

1           **Investigating the involvement of a *Midichloria* -like organism (MLO) in red mark**  
2   **syndrome in rainbow trout *Oncorhynchus mykiss***

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14           **Justification:** Red mark syndrome is a disease of economic concern that impacts on the  
15           marketability of rainbow trout stocks. The disease is still of unknown cause meaning that  
16           treatment is still symptomatic. Insight in the aetiology will increase treatment options and the  
17           welfare of the fish.

18           **Key words:** Red mark syndrome; *Oncorhynchus mykiss*; *Midichloria* -like organism; disease  
19           correlation, epidemiology, immunohistochemistry, quantitative PCR.

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21 **Abstract**

22 Red mark syndrome (RMS) is a skin condition in Rainbow trout *Oncorhynchus mykiss* that has  
23 been reported worldwide but was first seen in the United Kingdom (UK) in 2003. The current  
24 study was conducted to examine if there was an association between a *Midichloria*-like  
25 organism (MLO) and RMS using a statistically appropriate sample set, whilst determining if  
26 there is a lack of association with *Flavobacterium psychrophilum* implicated in disease in  
27 previous studies. Fish in this study were obtained from three sites positive for RMS in the UK  
28 and United States (US), and three sites in the UK and the Netherlands that had no previous  
29 history of this condition. Samples taken from RMS-affected sites were found to show typical  
30 RMS pathology. Analysis of the major organs of affected fish by quantitative polymerase chain  
31 reaction (qPCR) demonstrated a significantly higher presence of the MLO in the RMS-affected  
32 tissues. Although most of the tissues were positive for the MLO, the highest correlation was  
33 seen in the skin, whilst the tissues from the unaffected fish were all negative. Thus, a strong  
34 positive correlation was found between the MLO and RMS-affected fish, whilst no association  
35 was found between the RMS-affected fish and *F. psychrophilum* other than superficial presence  
36 in the skin. The use of immunohistochemistry showed positive staining of what was considered  
37 to be MLO-related antigens in the internal organs of most RMS-affected fish. Attempts were  
38 made to culture the MLO, but no MLO was isolated.

## 39 1. Introduction

40 Red mark syndrome (RMS) is a skin condition affecting rainbow trout (*Oncorhynchus mykiss*)  
41 in freshwater. Although the disease does not result in significant mortalities, it does cause  
42 considerable economic losses to the trout industry due to the downgrading of the fish at the  
43 time of harvest. RMS first appeared in the United Kingdom (UK) in late 2003 and has since  
44 spread to over more than 50% of trout farms in the UK (Verner-Jeffreys *et al.*, 2006). It has  
45 also now been reported in many European countries, and further afield as far as Iran and Chile  
46 (Sandoval *et al.*, 2016; Sasani *et al.*, 2016). A similar skin condition to RMS, known as  
47 strawberry disease (SD), has been present in rainbow trout in the United States (US) since the  
48 mid-1940s (Davis, 1946). The initial outbreak of RMS was linked to an egg importation from  
49 the US to the UK (Verner-Jeffreys *et al.*, 2006). Red mark syndrome is characterised by the  
50 clinical presentation of lesions that appear as slightly raised and well demarcated. The lesions  
51 are often located across or below the lateral line and can appear haemorrhagic with scales  
52 missing from the centre of the lesion. Acute inflammation with the presence of neutrophils can  
53 be observed around the area of scale loss together with high protein oedema (Ferguson, 2006;  
54 Oidtmann, 2013).

55 The aetiology of RMS has not been definitively identified to date, but the cause is  
56 believed to be bacterial in nature (Ferguson *et al.*, 2006; Verner-Jeffreys *et al.*, 2008). Ferguson  
57 *et al.* (2006) studied a small number of SD and RMS affected fish from the US and UK,  
58 respectively and suggested an association between RMS in Scottish fish and *Flavobacterium*  
59 *psychrophilum* using a polymerase chain reaction (PCR). Lloyd *et al.* (2008) later found a  
60 strong correlation between SD lesions and the presence of a Rickettsia-like organism (RLO)  
61 16S rRNA sequence. Samples of RMS from UK gave similar results to those obtained for SD  
62 samples from the US when examined by quantitative PCR (qPCR) (based on the RLO 16S  
63 rRNA sequence) and immunohistochemistry (IHC) (Metselaar *et al.*, 2010). It is therefore,

64 considered that RMS and SD are the same condition in different geographic locations  
65 (Metselaar *et al.*, 2010; Oidtmann *et al.*, 2013) and therefore only refer to the condition as RMS  
66 in the present study. More recently a *Midichloria* -like organism (MLO), belonging to the  
67 family Midichloriaceae within the Order Rickettsiales, has been associated with RMS (Cafiso  
68 *et al.*, 2015) and we will therefore subsequently refer to RLO as an MLO.

69         The exact involvement of the MLO and *F. psychrophilum* in the pathology of RMS is  
70 still unclear. The aim of the current study was to investigate this relationship further. In order  
71 to have a clearer understanding of their role in RMS, 30 fish were sampled from RMS-affected  
72 fish from sites in the US and UK with a history of the condition (positive farms) and compared  
73 with 30 fish from unaffected farms that have never experienced RMS outbreaks and which  
74 were located on water systems free of RMS-affected farms (negative farms) in the Netherlands  
75 and UK. To confirm the disease status of the fish, tissues were examined for the presence of  
76 RMS-related pathology. Primers for the MLO (Lloyd *et al.*, 2008; Lloyd *et al.*, 2011; [Montagna](#)  
77 [et al.](#), 2013) were used to investigate the association between the MLO and RMS in the samples  
78 from the two sets of farms. As the qPCR allows the quantity of MLO DNA present in the tissue  
79 to be determined, this can be correlated to the disease status of the animal, adding to the  
80 evidence that the MLO is the causative agent of RMS. Immunohistochemistry with anti-  
81 *Piscirickettsia salmonis* monoclonal antibodies (MAbs), shown to cross-react with RMS-  
82 affected tissue (Metselaar *et al.*, 2010), and anti-*F. psychrophilum* polyclonal antibodies (PAb)  
83 (Faruk *et al.*, 2002) were used to determine if there was an association of these pathogens with  
84 the pathology. Several agars known to support the growth of intracellular bacteria were also  
85 tried to establish if it was possible to culture any other bacterial agents from the fish. Standard  
86 bacteriology was used to ascertain if *F. psychrophilum* was present, with predominant yellow-  
87 pigmented bacteria recovered from the fish identified through conventional 16s partial rRNA

88 gene sequencing. Finally, a serological analysis was performed to evaluate the fish's antibody  
89 response to *F. psychrophilum* and MLO by ELISA.

## 90 **2. Material and methods**

### 91 **2.1 Fish sampling**

92 Sixty fish (ten per farm) were randomly selected from six unrelated rainbow trout farms (details  
93 of each site are withheld for confidentiality). Sample size was based on OIE recommendations  
94 for detection of disease prevalence of >10% with 95% confidence (OIE - Aquatic Animal  
95 Health Code, 2015). Tissue samples from skin (affected as well as unaffected skin), heart, liver,  
96 gill, kidney and spleen were collected for histology. Four farms in the UK were sampled two  
97 farms that had never experienced an outbreak of RMS and were not located on water systems  
98 with farms with a history RMS outbreaks (Farms 1 and 2) and two farms with on-going clinical  
99 RMS (Farms 4 and 5). In addition, samples were collected from one farm in the USA (Farm 6),  
100 where fish were exhibiting clinical signs, and, from a single farm (Farm 3) in the Netherlands,  
101 regarded as RMS-free. Bacteriological samples were taken from the skin lesions or unaffected  
102 skin where appropriate. Tissue samples were also collected from lesions, unaffected skin  
103 (opposite side of the body, distal to the lesion), heart, liver, gill, kidney and spleen and stored  
104 in 95 % ethanol (Farm 1-6) and formalin (Farms 1, 2, 4 and 5). Serum was also collected from  
105 the fish sampled in the UK for serology.

### 106 **2.2 Histology**

107 Tissues from the farms sampled in UK were processed for histology using standard protocols  
108 and five µm sections were stained with haematoxylin and eosin (H&E) and examined for the  
109 presence of RMS-associated pathology as described by Ferguson *et al.* (2006) and Oidtmann  
110 *et al.* (2013).

### 111 **2.3 Immunohistochemistry**

112 Additional five µm wax tissue sections were placed on glass slides and incubated overnight at  
113 60°C. Tissues were dewaxed and encircled with a wax pen. Slide were quenched for  
114 endogenous peroxidase activity following the procedures detailed in Metselaar *et al.* (2010).  
115 All sections were washed in 0.1M phosphate-buffered saline (PBS) and non-specific binding  
116 sites were blocked using a 1/10 dilution of goat serum for 30 min at 22°C. To investigate the  
117 presence *F. psychrophilum*, a cocktail of 2 rabbit anti *F. psychrophilum* sera (Faruk *et al.*, 2002)  
118 were used at a 1/5000 dilution. The slides were incubated with the PABs for 1 h at 22°C before  
119 capture with anti-rabbit IgG-HRP diluted 1/250 in PBS and visualised with a VIP chromogen  
120 kit with a methyl green counterstain.

121 The presence of the MLO was investigated using an anti- *P. salmonis* MAb (Metselaar  
122 *et al.*, 2010) at 10 µg/ml incubated for 1 h at 22°C. This was captured with an anti-mouse IgG-  
123 biotin conjugate, diluted 1/200 in PBS and streptavidin-HRP diluted 1/200 in PBS for 30 min  
124 at RT. This reaction was developed as described above. Negative controls were incubated with  
125 either an isotype-matched MAb (anti-white spot virus Mab 4), normal rabbit serum (1/5000) or  
126 PBS where appropriate. Negative tissue was obtained from the samples taken from negative  
127 farms (Farm 1 and 2) and unaffected skin on RMS-affected fish.

### 128 **2.4 Bacteriology**

129 Bacteriology samples were collected from 10 fish from all of the UK farms (Farms 1, 2, 4 and  
130 5). Swabs were taken from the rainbow trout at the site of the lesion (on negative fish in a  
131 similar location mid flank) and cultured on tryptic soya agar (TSA); Veggietone Agar (MVA)  
132 (Ngo *et al.*, 2017); and Austral-TSFe (Yanez *et al.*, 2012). Skin from unaffected fish was  
133 sampled from a similar position as that of the lesions. The agar plates were incubated at 10°C  
134 for 9 days, and the colony morphology described. Yellow pigmented colonies from the MVA  
135 plates, which resembled *Flavobacterium* sp., were selected and sub-cultured onto fresh MVA.

136 Gram staining was performed on all passaged bacteria and all Gram-negative rods, again  
137 resembling *Flavobacterium* sp., were sub-cultured onto fresh MVA plates. Small white  
138 colonies were selected from the Austral-TSFe media.

## 139 **2.5 Sequencing of the bacteria isolates**

140 In order to confirm the identity of the bacterial isolates conventional 16s rRNA gene sequencing  
141 was performed on fresh cultures of selected isolates (see Table 1 for details). The DNA was  
142 extracted using Proteinase-K and amplified using KBiosciences standard protocols. The  
143 resulting PCR products were sent to GATC-Biotech Ltd. for Sanger sequencing. The forward  
144 and reverse sequences were aligned using SeqMan® software and compared against the  
145 EMBL/GenBank sequence databases using the BLAST algorithm  
146 (<http://www.ncbi.nlm.nih.gov/BLAST>).

## 147 **2.6 Quantitative PCR for MLO**

148 In order to investigate levels of the MLO in the fish tissues qPCR was performed. DNA was  
149 extracted from all seven tissues (affected skin, unaffected skin, heart, liver, gill, kidney and  
150 spleen). Tissues were homogenised 1:10 in G2 buffer (Qiagen, UK) and digested with 6 milli-  
151 Anson units of proteinase K at 55°C for 3 h. DNA was then extracted from 200 l of  
152 homogenized tissue using an EZ1 DNA tissue kit in an EZ1 extraction robot (Qiagen). DNA  
153 was eluted in 50 l of elution buffer, according to the manufacturer's instructions. Insulin growth  
154 factor (IGF-1) was used to normalise the MLO copy numbers and a standard ( $2.5 \times 10^6$  to 250  
155 copies) was made from liver tissue from a specific pathogen free (SPF) fish. For the MLO  
156 standard, a 149 bp segment of the MLO was cloned using a PGMT-easy plasmid kit (Promega).  
157 The SPF fish DNA (used as the IGF-1 standard) was added to MLO standard at 375 ng/reaction.

158 The qPCR was performed using the primers (Table 1) and protocol as described the  
159 method of Lloyd *et al.* (2011) with modifications as following: 2 min at 50°C, 10 min at 95°C,  
160 40 cycles of 95°C for 15 sec, 55°C for 30 sec and 60°C for 30 sec. These were used to represent

161 the MLO (Cafiso et al., 2015). The Cq values were measured with the Applied Biosystems  
162 StepOne Plus® and downloaded into the StepOne® software and normalised against IGF-1.  
163 Negative DNA extractions and non-template were included as controls. MLO copy number  
164 was plotted against the corresponding Cq value and standard curves were generated by linear  
165 regression of plotted points. The amplification efficiency was assessed using the calculation  $E$   
166  $= -1 + 10(-1/\text{slope})$ . Efficiency was found to be 98%. The results were calculated as an average  
167 from three replicates and normalised against the IGF1 according to Lloyd et al., (2011). If only  
168 one of three replicates showed amplification, the result was considered to be insignificant or  
169 too near to the detection level to be considered positive. Only Cq values below <35 were  
170 included in this study.

## 171 **2.7 ELISA**

172 Fish serum was collected from four farms (Farms 1, 2, 4 and 5) and analysed by ELISA to  
173 determine the host antibody response to *F. psychrophilum* and the MLO. The MLO associated  
174 with RMS has not yet been isolated therefore it was postulated that, as the anti-*P. salmonis*  
175 MAbs reacted with RMS-affected tissue, *P. salmonis* could be used as the capture antigen.  
176 Capture antigens comprised of *F. psychrophilum* (type strain NCIMB 1947<sup>T</sup>) and three related  
177 *Flavobacterium* spp. (*F. hydatidis* ARF07; *Flavobacterium* sp. BGARF; *Chryseobacterium*  
178 *piscium* MOF25P) and *P. salmonis* (ATCC VR 1369<sup>T</sup>). ELISA reactions were optimised  
179 according to standard protocols (Cobo Labarca *et al.*, 2015). Negative wells incubated with  
180 PBS were used to calculate the background and the threshold of the ELISA was taken as three  
181 times the background absorbance value obtained with PBS.

## 182 **2.8 Statistical analysis**

183 To examine which tissue had the highest PCR results, negative results below the limits of  
184 detection were replaced with a value of half the smallest value found elsewhere in the dataset.  
185 All data were then converted into base-10 logarithms. Since the resulting distributions would



186 not satisfy ANOVA assumptions (bimodality due to the negative test results), A Friedman test  
187 was then used to compare tissues, accounting for fish as a blocking factor. This test was then  
188 followed by post-hoc comparisons using the `frdAllPairsExactTest` function provided by the  
189 `PMCMRplus` package in R. In all tests, a significance level of  $P=0.05$  was taken. An alternative,  
190 robust, approach using a chi-square test with post-hoc pairwise Fisher exact tests was used.  
191 This provided similar results and only the Friedman test results are presented here.

192 Of interest is the potential for clustering of test results amongst tissues. To investigate this, PCR  
193 results were converted to simple presence/absence and analysed with multiple correspondence  
194 analysis (MCA) in R using the `FactoMineR` and `FactoExtra` packages. Similar results were  
195 found using principal components analysis on the numerical results, due to the bimodality of  
196 the tissue-level data, and therefore the more robust MCA results are presented here.

197 To test for differences in isolate presentation between farm statuses, the ELISA data were  
198 analysed by fitting linear mixed-effect models using R (R Core Team, 2020) and the `lme4`  
199 package, since initial analysis indicated significant farm-level variation for some isolates. Three  
200 models were fitted for each isolate. First, the null model. Second, a model containing farm only,  
201 as a random effect. Third, a model containing farm status as an additional fixed effect. These  
202 nested models were compared using likelihood ratio tests to determine the significance of farm  
203 and status for each isolate. No correction was made for multiple comparisons here, but  
204 additionally, a MANOVA was performed on all isolates, with farm ID nested within status.

### 205 **3. Results**

#### 206 **3.1 Clinical signs and histopathology**

207 The fish from four farms sampled (Farms 1, 2, 4 and 5) from the UK were examined for gross  
208 pathology and histopathology (Table 2). There was a difference in the severity in the clinical  
209 signs between fish from the RMS-affected farms with fish from Farm 4 showing no signs of  
210 RMS in three fish and mild to moderate lesions in the remaining fish, while all ten fish from

211 Farm 5 had moderate to severe gross signs of RMS. There were signs of the skin swelling at  
212 the site of the lesion and with scale loss. A haemorrhagic dermatitis was evident, with the  
213 lesions often present around the lateral line. Histopathology typical of that previously reported  
214 for RMS was evident in the majority of fish from Farms 4 and 5 (Metselaar *et al.*, 2010). Skin  
215 showed multifocal lymphocytic intra-epithelial infiltration at the site of the lesion with evident  
216 acanthosis (with high mitotic numbers) and erosion and ulceration of the epithelium. The  
217 subcutis showed necrosis with fibro-angioblastic tissue and inflammatory reaction with  
218 heterophilic granulocytes and macrophages. There was very extensive necrosis of underlying  
219 musculature with a severe non-suppurative inflammatory response along the intermuscular  
220 septa. The spleen was hyperaemic with hyperplasia of the white pulp and perivascular  
221 histiocytic cell proliferation. The kidneys had moderate to severe diffuse histiocytic  
222 proliferation of interstitial haematopoietic tissue, with the liver showing no pathological  
223 changes. The gills showed fusion of the secondary lamellae in the distal areas of the primary  
224 lamellae, hyperplasia of goblet cells and interstitial oedema with heterophilic granulocytes.  
225 This may be unrelated and within normal limits and was classed incidental. Subepithelial  
226 multifocal non-suppurative infiltrates were present in the gill arch.

227         None of the tissues sampled from the RMS-negative farms had any pathology indicative  
228 of RMS. One fish did have mild focal myositis, but this was considered as an incidental finding  
229 and not related to RMS (Oidtmann, 2013).

### 230 **3.2 Immunohistochemistry**

231 A positive reaction was seen with the anti-*P. salmonis* MAb in most of the affected fish from  
232 Farms 4 and 5 (Table 3), with staining mostly seen in internal organs. The rainbow trout  
233 sampled from Farm 4 were in the final stage of the condition, as the infection had started to  
234 regress on this farm. One fish from one of the unaffected farms (Farm 1) had mild staining in  
235 its kidney and four fish from the other unaffected farm (Farm 2) also had mild staining in their

236 spleen, although this was much less severe than the reactions seen in the RMS-affected fish.  
237 Staining can be seen in Figure 1

238 The fish screened for *F. psychrophilum* showed a positive reaction in the skin and one  
239 of the internal organs of two of the RMS-affected fish. Positive staining was also seen in  
240 unaffected skin from one of these fish. There was a mild reaction within the skin, kidney and  
241 gill of three non-RMS-affected fish (Farms 1, 2 and 3). Staining can be seen in Figure 2.

242 The negative controls with normal rabbit serum or PBS were negative, while the *F.*  
243 *psychrophilum*-positive control tissue was positive. The isotype matched control was also  
244 negative.

### 245 **3.3 Bacteriology**

246 The TSA plates were discarded after nine days due to non-specific overgrowth by bacteria  
247 and/or fungus. No notable colonies were obtained on the Austral-TSFe agar. Yellow and  
248 yellow/white pigmented bacteria on the MVA plates were stained with Gram stain after 27 days  
249 of culture, and all gram-negative rods were sub-cultured onto fresh agar plates. In total 116  
250 gram negative isolates were cultured from mucus, internal and external surfaces of skin from  
251 the RMS-affected or unaffected rainbow trout from the four fish farms (Table 4).

### 252 **3.4 Sequencing of 16s rRNA gene**

253 In general, it was found that the forward and reverse sequences resulted in >1000 bp. These  
254 were aligned and submitted to the NCBI website for identification, after which the best  
255 identification match was selected. Various *Flavobacterium* isolates were seen in both RMS-  
256 affected and unaffected groups of fish and although some bacteria were only found in either  
257 affected or non-affected fish, there was no discernible association with *F. psychrophilum* and  
258 RMS (Figure 3).

259 **3.5 Quantification of the MLO in tissue of fish using a qPCR**

260 Quantification of the MLO in the various tissues of fish from the unaffected farms (Farms 1-3)  
261 and the affected fish (Farms 4-6) by qPCR can be seen in Table 5 and Table 6 respectable and  
262 summarised in Figure 4. Four fish from the unaffected farms had amplification in one of the  
263 three replicates in the qPCR but were not considered to be positive for the MLO. With the  
264 affected farms almost, all fish showed amplification of the MLO in one or more of their tissues.  
265 The detection limit of the qPCR assay used in this study is estimated to be at least 10 copies.

266 **3.6 ELISA using serum from RMS affected fish**

267 Antibody titres against *P. salmonis* and four *F. psychrophilum* isolates were generally very low  
268 and ranged between 1/64 and 1/256 (Table 7).

269 **3.7 Statistical analysis**

270 Looking at differences in PCR data between tissues for diseased fish, the Friedman test  
271 indicated that significant differences were present amongst tissues ( $\chi^2_{26}=54.2$ ;  $P<0.001$ ).  
272 Pairwise post-hoc tests then showed there to be significant differences between higher levels  
273 in diseased skin and several other tissues: normal skin ( $P=0.12$ ), heart ( $P<0.001$ ), liver  
274 ( $P<0.001$ ), kidney ( $P=0.026$ ) and spleen ( $P=0.014$ ). There were no other significant differences  
275 between any tissues.

276 Multiple correspondence analysis was used to investigate patterns of PCR positive results  
277 amongst fish and tissues. No strong signal here was in evidence, with the first two of seven  
278 dimensions accounting for 25.2 and 22.8% of variance, respectively. There is some suggestion  
279 that liver, heart, and gill may contribute towards one dimension preferentially, and kidney, skin  
280 and spleen to another. (Fig. 5)

281 With the ELISA data, MANOVA indicated significant contributions across isolates of both  
282 farm status ( $F_{5,32} = 6.88$ ;  $P<0.001$ ; Pillai's Trace = 0.52) and farm ID nested within status  
283 ( $F_{10,66}=3.36$ ;  $P=0.001$ ; Pillai's Trace=0.67). The linear mixed-effect models found farm ID to

284 be significant for two isolates (NCIMB fp and BG ARF) but in no case was farm status  
285 significant (Table 8). Effect sizes were small and only a small proportion of deviance explained  
286 by the models. Normality plots were checked for the residuals of each model and found to be  
287 satisfactory.

#### 288 **4. Discussion**

289 The current study appears to suggest a degree of involvement of MLO's with RMS, however,  
290 no MLO-like bacteria were described in any of the H&E stained tissues sampled from RMS-  
291 affected fish in the publications to date. The MLO's have been seen in fresh spleen smears of  
292 affected rainbow trout in Italy using Giemsa staining (Galeotti, *et al.*, 2011) and by TEM with  
293 the presence of intracytoplasmic microorganisms resembling Rickettsiales observed within  
294 macrophages, fibroblasts and erythrocytes of rainbow trout. The microorganisms observed  
295 were oval/short rod in shape (400–800 nm in length and 100–200 nm in width) (Galeotti *et al.*,  
296 2016). In the histology, although all fish from Farm 5 displayed classical gross signs of RMS,  
297 not all the fish from Farm 4 (an RMS-affected farm) exhibited typical histopathological signs.  
298 This was not unexpected as the RMS lesions had started to resolve on this farm and many of  
299 the lesions on these fish were in an advanced stage of healing, with three fish having no signs  
300 of the disease when sampled. It was noted that when RMS lesions were more advanced and  
301 showing signs of healing that lesions were often covered with a protective film of clear mucus.  
302 There was also no sign of clinical bacterial cold-water disease, caused by *F. psychrophilum*,  
303 in these fish.

304         There was a clear difference in the degree of staining in the IHC with the anti-*P.*  
305 *salmonis* MAb between the affected and the non-affected groups, with a strong association seen  
306 between the affected tissue and the staining, thought to represent the presence of the MLO  
307 (Metselaar *et al.*, 2010). There was mild staining in the kidney of one RMS-unaffected fish and  
308 the spleen of another, but this staining was much weaker than that seen with affected fish. The

309 anti-*F. psychrophilum* PABs used reacted minimally with a few of the samples, which were  
310 from both affected and unaffected fish. It has been suggested that a particular strain of *F.*  
311 *psychrophilum* may be involved in RMS and may differ from other strains involved in RTFS  
312 (Ferguson *et al.*, 2006), although authors did not specify or report any differences. It is known  
313 that these PABs react with many different *F. psychrophilum* isolates, but not with all *F.*  
314 *psychrophilum* isolates (Faruk *et al.*, 2002), and therefore a cocktail of the two sera made by  
315 Faruk *et al.* (2002) was used. It is possible that for some *F. psychrophilum* isolates, if present,  
316 may not have been recognised by these antisera, however this study found no association with  
317 *F. psychrophilum* in RMS-affected tissue using the described techniques.

318 The MLO associated with RMS has recently been allocated to the family  
319 Midichloriaceae, within the order Rickettsiales (Cafiso *et al.*, 2015) which requires host cells  
320 to support growth. No bacterial growth was noted on the Austral-TSFe agar. We have  
321 previously tried to culture the MLO in fish cell lines unsuccessfully (data not presented). The  
322 authors believe that the Austral-TSFe agar can support intracellular organisms such as the  
323 MLO, but we cannot be certain of this.

324 The original qPCR assay reported by Lloyd *et al.*, (2011) was able to detect 100 copies  
325 of the MLO. The detection limit of the qPCR assay used in this study is estimated to be at least  
326 10 copies in the presence of background rainbow trout DNA. The results of the qPCR showed  
327 that a single MLO was present for every 10,000 host cells. The previous studies by by Lloyd *et*  
328 *al.*, (2011) showed much higher copy numbers, believed to be unusually high and the current  
329 ratio does support the lack of visualisation. In the original study by Lloyd *et al.* (2011) related  
330 to the development of the qPCR, there was a significant difference in copy number across the  
331 range of RMS lesion severity with the qPCR. A significant difference was seen in the results  
332 of the qPCR here, with highly significant differences in the presence of the MLO detected in  
333 the affected fish between the different tissues. In the MCA numbers of data were small. It

334 should be noted that there is no test of significance presented here for this approach. The  
335 approach shown here suggests that there might be clustering amongst the skin, kidney and  
336 spleen where there was no significant difference with the affected skin, compared to liver and  
337 heart ( $P < 0.001$ ). The former tissues are often target for bacterial infections and thus can form  
338 the basis of future, more targeted attempts for isolation.

339 Serology is regularly used in both clinical and veterinary medicine to indicate previous  
340 exposure to pathogens by the detection of specific antibodies in the serum of animals (Yuce *et*  
341 *al.*, 2001; Fournier & Raoult, 2003). Serology can be useful when it is not possible to isolate  
342 the pathogen by traditional methods or when rapid tests to identify the pathogen have not yet  
343 been developed (Adams & Thompson, 2006). Sero-conversion can be used to confirm that  
344 certain pathogens are related to the symptoms seen. The preferred method to examine this is by  
345 ELISA, but this requires purified pathogen to coat the ELISA plate, which was not possible for  
346 RMS. As such *P. salmonis*, based on potential cross binding, was used to establish if there was  
347 a difference between samples obtained from the two sets of farms. Antibody titres obtained  
348 against *Flavobacterium* isolates and *P. salmonis* were low and did not appear to demonstrate  
349 any differences in antibody responses between RMS-affected and non-affected sites. The  
350 MANOVA does not treat Farm ID as a random factor, only as a fixed one, as a limitation of  
351 the approach used, however it is useful as an indication that some differences exist within the  
352 dataset. The apparent differences are between farm status; however, this appears to be due to  
353 differences in individual farms and the small number of farms sampled.

## 354 **5. Conclusion**

355 In conclusion, the results of this study strongly indicate that the MLO are involved in RMS  
356 rather than *F. psychrophilum*. Analysis, of a statistically appropriate sample set, appears to  
357 confirm a positive correlation between the MLO's and RMS-affected tissue, mainly in the skin,  
358 further supported by the results of IHC tests. It was not possible to confirm whether the inferred

359 presence of MLO had a primary or secondary role in RMS. However, it may be important to  
360 note that the finding of MLO's was a consistent feature even when sampling RMS-affected fish  
361 from several discrete geographical areas. The failure to date to directly culture any MLO's  
362 warrants further investigation. No association was found between the RMS-affected fish and  
363 *F. psychrophilum* other than superficial presence in the skin.

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481

Table 1. Overview of primers in polymerase chain reactions

| Name             | Sequence (5' - 3')                               | Reference                   |
|------------------|--|-----------------------------|
| RLO qPCR probe   | (6FAM) CCC AGA TAA CCG CCT<br>TCG CCT CCG (BHQ1) | Lloyd <i>et al.</i> (2011)  |
| RLO qPCRf        | GGC TCA ACC CAA GAA CTG<br>CTT                   | Lloyd <i>et al.</i> (2011)  |
| RLO qPCRr        | GTG CAA CAG CGT CAG TGA CT                       | Lloyd <i>et al.</i> (2011)  |
| IGF-1 qPCR probe | (6FAM)CCG TGG TAT TGT GGA<br>CGA GTG CTG C(BHQ1) | Kelley <i>et al.</i> (2004) |
| IGF-1 qPCRf      | CAG TTC ACG GCG GTC ACA T                        | Kelley <i>et al.</i> (2004) |
| IGF-1 qPCRr      | CCG TAG CTC GCA ACT CTG G                        | Kelley <i>et al.</i> (2004) |

482

483 Table 2. Histopathology of *Oncorhynchus mykiss* from red mark syndrome (RMS)-affected and  
 484 non-affected farms

| <b>Farm</b> | <b>Fish</b> | <b>RMS Status</b> | <b>Histopathology</b>   |
|-------------|-------------|-------------------|---|
| <b>1</b>    | 1-10        | -                 | No pathology in all but 1 fish; mild focal mysositis (not in <i>stratum compactum</i> ). Considered an incidental finding and not RMS |
| <b>2</b>    | 1-10        | -                 | No pathology  |
| <b>4</b>    | 1           | +                 | RMS confirmed   |
|             | 2           |                   | Possibly incipient RMS  |
|             | 3-4         |                   | Moderate/Marked RMS involving the musculature   |
|             | 5           |                   | Moderate RMS with loss of epidermis   |
|             | 6-7         |                   | Mild RMS  |
|             | 8-10        |                   | No pathology  |
| <b>5</b>    | 1-3,5,8-9   | +                 | Marked RMS  |
|             | 4,6         |                   | Moderate RMS  |
|             | 7           |                   | Mild/Moderate RMS   |
|             | 10          |                   | Marked RMS with pericarditis  |

485

486 Table 3. Immunohistochemistry reaction in red mark syndrome-affected and unaffected  
 487 *Oncorhynchus mykiss* tissue using an anti-*Piscirickettsia salmonis* MAb 23.  
 488

| Farm | Fish | RMS<br>Status | Non<br>affected<br>skin | Affected<br>skin | Kidney | Spleen | Liver  | Heart  |
|------|------|---------------|-------------------------|------------------|--------|--------|--------|--------|
| 1    | 1-10 | -             | -                       | -                | 1x +/- | -      | -      | -      |
| 2    | 1-10 | -             | -                       | -                | -      | -      | -      | -      |
| 4    | 1-10 | +             | -                       | 1x +             | 3x +   | 2x +/- | 6x +/- | 1x +/- |
| 5    | 1-10 | +             | -                       | -                | 1x +/- | 1 x +  | 5x +   | 1x +   |

489

490 Table 4. The number of yellow, Gram negative bacteria isolated from *Oncorhynchus mykiss*  
491 sampled from the farms in the UK and the location of their isolation on the fish.

| Farm     | RMS status | Mucus | Exterior | Interior | Total |
|----------|------------|-------|----------|----------|-------|
| <b>1</b> | -          | (9)   | (10)     | (7)      | (26)  |
| <b>2</b> | -          | (12)  | (11)     | (4)      | (27)  |
| <b>4</b> | +          | (5)   | (16)     | (9)      | (30)  |
| <b>5</b> | +          | (12)  | (13)     | (7)      | (33)  |

492



Table 5 Quantitative polymerase chain reaction results (average of 3 replicates) for RMS unaffected Farm 1-3. Relative MLO presence is presented as the MLO copy number divided by the IGF-1 copy number to normalise the results.

|                       | no. | Affected skin | Non affected skin | Heart | Liver | Gill                       | Kidney                     | Spleen                     |
|-----------------------|-----|---------------|-------------------|-------|-------|----------------------------|----------------------------|----------------------------|
| Farm 1 (RMS negative) | 1   | -             | 0.17**            | -     | -     | -                          | -                          | -                          |
|                       | 2   | -             | -                 | -     | -     | -                          | -                          | -                          |
|                       | 3   | -             | -                 | -     | -     | -                          | -                          | -                          |
|                       | 4   | -             | -                 | -     | -     | -                          | -                          | -                          |
|                       | 5   | -             | -                 | -     | -     | -                          | -                          | -                          |
|                       | 6   | -             | -                 | -     | -     | -                          | -                          | -                          |
|                       | 7   | -             | -                 | -     | -     | -                          | -                          | -                          |
|                       | 8   | -             | -                 | -     | -     | -                          | -                          | -                          |
|                       | 9   | -             | -                 | -     | -     | -                          | -                          | -                          |
|                       | 10  | -             | -                 | -     | -     | -                          | -                          | -                          |
| Farm 2 (RMS negative) | 1   | -             | -                 | -     | -     | -                          | -                          | -                          |
|                       | 2   | -             | -                 | -     | -     | -                          | -                          | -                          |
|                       | 3   | -             | -                 | -     | -     | 3.35*<br>x10 <sup>-6</sup> | 3.97*<br>x10 <sup>-7</sup> | -                          |
|                       | 4   | -             | -                 | -     | -     | -                          | -                          | -                          |
|                       | 5   | -             | -                 | -     | -     | -                          | -                          | -                          |
|                       | 6   | -             | -                 | -     | -     | -                          | -                          | -                          |
|                       | 7   | -             | -                 | -     | -     | -                          | -                          | -                          |
|                       | 8   | -             | -                 | -     | -     | -                          | -                          | -                          |
|                       | 9   | -             | -                 | -     | -     | -                          | -                          | -                          |
|                       | 10  | -             | -                 | -     | -     | -                          | -                          | -                          |
| Farm 3 (RMS negative) | 1   | -             | -                 | -     | -     | -                          | -                          | -                          |
|                       | 2   | -             | -                 | -     | -     | -                          | -                          | -                          |
|                       | 3   | -             | -                 | -     | -     | -                          | -                          | -                          |
|                       | 4   | -             | -                 | -     | -     | -                          | -                          | -                          |
|                       | 5   | -             | -                 | -     | -     | -                          | -                          | -                          |
|                       | 6   | -             | -                 | -     | -     | -                          | -                          | -                          |
|                       | 7   | -             | -                 | -     | -     | 1.79*<br>x10 <sup>-6</sup> | -                          | -                          |
|                       | 8   | -             | -                 | -     | -     | 5.94*<br>x10 <sup>-6</sup> | -                          | 2.46*<br>x10 <sup>-6</sup> |
|                       | 9   | -             | -                 | -     | -     | -                          | -                          | -                          |
|                       | 10  | -             | -                 | -     | -     | -                          | -                          | -                          |

\*Not significant; one of three replicates amplified

\*\* Outlier; one out 3 replicates significantly higher

- No amplification of the *Midichloria* -like organism

Table 6 Quantitative polymerase chain reaction results (average of 3 replicates) for RMS-affected Farm 4-6. Relative MLO presence is calculated by dividing the MLO copy numbers by the IGF-1 copy number to normalise the results.

|                       | n  | Affected skin          | Non affected skin      | Heart                  | Liver                  | Gill                   | Kidney                 | Spleen                 |
|-----------------------|----|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|
| Farm 4 (RMS positive) | 1  | 3.29 x10 <sup>-5</sup> | -                      | -                      | -                      | -                      | -                      | -                      |
|                       | 2  | -                      | -                      | -                      | 9.61*x10 <sup>-7</sup> | -                      | 1.96*x10 <sup>-6</sup> | 1.26 x10 <sup>-6</sup> |
|                       | 3  | 1.87 x10 <sup>-3</sup> | -                      | -                      | -                      | -                      | -                      | 1.78*x10 <sup>-6</sup> |
|                       | 4  | 1.58 x10 <sup>-4</sup> | 9.35 x10 <sup>-6</sup> | 1.26*x10 <sup>-6</sup> | -                      | 7.22*x10 <sup>-7</sup> | 9.13 x10 <sup>-7</sup> | 2.21 x10 <sup>-6</sup> |
|                       | 5  | 3.14 x10 <sup>-5</sup> | 6.98 x10 <sup>-5</sup> | -                      | -                      | -                      | 1.52 x10 <sup>-6</sup> | -                      |
|                       | 6  | 6.56 x10 <sup>-4</sup> | 1.17 x10 <sup>-5</sup> | -                      | -                      | -                      | 9.01 x10 <sup>-7</sup> | 1.42*x10 <sup>-6</sup> |
|                       | 7  | 1.55 x10 <sup>-4</sup> | -                      | 4.62 x10 <sup>-6</sup> | 6.65 x10 <sup>-6</sup> | 3.69 x10 <sup>-4</sup> | 1.21* 10 <sup>-6</sup> | -                      |
|                       | 8  | 5.53 x10 <sup>-6</sup> | -                      | -                      | 3.59*x10 <sup>-6</sup> | -                      | -                      | -                      |
|                       | 9  | -                      | -                      | -                      | -                      | 6.26*x10 <sup>-7</sup> | -                      | -                      |
|                       | 10 | 1.34*x10 <sup>-5</sup> | 3.41*x10 <sup>-5</sup> | 1.42 x10 <sup>-6</sup> | 1.20 x10 <sup>-6</sup> | 1.55*x10 <sup>-6</sup> | -                      | -                      |
| Farm 5 (RMS positive) | 1  | 8.38 x10 <sup>-4</sup> | 2.51 x10 <sup>-5</sup> | 5.03 x10 <sup>-6</sup> | -                      | 1.40 x10 <sup>-4</sup> | 8.57 x10 <sup>-6</sup> | 1.30**                 |
|                       | 2  | 0.68**                 | -                      | 4.00*x10 <sup>-6</sup> | 2.75*x10 <sup>-6</sup> | 1.52 x10 <sup>-6</sup> | 9.38 x10 <sup>-7</sup> | 1.63**                 |
|                       | 3  | 6.07 x10 <sup>-7</sup> | -                      | 8.40*x10 <sup>-9</sup> | -                      | -                      | 5.56 x10 <sup>-9</sup> | -                      |
|                       | 4  | 1.37 x10 <sup>-6</sup> | 1.44 x10 <sup>-4</sup> | 1.36*x10 <sup>-6</sup> | 8.80*x10 <sup>-7</sup> | 9.40 x10 <sup>-7</sup> | 3.15 x10 <sup>-6</sup> | 1.79 x10 <sup>-6</sup> |
|                       | 5  | 4.71 x10 <sup>-4</sup> | 5.96 x10 <sup>-5</sup> | -                      | -                      | 3.26*x10 <sup>-7</sup> | 2.93*x10 <sup>-7</sup> | -                      |
|                       | 6  | 1.22 x10 <sup>-4</sup> | 1.18*x10 <sup>-5</sup> | 2.55 x10 <sup>-6</sup> | -                      | 1.15*x10 <sup>-6</sup> | 3.68 x10 <sup>-6</sup> | 2.71 x10 <sup>-6</sup> |
|                       | 7  | -                      | -                      | -                      | -                      | 1.24 x10 <sup>-6</sup> | -                      | 4.60*x10 <sup>-6</sup> |
|                       | 8  | -                      | 6.26 x10 <sup>-5</sup> | -                      | -                      | -                      | -                      | -                      |
|                       | 9  | 7.35 x10 <sup>-6</sup> | -                      | -                      | -                      | 8.40 x10 <sup>-7</sup> | -                      | -                      |
|                       | 10 | 1.64 x10 <sup>-4</sup> | 1.38 x10 <sup>-5</sup> | 1.11*x10 <sup>-6</sup> | 5.20*x10 <sup>-7</sup> | 5.07*x10 <sup>-7</sup> | 4.14*x10 <sup>-7</sup> | 4.70*x10 <sup>-3</sup> |
| Farm 6 (RMS positive) | 1  | 7.41 x10 <sup>-4</sup> | 1.81 x10 <sup>-5</sup> | -                      | -                      | 5.43*x10 <sup>-6</sup> | -                      | 5.92 x10 <sup>-6</sup> |
|                       | 2  | 3.93 x10 <sup>-4</sup> | -                      | -                      | -                      | -                      | 7.86 x10 <sup>-6</sup> | 1.49 x10 <sup>-3</sup> |
|                       | 3  | 1.86**                 | 2.89 x10 <sup>-5</sup> | -                      | 4.55 x10 <sup>-6</sup> | -                      | 1.10 x10 <sup>-5</sup> | 7.39 x10 <sup>-6</sup> |
|                       | 4  | 1.06 x10 <sup>-3</sup> | -                      | -                      | -                      | 1.76*x10 <sup>-6</sup> | -                      | 1.55 x10 <sup>-6</sup> |
|                       | 5  | 2.28 x10 <sup>-4</sup> | -                      | -                      | 2.92*x10 <sup>-6</sup> | 39.23**                | 1.9 x10 <sup>-6</sup>  | 3.72 x10 <sup>-6</sup> |
|                       | 6  | 3.83 x10 <sup>-5</sup> | 3.3* x10 <sup>-5</sup> | -                      | -                      | -                      | -                      | -                      |
|                       | 7  | 1.78 x10 <sup>-4</sup> | 1.57*x10 <sup>-5</sup> | -                      | -                      | 3.93 x10 <sup>-6</sup> | 1.02 x10 <sup>-6</sup> | 1.17 x10 <sup>-5</sup> |
|                       | 8  | 1.67 x10 <sup>-4</sup> | 4.51 x10 <sup>-4</sup> | 3.83*x10 <sup>-5</sup> | 5.25 x10 <sup>-6</sup> | 1.19 x10 <sup>-5</sup> | 3.69 x10 <sup>-5</sup> | 7.11 x10 <sup>-5</sup> |
|                       | 9  | 4.69 x10 <sup>-5</sup> | 1.25 x10 <sup>-4</sup> | -                      | -                      | 1.00*x10 <sup>-5</sup> | 3.22*x10 <sup>-6</sup> | 2.09 x10 <sup>-6</sup> |
|                       | 10 | 1.20 x10 <sup>-4</sup> | 2.04*x10 <sup>-4</sup> | 1.23 x10 <sup>-5</sup> | -                      | -                      | 1.26 x10 <sup>-5</sup> | 7.64 x10 <sup>-6</sup> |

\*Not significant; one of three replicates amplified

\*\* Outlier; one out 3 replicates significantly higher

- No amplification of the *Midichloria* -like organism

Table 7. Enzyme-linked immunosorbent assay to measure the antibody titre ( $-\log_2+1$ ) of with serum from red mark syndrome (RMS) negative (Farm 1 & 2) and RMS-positive fish (Farm 4 & 5) to different *Flavobacterium* spp. and *Piscirickettsia salmonis*.

| Farm     | RMS Status | <i>Flavobacterium</i> sp.   |           |           |           | <i>P. salmonis</i> |
|----------|------------|-----------------------------|-----------|-----------|-----------|--------------------|
|          |            | NCIMB <i>Fp<sup>T</sup></i> | ARF07     | MOF25     | BGARF     |                    |
| <b>1</b> | -          | 5.8 ± 1.4                   | 8.1 ± 1.2 | 7.0 ± 1.2 | 7.9 ± 0.7 | 7.7 ± 0.8          |
| <b>2</b> | -          | 7.5 ± 1.0                   | 7.1 ± 0.6 | 7.6 ± 1.2 | 7.3 ± 0.5 | 7.4 ± 0.5          |
| <b>4</b> | +          | 7.3 ± 2.4                   | 6.9 ± 2.4 | 7.1 ± 2.3 | 6.9 ± 2.4 | 7.0 ± 2.4          |
| <b>5</b> | +          | 8.0 ± 0.5                   | 7.0 ± 0.5 | 7.5 ± 0.5 | 7.0 ± 0.5 | 7.8 ± 0.4          |

Results represent the mean of 10 duplicate samples per farm ±SD

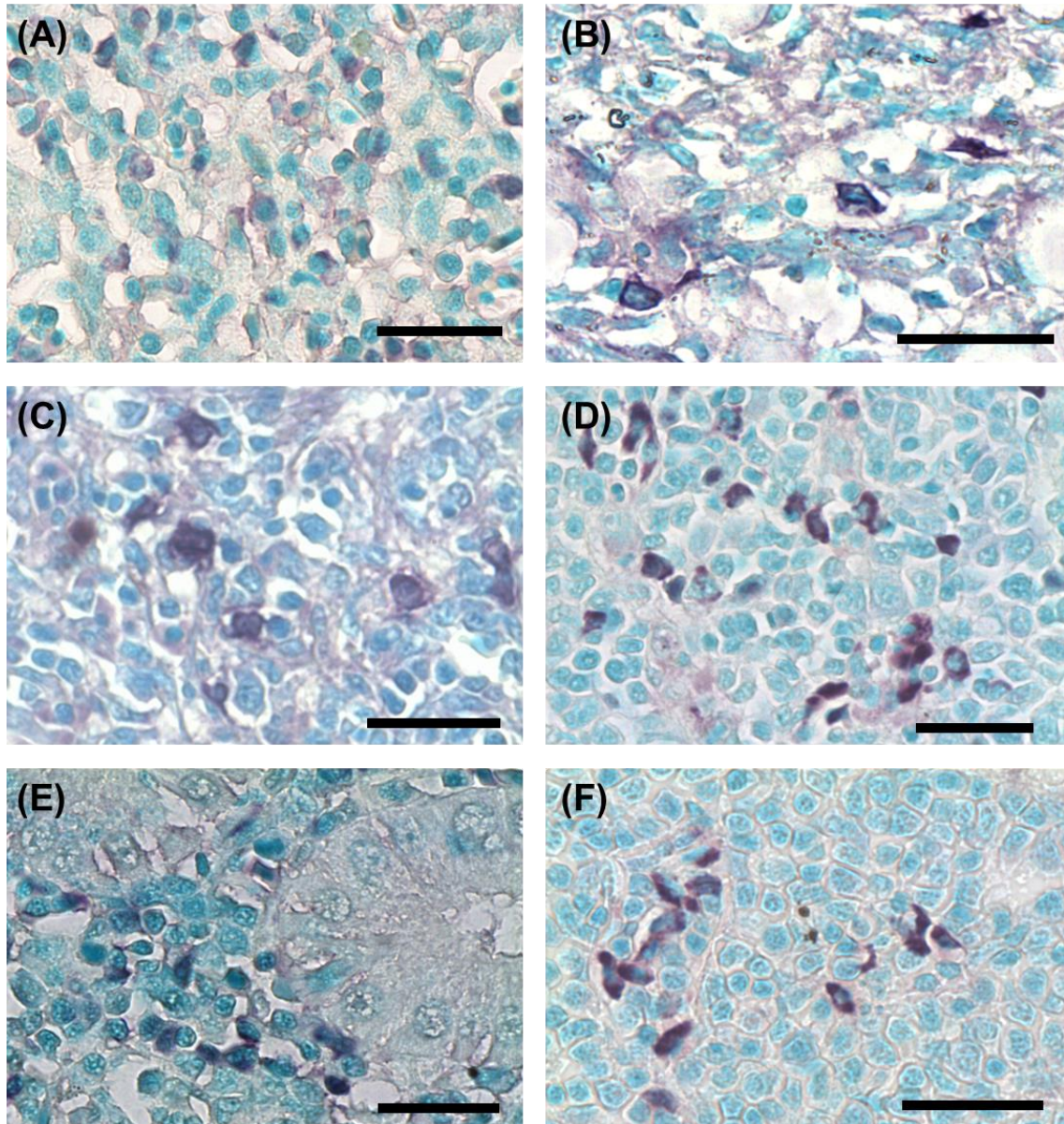
Table 8. Linear mixed-effect models for isolate ELISA results, accounting for farm status and farm ID. Coefficients are illustrated from the full models. Likelihood ratio tests compare the nested models.

| Isolate         | Model                            | Coefficient <sup>b</sup> | SE <sup>b</sup> | t <sub>36</sub> <sup>b</sup> | (Δ) Deviance | P        |
|-----------------|----------------------------------|--------------------------|-----------------|------------------------------|--------------|----------|
| <b>NCIMB fp</b> | Intercept                        | 6.90                     |                 |                              | 126.61       |          |
|                 | + Farm <sup>a</sup>              |                          | 0.60            |                              | -9.69        | 0.001 ** |
|                 | + Status (affected) <sup>a</sup> | 0.70                     | 0.67            | 1.04                         | -0.96        | 0.33     |
| <b>ARF 7</b>    | Intercept                        | 7.55                     |                 |                              | 109.27       |          |
|                 | + Farm                           |                          | 0.15            |                              | -1.39        | 0.24     |
|                 | + Status (affected)              | -0.55                    | 0.32            | -1.73                        | -2.23        | 0.14     |
| <b>PS</b>       | Intercept                        | 7.55                     |                 |                              | 89.60        |          |
|                 | + Farm                           |                          | 0.14            |                              | -0.24        | 0.62     |
|                 | + Status (affected)              | -0.10                    | 0.27            | -0.37                        | -0.14        | 0.71     |
| <b>MCF 25p</b>  | Intercept                        | 7.55                     |                 |                              | 113.49       |          |
|                 | + Farm                           |                          | 0.28            |                              | -0.94        | 0.33     |
|                 | + Status (affected)              | -0.15                    | 0.41            | -0.36                        | -0.13        | 0.72     |
| <b>BG ARF</b>   | Intercept                        | 7.7                      |                 |                              | 100.8        |          |
|                 | + Farm                           |                          | 0.15            |                              | -3.86        | 0.050 *  |
|                 | + Status (affected)              | -0.70                    | 0.28            | -2.48                        | -3.71        | 0.054    |

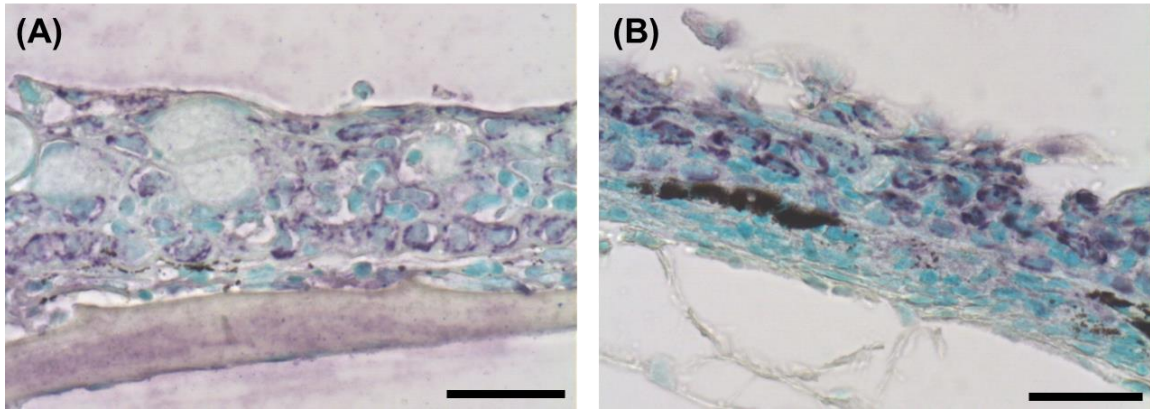
<sup>a</sup> All contrasts use one degree of freedom.

<sup>b</sup> Derived from the third model in each set.

d



**Figure 1** Reaction of rabbit anti-*P. salmonis* serum in immunohistochemistry of tissues affected with red mark syndrome (RMS) from the United Kingdom (UK) and strawberry disease (SD) in United States of America (USA). (A) Skin RMS UK; (B) Skin SD USA; (C) Spleen RMS UK; (D) Spleen SD USA; (E) Kidney RMS UK; (F) Kidney SD USA. (Magnification 400x, Bar is 25  $\mu$ m)



**Figure 2** Reaction of rabbit anti-*F. psychrophilum*. (A) positive staining seen in the epidermis, around the lesion in affected skin and (B) normal skin. No staining was seen in the dermis or muscle layer. (magnification 400x, Bar is 25 µm)

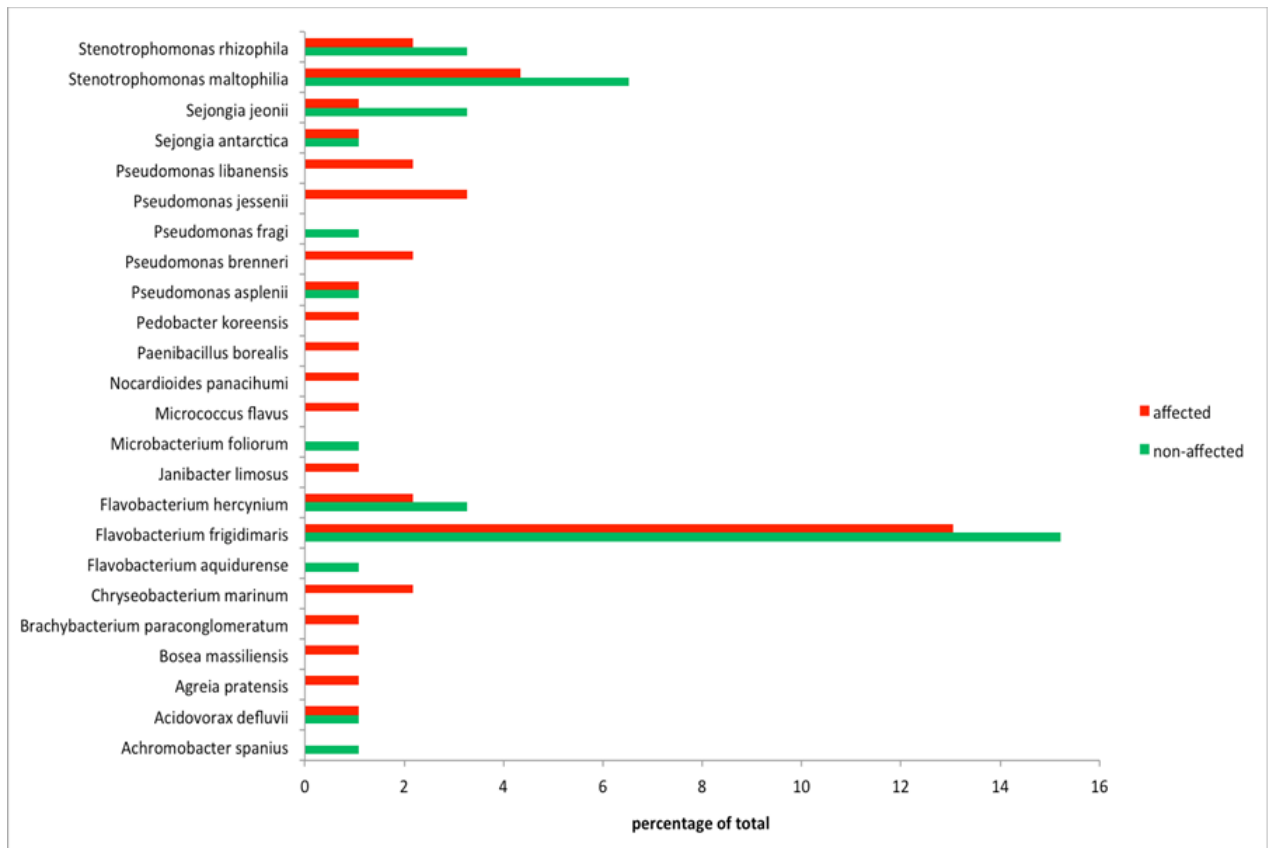


Figure 3. Identification using 16s rRNA gene sequencing of isolates cultures from red mark syndrome (RMS)-affected (red) and unaffected (green) *Oncorhynchus mykiss*, although some bacteria are only found in either affected or non-affected with no strong association apparent with *Flavobacterium psychrophilum*.

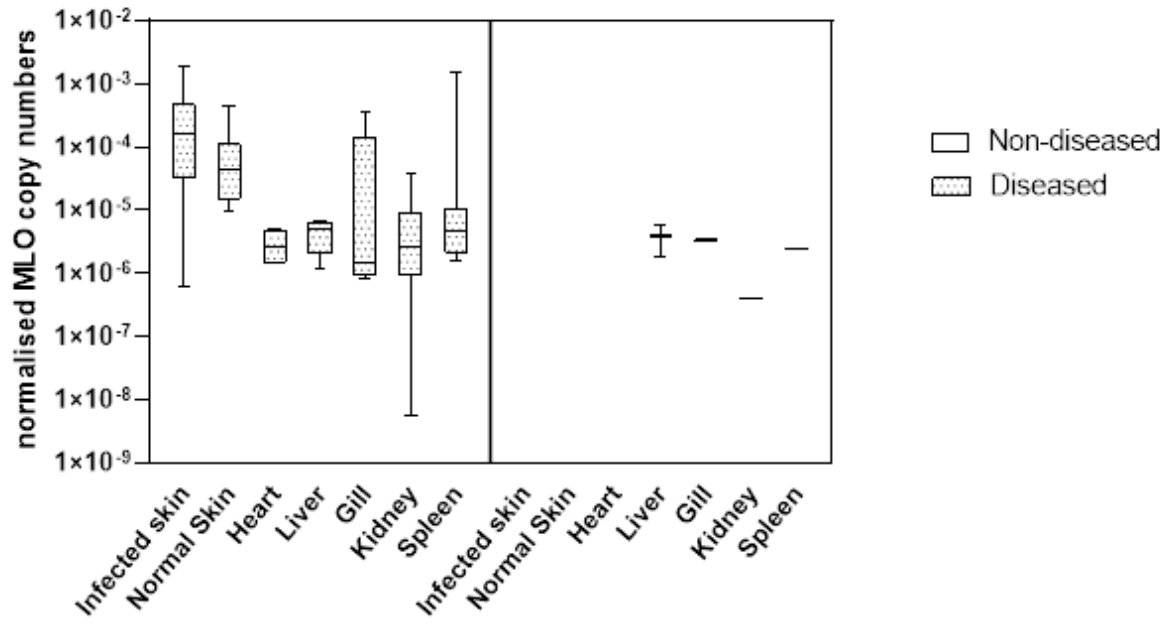


Figure 4 Boxplot of the normalised MLO copy numbers for seven different tissues in both affect (left) and unaffected farms (right) combined.



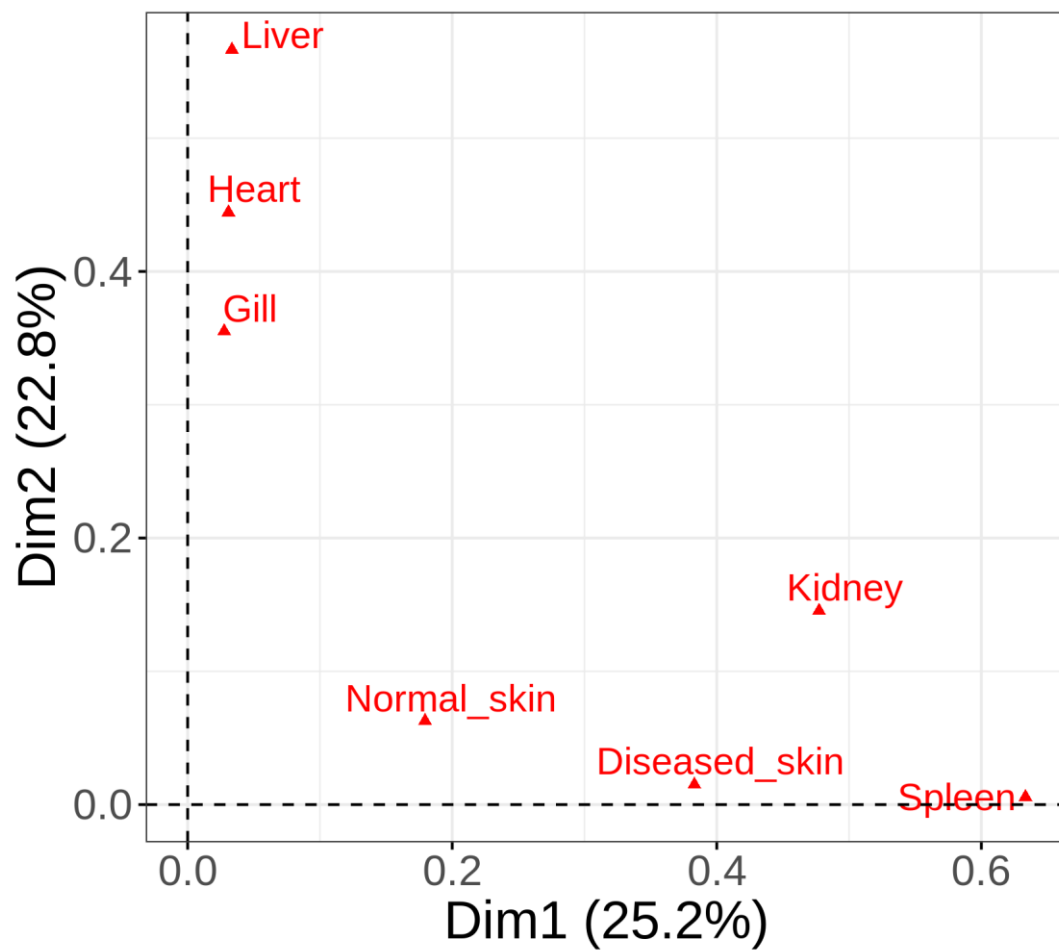


Figure 5. Multiple correspondence analysis on tissue-level PCR results for positive fish.

Variable weightings for the first two dimensions are shown.