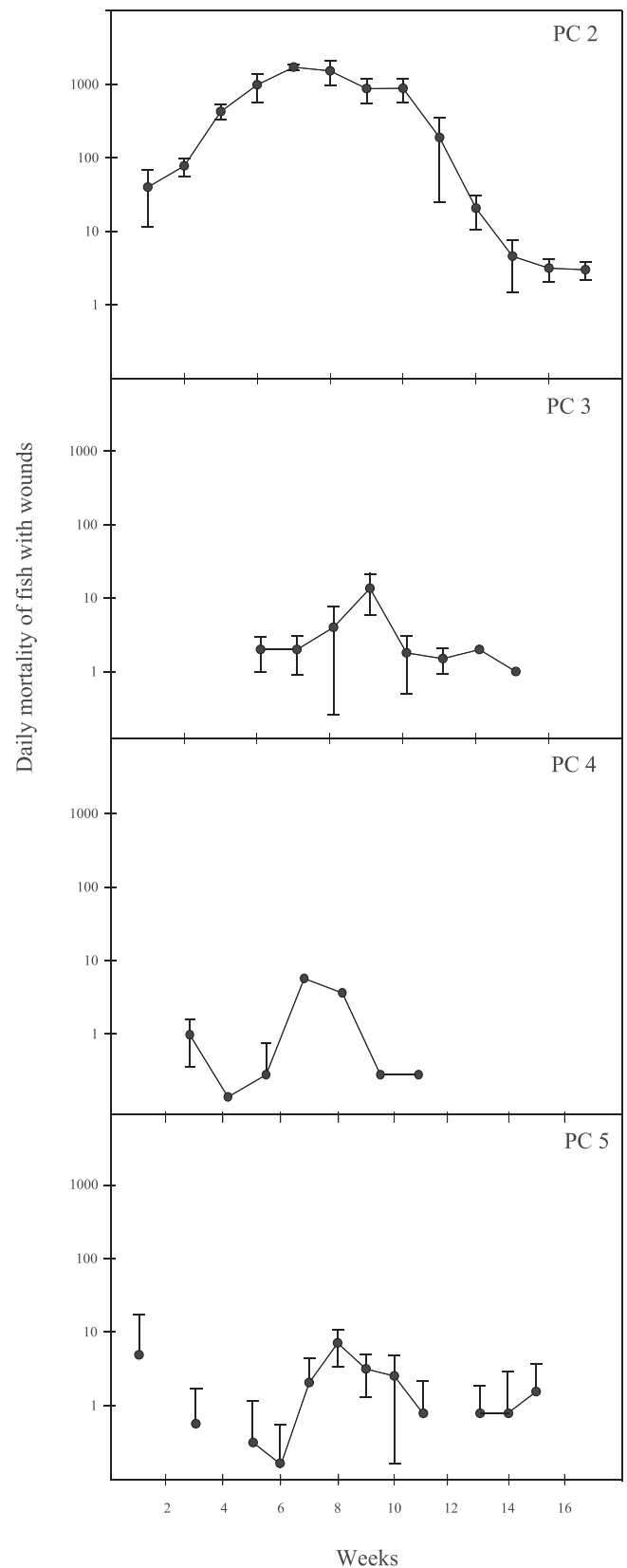
Fig. 4. Death of post-smolt during four production cycles in log scale. Each point represents the average of daily death per week, and the error bars shows the corresponding standard deviation. Black curve; Total number of dead fishes. Red curve; Dead fishes with skin ulcers. During the second production cycle, all dead fishes were register with ulcer. PC; Production cycle.

H₂S, trimethylamine (TMA), indole or have proteolytic activity (Betts, 2006).

The taxon, *Pseudoalteromonas*, did show a modest association to fish skin in the marine RAS (Fig. 3). A few *Pseudoalteromonas* members has been associated due to virulence factors, such as the *Pseudoalteromonas* strain that efficiently hydrolyze fish skin collagen (Dutilh, 2016) and the four *Pseudoalteromonas* strains that were isolated from internal organs of cultured sea beam and sea bass with fish with disease symptoms (Pujalte et al., 2007). This genus has previously been observed on post-smolt skin in a marine flow-through system, but were absent on post-smolt skin in a marine RAS test station (Lokesh and Kiron, 2016; Minniti et al., 2017). Cultivated *Pseudoalteromonas* strains can utilize a wide range of substrates, including carbohydrates, alcohols, organic acids and amino acids. In a study by Minniti et al. 2019, *Pseudoalteromonas* strongly expressed proteins associated with metabolic processes (proteolysis) and transport (siderophore transport) in the fish mucus, and the increase of bacterial proteins was correlated with a decrease of salmon proteins available in the mucus feed. This suggested bacterial utilization of salmon proteins as a nutrient source, indicating bacterial growth in mucus. The *Pseudoalteromonas* genera was not associated with fish skin ulcers in present study.

Although the *Mycobacterium* genus was present upon intact skin at three occasions (below 0.1 % relative abundance), the skin refugium was less selective for this genus as compared to water and biofilm. In general, the *Aliivibrio* and the *Psychrobacter* genera were the most abundant pathogen-relatives upon fish skin.

3.7. Correlation analysis between the identified pathogen-relatives

So far, we have discussed the identified pathogen-relatives in the marine RAS with respect to the sites where they were most abundant. A possible correlation between the pathogen relatives was still adequate to address, so a Pearson correlation analysis was performed to all the OTUs defined in the 16 S rRNA gene library in study. The correlation values for the pathogen-relatives were retrieved as reported in Table 1 of the second co-submitted Data in Brief article (Dronen et al. 2022c). Most correlation values were close to zero, but between *Aeromonas* and *Francisella* there was a strong correlation (0.74). The analysis reported also a relatively strong correlation between *Flavobacterium* and *Pseudoalteromonas*. (0.31). In one way, it was not surprising that the two pathogen-relatives that originated from the inoculum were strongly correlated, given their affiliation to the same consortium formed by selection. One the other hand, *Aeromonas* was identified in 5 samples, whereas *Francisella* was identified in 50 samples, making it difficult to see this correlation by eye. However, the Pearson correlation formula considers only average values and their variance, making the high correlation value possible to achieve. Thus, environments selecting for *Francisella* occasionally also select for *Aeromonas*. Present data suggest that this can happen both in the production water and upon the fish skin in RAS. In opposite, a co-selection between *Flavobacterium* and *Pseudoalteromonas* seemed to occur solely in the production water in the RAS.

4. Summary

This initial study was performed to provide knowledge for establishing microbial monitoring routines for a marine post-smolt RAS by use of next generation sequencing and 16S amplicon library technology. Sequences from the monitoring data (250 bp) were examined for identity to sequences of hitherto recognized fish pathogens (750-1500 bp)
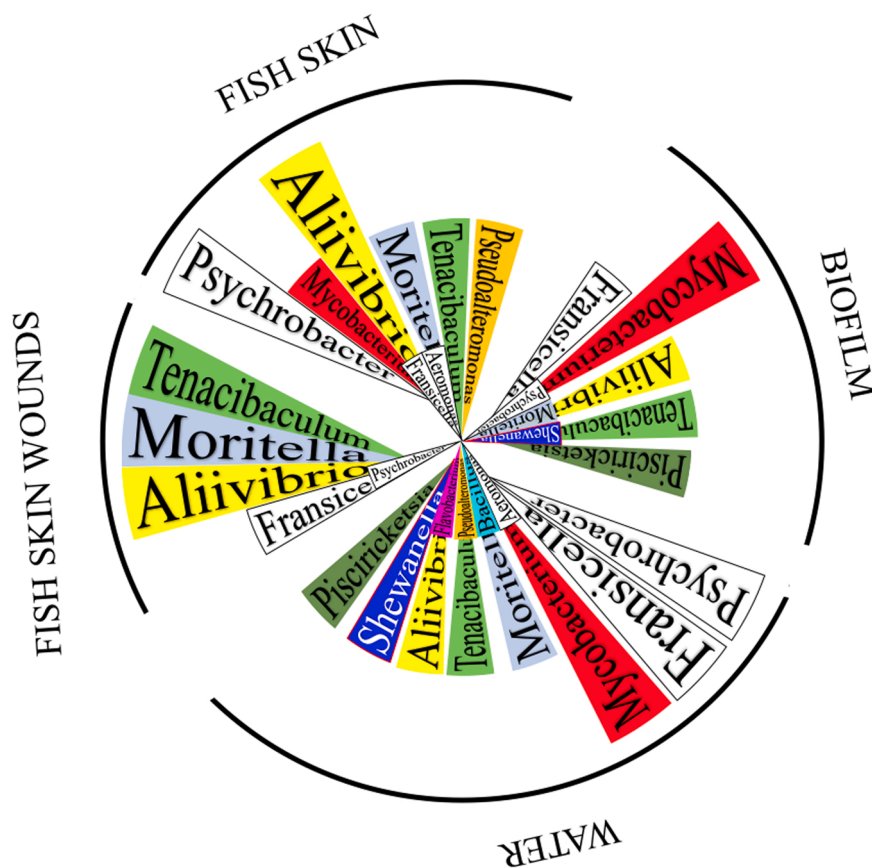

Fig. 5. Visual summary of the main findings of the article. The graphic depicts trends in association to the sampling sites of production water, biofilter biofilm carriers, intact fish skin and skin ulcers for the identified pathogen-relatives in the RAS. The categorization into low, modest or high association, as given by size of tabs, was evaluated upon number of times detected and relative abundance upon detection.

retrieved from GenBank and gathered into a Fish Pathogen Database (FPD). In total of 12 RAS library OTUs had 90-100 % similarity to the FPD sequences, and these were termed pathogen-relatives with a given percent similarity. The trends in hosting of the different pathogen-relatives based on times of appearance and relative abundance observed at the different sampling sites are summarize in Fig. 5. The biofilter biofilm was a unique site due to its remarkable low abundance of the pathogen-relatives. Fish skin wounds and fish skin as such did habitat three pathogen-relatives with high similarity to the strains of classical salmon winter ulcers, i.e., *Moritella viscosa*, *Tenacibaculum dicentrarchi* and *Aliivibrio wodanis*. The dominant role of usually secondary pathogens during severe ulceration became an unsolved enigma. All pathogen-relatives were detectable at least once in the production water, but only members of the genera *Francisella*, *Mycobacterium* and *Psychrobacter* became highly abundant. Thus, the conditions supporting their growth should be investigated further. We recommend the production water and fish skin as the best sampling sites for pathogen-relatives monitoring and pathogens early warning in the RAS system.

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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