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2 **Running title: Salmon gut microbiota**

3 A stable core gut microbiota across fresh- to saltwater transition 4 for farmed Atlantic salmon

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10

11 ABSTRACT

12 Gut microbiota associations through habitat transitions are fundamentally important, yet poorly
13 understood. One such habitat transition is the migration from fresh to salt water for anadromous
14 fish such as salmon. The aim of the current work was therefore to determine the fresh- to
15 saltwater impact on the gut microbiota in farmed Atlantic salmon, with dietary interventions
16 resembling that of fresh- and salt water diets with respect to fatty acid composition. Using deep
17 16S rRNA gene sequencing, and quantitative PCR, we found that the fresh- to salt water
18 transition both had a major association with the microbiota composition and quantity, while
19 diet did not show significantly associations with the microbiota. In salt water there was a 100-
20 fold increase in bacterial quantity, with a relative increase of *Firmicutes* and a relative decrease
21 of both *Actinobacteria* and *Proteobacteria.* Irrespective of an overall shift in microbiota
22 composition from fresh to salt water we identified three core clostridia and one *Lactobacillus*-
23 affiliated phylotype with wide geographic distribution that were highly prevalent and co-

24 occurring. Taken together, our results support the importance of the dominating bacteria in the
25 salmon gut, with the fresh water microbiota being immature. Due to the low number of
26 potentially host associated bacterial species in the salmon gut, we believe farmed salmon can
27 represent an important model for future understanding of host-bacterial interactions in aquatic
28 environments.

29

30 **IMPORTANCE**

31 Little is known about factors affecting the inter-individual distribution of gut bacteria in aquatic
32 environments. We have shown that there is a core of four highly prevalent and co-occurring
33 bacteria irrespective of feed and fresh- to saltwater transition. The potential host interactions of
34 the core bacteria, however, need to be elucidated further.

35

36 **INTRODUCTION**

37 Gut bacteria are a key part of both terrestrial and aquatic animal life. However, these contrasting
38 host-associated environments are fundamentally different with respect to dispersal and survival
39 of microorganisms (1). We are starting to understand the dispersal and importance of gut
40 bacteria in terrestrial environments (2), while our knowledge about gut bacteria in aquatic
41 environments is still very limited. In particular, little is known about the effect of environmental
42 factors such as water salinity on the inter-individual distribution of gut bacteria (3, 4).

43 For anadromous fish, fresh- to saltwater migration both represents a major shift in
44 environmental microbial exposure (3, 4) and nutrient availability – in particular lipid sources
45 which are low in long-chain polyunsaturated fatty acids (LC-PUFA) in freshwater and high in
46 saltwater (5). It has recently been shown that fresh- to saltwater transition has a major impact

47 on the skin mucosal microbiota for the anadromous Atlantic salmon (*Salmon salar*) (6).
48 However, current studies on the gut microbiota of farmed Atlantic salmon have not yet
49 addressed the impact of this transition (7-14), and how the environmental exposure and/or
50 nutrient availability affects the composition, and inter-individual distribution of the gut
51 microbiota.

52 Accordingly, the aim of our work was to investigate the effect of fresh- to saltwater transition
53 under two contrasting diets that have a freshwater-type lipid composition low in LC-PUFA, and
54 a high LC-PUFA marine-like lipid composition. In order to explore the microbiota we used a
55 combination of quantitative PCR and 16S rRNA gene deep sequencing.

56 We present results showing a distinct shift in overall microbiota potentially associated with the
57 fresh- to saltwater transition, while there were four co-occurring core bacterial with wide
58 geographic dispersal exerting stability across this transition.

59

60 RESULTS

61 **Characterization of microbiota composition and distribution.** By deep sequencing we
62 obtained a total number 13 752 775 of paired-end merged 16S rRNA gene sequences passing
63 the quality filter. For these we identified a total of 1179 prokaryote OTUs belonging to 20 phyla,
64 with 5 phyla constituting > 90% of the microbiota.

65 The overall microbiota composition differed clearly between fresh and salt water type, as seen
66 in Figure 2 A and B, and from the ANOVA, where this effect was very clear ($p < 10^{-10}$). There
67 were 413 OTU's that were significantly affected by the fresh- to salt water transition ($p<0.05$,
68 BH FDR corrected), for which a majority (76.5%) showed decrease in salt water. The frequency
69 of OTUs with high relative quantity, on the other hand, increased in salt water (Suppl. Fig. 1).
70 The main taxonomic shift from fresh to salt water was a decrease in both *Actinobacteria*

(median 4.4% vs 3.5%, $p<0.0005$) and *Proteobacteria* (median 7.6% vs 5.4%, $p=0.002$), while *Firmicutes* showed a major increase (median 48.5% vs 72.7%, $p<0.0005$). Both the classes *Clostridia* (median 33.6% vs 50.2%, $p<0.0005$) and *Bacilli* (median 14.9% vs 20.5%, $p<0.0005$) increased. *Alphaproteobacteria* increased (median 0.7% vs 1.2%, $p<0.0005$), despite the general decrease of *Proteobacteria.* Similarly, *Coriobacteriaceae* increased (1.6% vs 2.2%, $p<0.0005$), irrespective of the general decrease in *Actinobacteria*.

Fig. 3 illustrates the fresh- to saltwater shift in prevalence for the most abundant OTUs. Although OTU4 (classified as *Corynebacterium*) showed a major decrease in prevalence from fresh to salt water (44% vs 0.61%), this OTU did not show a significant relative quantitative decrease (0.087% vs 0.12%, $p=0.99$). OTU 18 (*Pseudomonas*) decreased in prevalence (65.8% vs 0.6%) as well as relative quantity (1.5% vs 0.0%, $p<0.0005$). The OTUs with the largest fresh to salt water increase were OTU 13 (*Bradyrhizobium*) with a prevalence (6% vs 52.4%) and relative quantity (0.01% vs 1.0%, $p<0.0005$), and OTU 21 (*Lactobacillus*) with a prevalence of (0.0% vs. 67.7%) and relative quantity (0.26 % vs 1.2 %, $p<0.0005$). All the OTUs showing major fresh to salt water shifts also had closely related sequences in the Scottish dataset (Suppl. Table 1).

There was a more even distribution of rarefaction curves for salt water, as compared to fresh water samples, with more high abundant OTUs in salt water (Suppl. Fig. 1). Water type also showed significant differences in alpha diversity, where salt water showed higher index levels than fresh water (Fig. 4A and B)., while beta diversity showed higher levels in fresh water compared to salt water (Fig. 4C). Using quantitative PCR, we also identified a major (> 100-fold) increase in the ratio of bacterial DNA to eukaryote DNA from fresh- to saltwater transition, as determined from SSU gene copies (Fig. 4D).

94 Amplicon sequencing of eukaryote SSU from fresh water revealed that > 95% of the eukaryote
95 sequences belong to salmon. By gel electrophoresis we found DNA with a size distribution with
96 bands about 180 bp apart, resembling DNA from apoptotic cells (Suppl. Fig. 3).

97 Diets (vegetable versus marine-oil based feed) and feed switch did not significantly affect the
98 microbiota composition, neither in the fresh- nor the saltwater phase. ANOVA showed no
99 significant main effects for any of the feeding regimes on the overall microbiota composition.
100 Furthermore, diet did not show any effect on alpha diversity (Fig. 4 A and B), while there was
101 a slight but significant effect on beta diversity for marine oil in fresh water (Fig. 4C).

102 **Overlap in microbiota across fresh and salt water.** For the overall overlap in OTUs we found
103 that 818 OTUs (69%) were shared across fresh and salt water. However, the number of unique
104 OTUs were higher for fresh water than for salt water with 245 (21%) vs 117 (10%),
105 respectively. Of the OTUs shared across fresh and salt water, a subset of 408 OTUs (34%)
106 were also shared with a Scottish freshwater dataset consisting of commercial and aquarium
107 breed parr kept under different feeding regimes (7). Furthermore, 38 (3.2%) of the Scottish
108 OTUs were uniquely shared with the freshwater dataset and 14 (1.2%) with salt water.

109 Overall, the abundant OTUs (> 1% within an individual) were more prevalent in salt water than
110 in fresh water (Fig. 5). There were four bacterial core OTUs (OTU1, OTU2, OTU6 and OTU10)
111 affiliated with the *Firmicutes* that were abundant in more than 90% of the fishes in both fresh
112 and salt water. All the core OTUs showed positive relative quantitative co-occurrence across
113 fishes in both fresh and salt water (Fig. 6A and B), in addition to a general increase in relative
114 quantity from fresh to salt water (Fig. 6B). All the core OTUs also showed close matches (>
115 97% identity) to OTUs from the Scottish dataset (Suppl. Table 1).

116

117

118 **DISCUSSION**

119 We found that the salt- to freshwater transition had a major effect on the microbiota
120 composition, while marine or vegetable oil in the diet only had a minor effect. Salinity
121 represents a major environmental barrier for microbes (15). The fresh water gut microbiota was
122 the least mature having lower bacterial load, lower alpha diversity and sharing of core OTU's,
123 in addition to higher levels of low abundant OTUs and higher beta diversity compared to salt
124 water. A recent study showed an apparent opposite diversity pattern for the salmon skin
125 microbiota, with higher alpha diversity in fresh water than salt water (6). For the skin
126 microbiota, the diversity difference is explained by the fresh water microbiota being more
127 mature than the salt water microbiota (6). A potential explanation for the salt water maturity
128 difference between skin and gut microbiota could be that the gut microbiota is more protected
129 towards the direct contact with the saltwater than the skin microbiota, which allows continued
130 maturation through the fresh to salt water transition.

131 Since LC-PUFA is required in high relatively quantity in fresh water (5), the low density
132 immature fresh water microbiota would most likely not be sufficient to support the LC-PUFA
133 requirement. We therefore find it unlikely that the gut microbiota play an important role in
134 alleviating limitations in LC-PUFA in freshwater ecosystems.

135 We found a dominance of *Firmicutes* at both the parr and post smolt stage, while wild salmon
136 was dominated by *Proteobacteria* for the corresponding life-stages (10). The difference in the
137 *Firmicutes* to *Proteobacteria* ratio between wild and farmed salmon resembled that of high and
138 low fat diets, where high fat diet increase the *Firmicutes* to *Proteobacteria* ratio (16). Thus, the
139 wild and farmed salmon differences in gut microbiota could partly reflect the high fat and
140 energy content in the farmed salmon feed, as compared to that of the natural diet (17).

141 A subset of 4 OTU's showed high stability for the fresh- to salt water transition. Stability across
142 the fresh- to saltwater transition may indicate strong host associations of the core OTUs in the
143 salmon gut, despite the major shift in the overall microbiota. The core genus *Vagococcus* is
144 related to mucin utilizing species (18). Mucin utilization could potentially explain a close host
145 association for the *Vagococcus*-affiliated core OTU (19), with the positive correlations for the
146 rest of the core OTUs may either indicate cross-feeding, syntrophy, or association with other
147 correlated factors. Specific mechanistic studies, however, are needed to determine the
148 underlying cause for the positive correlations of the core OTU's.

149 Previous studies on identifying core OTUs in the salmon gut of farmed salmon, however,
150 suggest a relatively high number and wide diversity of core OTUs (7, 8). These studies include
151 a relatively low number of fish (< 50), not covering the fresh- to saltwater transition. This may
152 have led to overestimation of core OTUs. However, although we identified the core OTUs in a
153 Scottish dataset, in both fresh and salt water and under different feeding regimes, the datasets
154 are still too limited to claim universal distribution.

155 In conclusion, we have shown a major shift microbiota composition, diversity and quantity for
156 the fresh to salt water transition, with four core bacteria showing high prevalence and co-
157 occurrence across this transition.

158

159 **MATERIALS AND METHODS**

160 **Fish maintenance and sampling procedure.** Fish were sampled from two replicate fish tanks
161 where they were fed vegetable oil (VO) or marine oil (MA) based feeds (total 4 tanks). VO
162 based feeds contained a combination of linseed oil and palm oil at a ratio of 1.8:1 and FO based
163 feeds contained only North Atlantic fish oil. A feed switch to the alternative diet was introduced
164 for half of the fish in fresh water (parr stage – approx. 50 g) and then repeated as the fish
165 transitioned into sea water (post smolt – approx. 200 g). Smoltification was triggered by 5

166 weeks of winter-like conditions with 12 hours of light per day followed by spring-like
167 conditions with 24 hours of light per day. Salmon were then immediately switched to salt water
168 and allowed to acclimate for 3 weeks before first sampling (5). Gut microbiota sampling was
169 conducted immediately before the feed switch (day 0) in both fresh and salt water, and at days
170 1, 2, 6, 9, 16 and 20 after the switches. The experimental setup is schematically outlined in Fig.
171 1.

172 **Sampling and DNA extraction.** Sampling procedure involved antiseptically squeezing out the
173 complete gut content by using tweezers. Gut content samples were collected in 2 ml sample
174 tubes (Sarstedt, Germany) prefilled with ~0.2 g acid washed beads (≤106 µm in diameter;
175 Sigma-Aldrich, Germany) and 400 µl Stool Transport and Recovery buffer (Roche, Germany)
176 before long term storage at -40°C .

177 Samples (n=180 from fresh water, n=169 from salt water) were thawed and homogenized by
178 bead beating in a MagNA Lyser instrument (Roche, Germany) for 2 x 20 sec at 6500 rpm with
179 a 1 min rest between runs. DNA was isolated using a LGC Mag Midi DNA extraction kit (LGC
180 Genomics, UK) according to the manufacturer's instructions. Extracted DNA was quantified
181 by Qubit dsDNA HS assay kit (Thermo Fisher Scientific, United States), and analyzed on 1%
182 agarose gel.

183 **Quantitative PCR.** To quantify the number of eukaryotic and prokaryotic SSU genes,
184 quantitative PCR was performed using LightCycler 480 II (Roche, Germany), with primer pairs
185 PRK341F (5'-CCTACGGGRBGCASCAG-3') / PRK806R (5'-GGACTACYVGGGTATCT-
186 AAT-'3) (20) targeting the V3-V4 region of the prokaryotic SSU gene, and 3NDF (5'-
187 GGCAAGTCTGGTGCCAG-3') (21)/V4EukR2 (5'-ACGGTATCTRATCRTCTTCG-3') (22)
188 targeting V4 region of the eukaryotic SSU gene. Reactions were performed in 20 µl volumes
189 containing 1× Hot FirePol EvaGreen qPCR Supermix (Solis BioDyne, Estonia), 0.2 µM of each

190 primer, and 1 µl genomic DNA (0.2-30 ng) . Thermal conditions involved initial denaturation
191 at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °
192 C (in PCR targeting prokaryotes) or 59 °C (in PCR targeting eukaryotes) for 30 sec, and
193 elongation at 72 °C for 45 sec.

194 **Illumina Sequencing.** The taxonomic composition of the microbiota was determined by
195 sequencing the resulting amplicons from a two-step PCR using the same primers as used in
196 quantitative PCR. Amplification was performed in 25 µl volumes containing 1x HotFirePol
197 Blend master mix ready to load (Solis BioDyne, Estonia), 0.2 µM of both primers (Thermo
198 Fisher Scientific, United States) and 2 µl (0.4-60 ng) genomic DNA. First PCR was performed
199 with initial denaturation at 95°C for 15 minutes, followed by 30 cycles of identical denaturation,
200 annealing and elongation steps as done in qPCRs. A final elongation at 72 °C for 7 min was
201 included. Resulting amplicons were purified with AMPure XP beads (Beckman-Coulter,
202 United States), following the manufacturer's instructions. For attachment of dual indices and
203 Illumina sequencing adapters, a second PCR was performed with Illumina-modified prokaryote
204 and eukaryote primers following same conditions as before, only with 12 cycles and an
205 increased annealing step to 1 min. Amplicon libraries were quantified by Qubit dsDNA HS
206 assay kit and normalized to a sequencing pool before purification by AMPure XP beads. Final
207 library was quantified in a QX200™ Droplet Digital™ PCR System (Bio-Rad, United States)
208 using primers targeting Illumina-adaptors, following the manufacturers recommendations.
209 Sequencing was performed on a MiSeq platform (Illumina, United States) using v3 chemistry
210 with 300 base pairs paired-end reads.

211 The resulting amplicon reads were processed (de-multiplexing, primer removal, merging,
212 filtering, de-replicating, OTU-clustering and filtering of chimeras) using a standard procedure
213 associated with the USEARCH 9.0 software (23), with taxonomic assignments using the RDP
214 database (24) and BLAST for eukaryote SSU genes (25). Comparison between this data and an

215 additional Scottish prokaryote SSU dataset (7) were done using BLAST with representative
216 sequences for the OTUs towards a database for the Scottish SSU sequences. A match was
217 assigned if the hit length was > 300 bp and identity > 97%. Read-counts and characteristic
218 sequences for OTUs are available at (www.fairdomhub.org/data_files/1585).

219 **Data analysis**. OTU data were analyzed in the R computing environment (https://www.r-
220 project.org/). For each sample we computed the taxonomic profile as follows: For sample i
221 ($i=1,\dots,N$) and OTU j ($j=1,\dots,P$) we have the read-count c_{ij}. For each sample we compute the
222 relative abundance

223
$$r_{ij} = \frac{c_{ij} + q}{\sum_{j=1}^{P}(c_{ij} + q)}$$

224 Where q is a pseudo-count added to all read-counts, required below. We used $q=1$ in this
225 analysis. The vector of relative abundances for a sample is an example of compositional data,
226 and for such data a commonly used transform is the Aitchison log-ratio transform (17):

227
$$x_{ij} = \log_2\left(\frac{r_{ij}}{\left(\prod_{j=1}^{P} r_{ij}\right)^{1/P}}\right)$$

228 Thus, the taxonomic profile value x_{ij} is the logarithm of the relative abundance divided by its
229 geometric mean. The pseudo-counts added are essential to avoid zeros in the denominator of
230 this transform. This transform is often beneficial when later using some kind of sum-of-squares
231 analysis (e.g. PCA, ANOVA, Euclidean distances) (17). For sample i the vector $\boldsymbol{x}_i=(x_{i1},\dots,x_{iP})$
232 was arranged as row number i in the OUT-matrix $\boldsymbol{X}$ of taxonomic profiles (N rows and P
233 columns).

234 Based on the matrix $\boldsymbol{X}$ we used Principal Component Analysis to get an overview of the
235 variations in taxonomic profiles. More specifically, the PCA-scores of the first components

236 were used in ANOVA to test for effects of water-type *W* (fresh, salt), diet *D* (vegetable-oil,
237 vegetable-to-fish-oil, fish-oil, fish-oil-to-vegetable-oil) and sampling day *S* (0,1,2,6,9,16,20)

238 $$y_{ijkl} = \mu + W_i + D_j + S_k + e_{ijkl}$$

239 Where $i=1,2$, $j=1,...,4$, $k=1,...,7$. As the response y_{ijkl} we used PCA-scores from components
240 1,2,…,5 in turn, reflecting different aspects of change in microbiota composition.

241 We used the Kruskal–Wallis test for non-parametric comparison of means. False discovery rate
242 (FDR) correction was done using the Benjamin and Hochberg approach (26).

243 **Accession number(s).** The raw data reads obtained from the 16S rRNA gene sequencing are
244 available in the Sequence Read Archive (SRA) database under accession number SRP119730
245 (https://trace.ncbi.nlm.nih.gov/Traces/sra/?study=SRP119730).

246

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252

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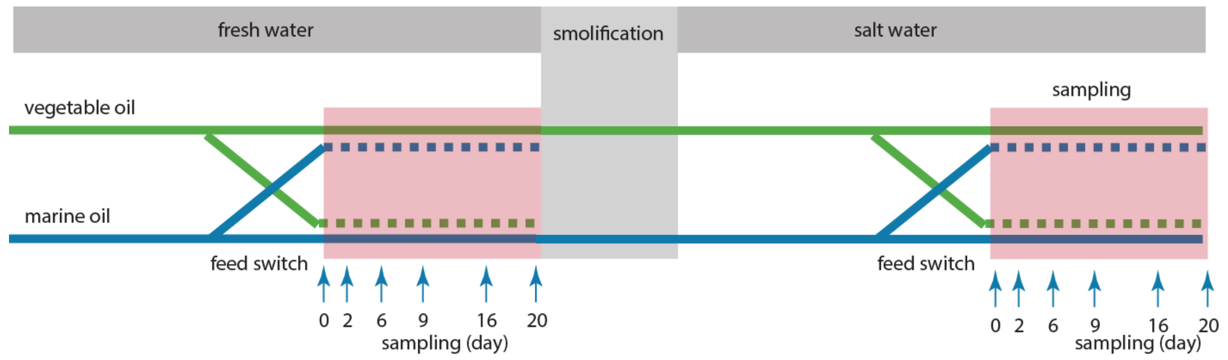
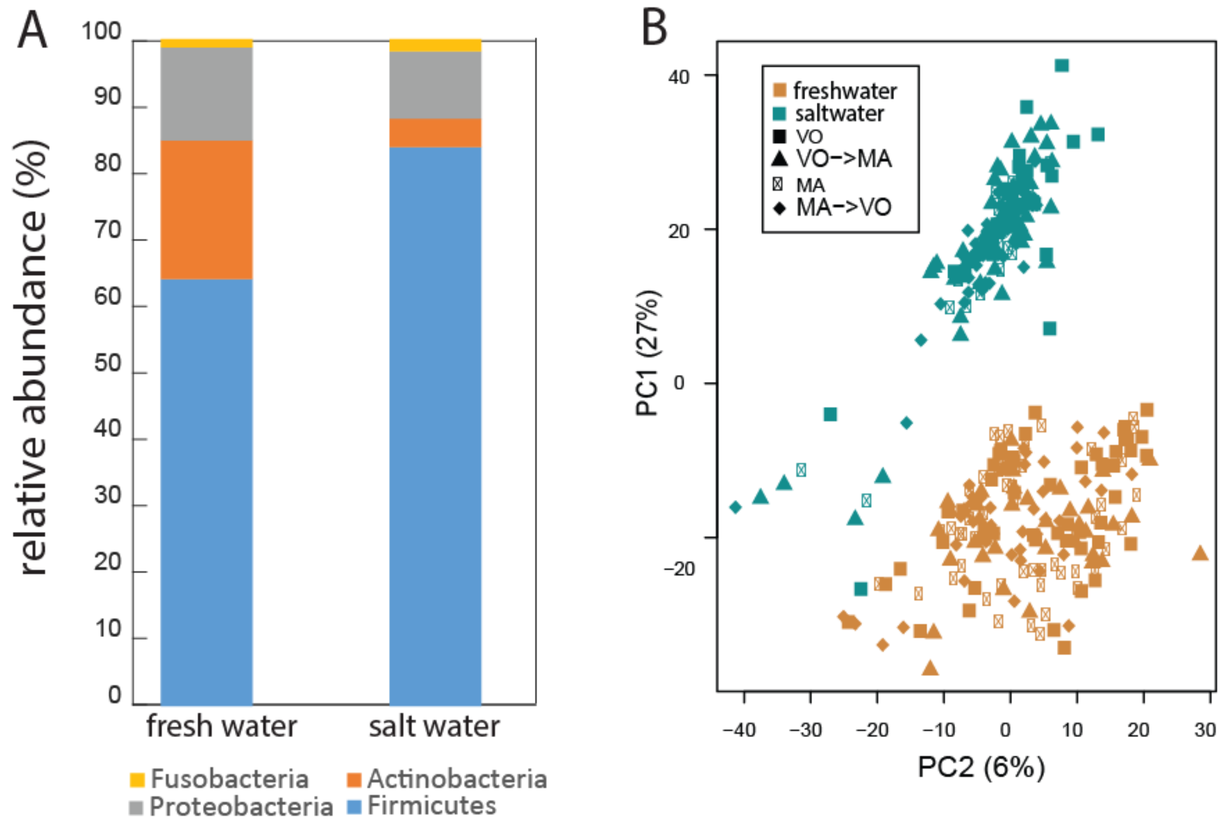
330

331 **FIGURES**



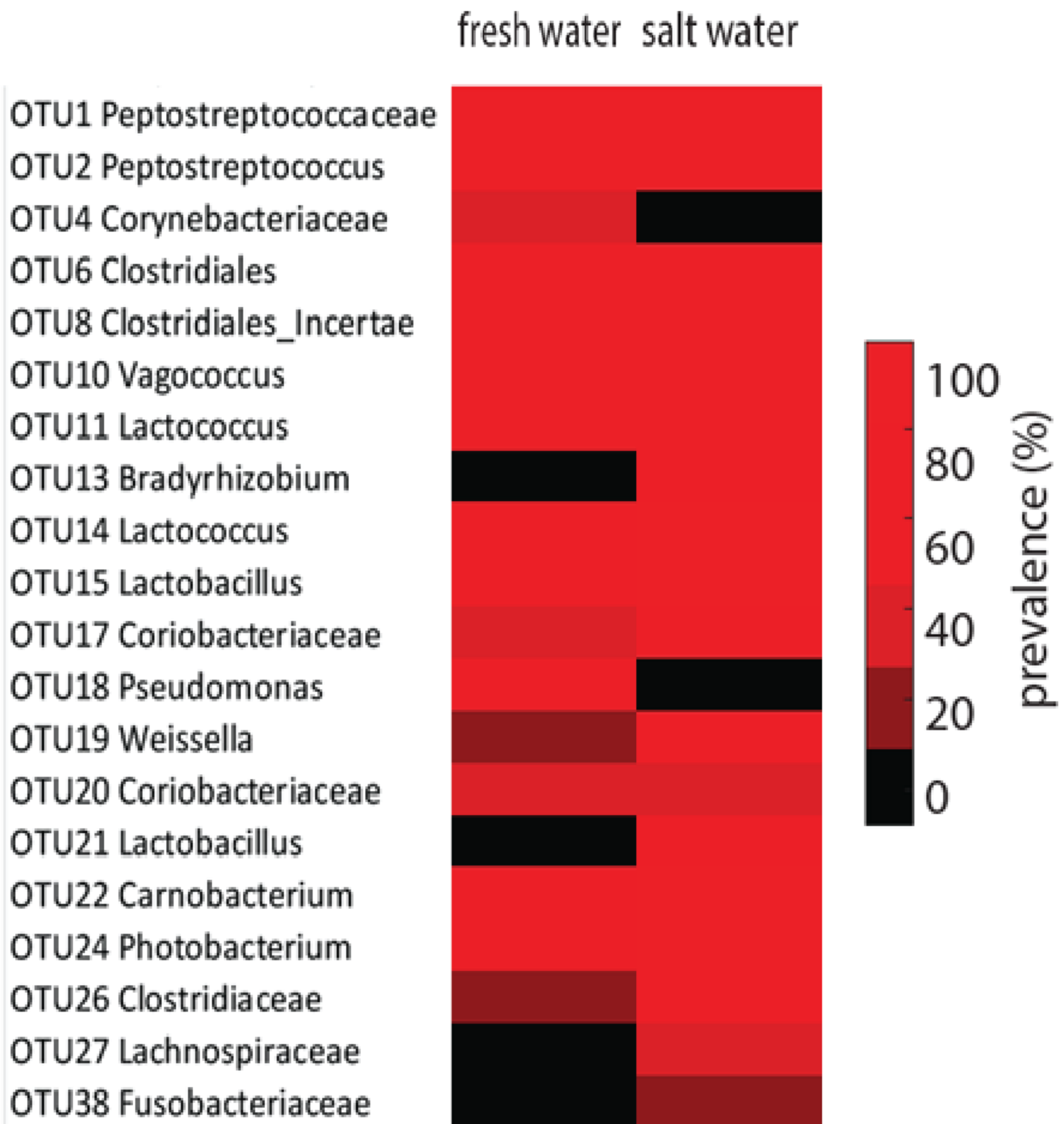
332

333 **Figure 1. Outline of the experimental setup.** For each experimental period the fishes were given either a diet
334 based on vegetable or marine oil. The numbers of samples (n) analyzed for each feeding category is included.



335

336 **Figure 2. Composition (A) and distribution (B) of the microbiota in salt and fresh water.** (A) Distribution in
337 fresh and salt water for dominant bacterial phyla. (B) The distribution across treatments, fresh and salt water are
338 illustrated by PCA analyses. VO; vegetable oil and MA; marine oil.



339

340 **Figure 3. Prevalence of OTUs in fresh and salt water across treatments**, measured as the proportion of samples
341 where each OTU made up more than 1% of reads. Only bacterial OTUs which was present in more than 10 % of
342 all samples are shown.

343

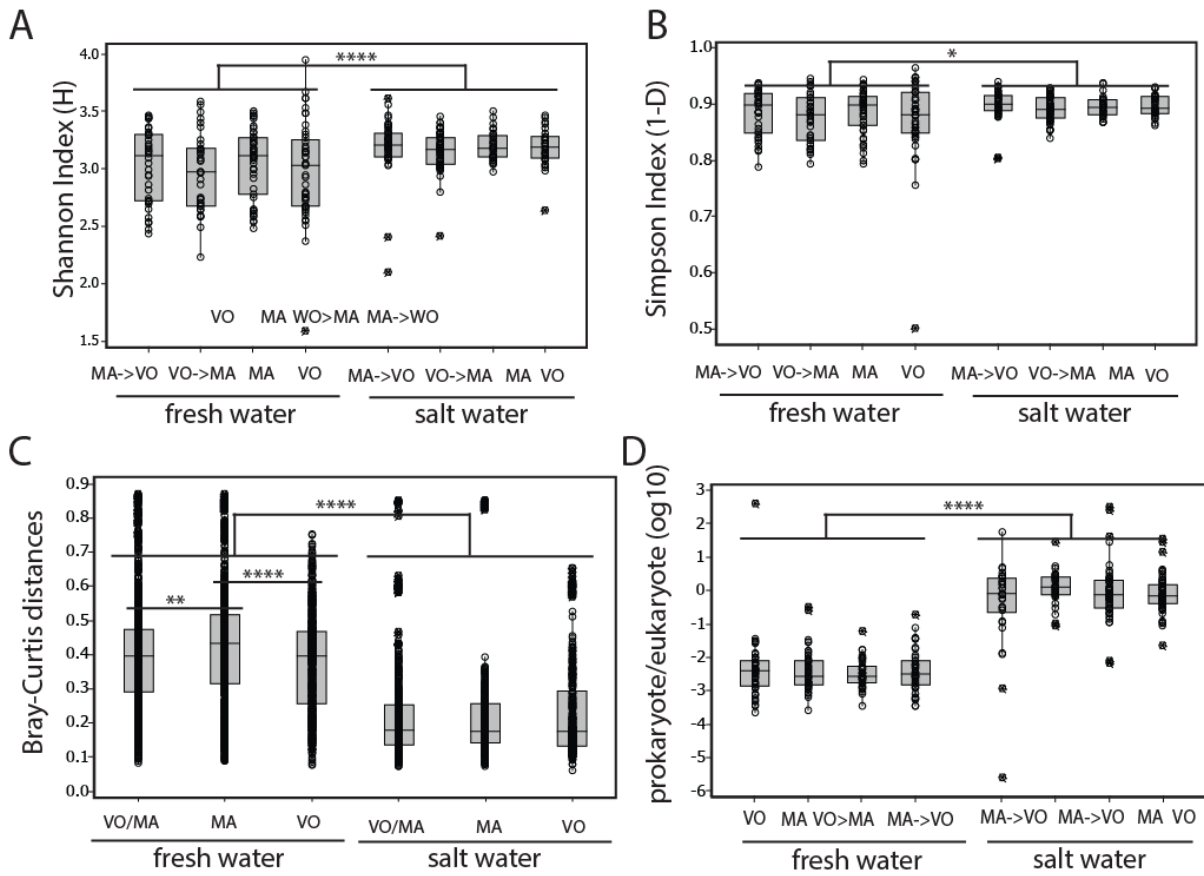
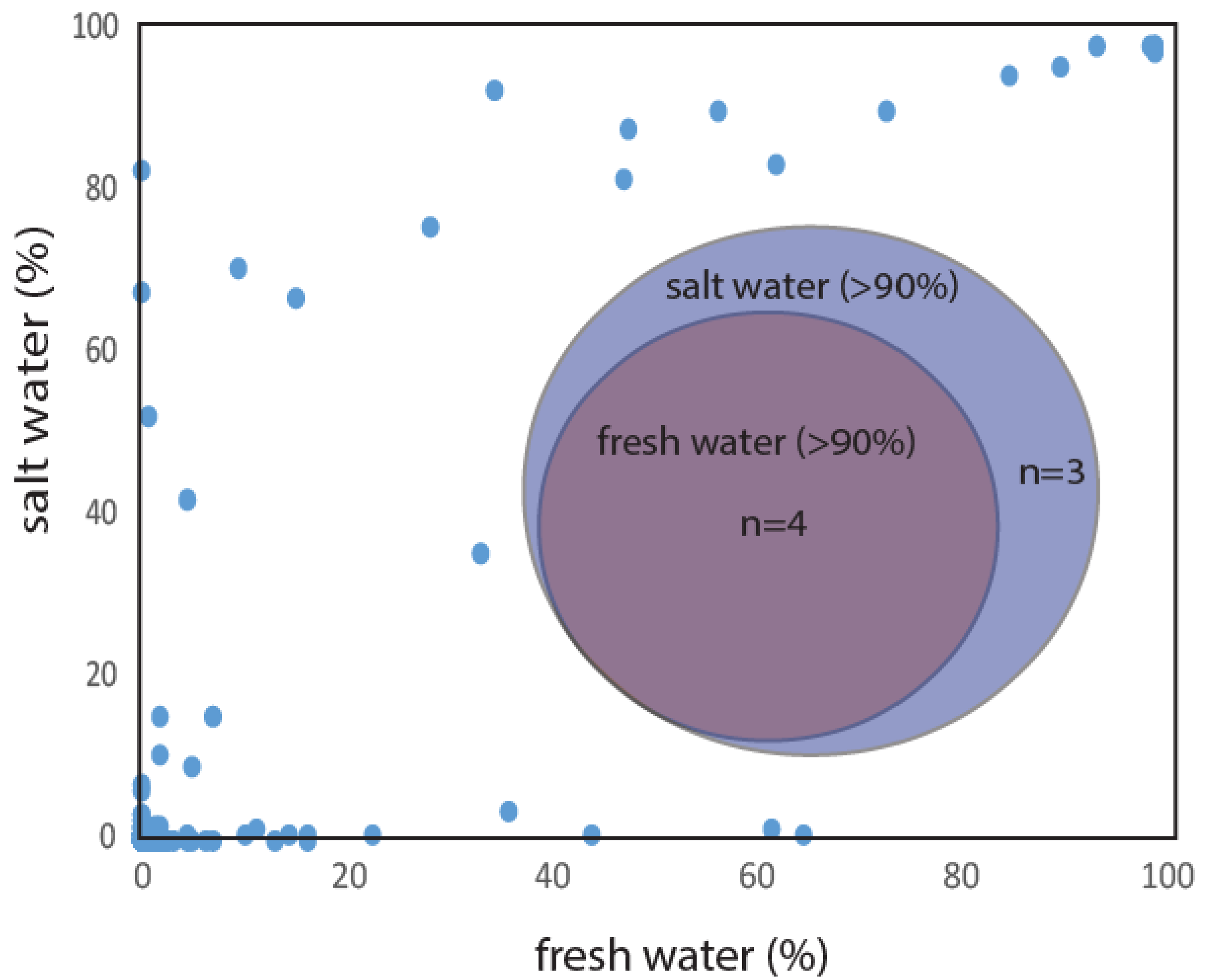


Figure 4. Alpha diversity (A and B), beta diversity (C) and quantity (E) in fresh and salt water of the microbiota. (A and B) Alpha diversity was determined respectively by Shannon – and Simpson index. (C) Bray-Curtis was used to determine beta diversity, and (D) the quantity of prokaryotes were determined relative to the level of eukaryote DNA based on SSU gene copies. The following abbreviations were used: VO; vegetable oil, MA; marine oil, and VO/MA comparison between vegetable and marine oil, VO->MA; switch from vegetable to marine oil, MA->VO; switch from marine to vegetable oil. P-values are indicated with the following symbols: **** p<0.0001, ** p<0.01, * p<0.05

352

353



354

355 **Figure 5. Distribution of OTUs across fresh and salt water.** Association between respective prevalence of
356 OTUs present > 1% in both salt and fresh water. Embedded circles indicate overlap between core OTUs found in
357 more than 90% samples in fresh and salt water.

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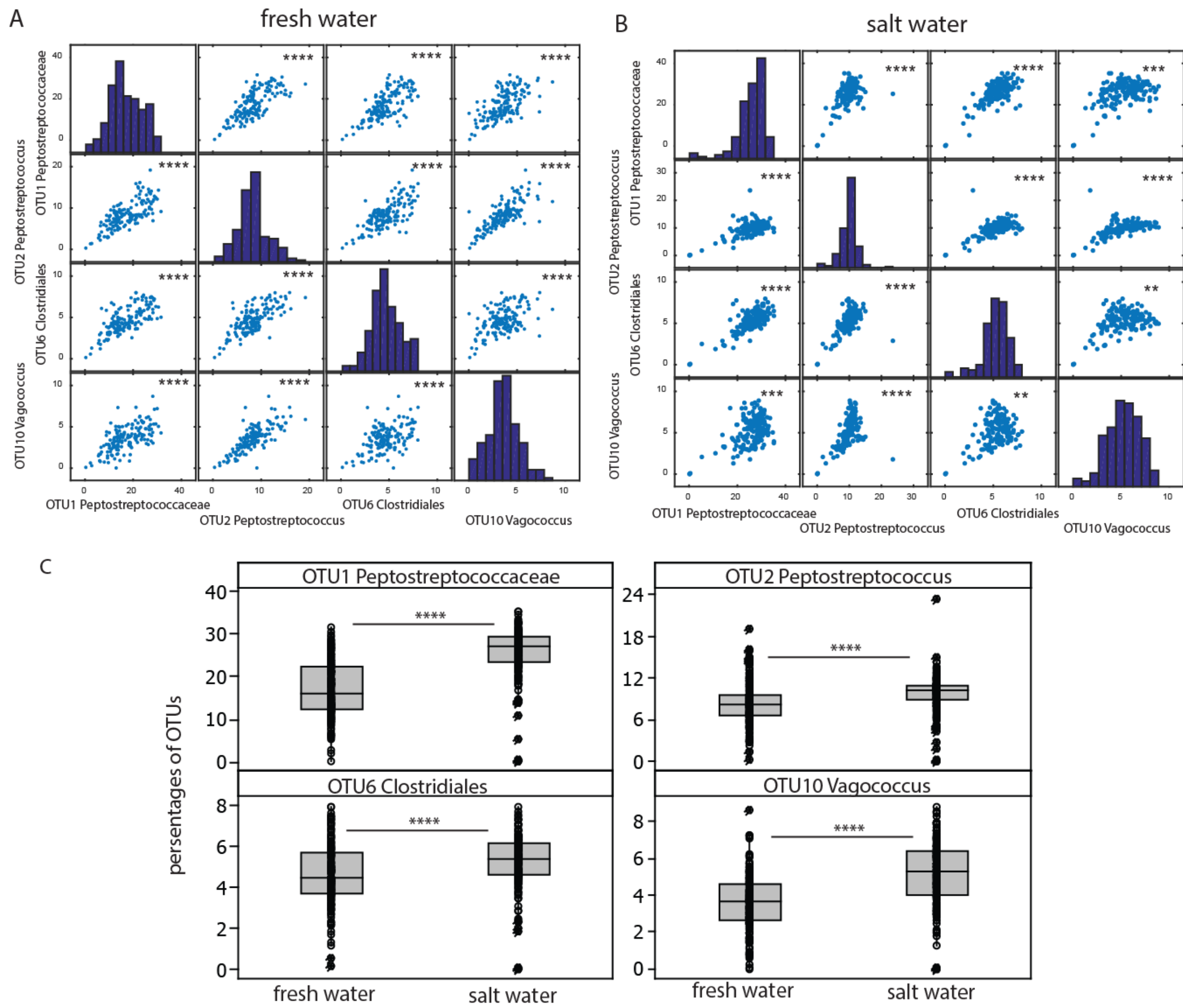

359

360 **Figure 6. Scatterplot matrices for percentages of core OTUs in fresh water (A) and in salt water (B), and**
361 **relative quantity (C).** Correlations between the relative abundance of core OTUs were determined using
362 Spearman correlations for fresh (A) and salt water (B). Differences in levels of OTUs were determined by Kruskal-
363 Wallis test C) P-values are indicated with the following symbols: **** p<0.0001, *** p<0.001, ** p<0.01

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365