Using fatty acid markers to distinguish between effects of salmon (*Salmo salar*) and halibut (*Hippoglossus hippoglossus*) farming on mackerel (*Scomber scombrus*) and whiting (*Merlangius marlangus*)

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Abstract

Presence of coastal aquaculture activities in marine landscapes is growing with impacts on the wild fish that share these habitats. However, it is difficult to disentangle subsequent ecological interactions between these activities and marine fish communities. We evaluated the impact of both salmon and halibut farms on mackerel (*Scomber scombrus*) and whiting (*Merlangius merlangus*) sampled near sea cages using condition indices and fatty acid (FA) biomarkers. Results of the stomach content analysis indicated that mackerel and whiting consumed waste feed which was also reflected in their modified FA profiles. Both mackerel and whiting had elevated levels of FAs that are of vegetable oils origin. The use of vegetable oils as replacement for marine oils is a lot more common in salmon farming than halibut farming. Additionally,

the overall effects of the two fish farms were more pronounced in whiting than in mackerel sampled near the sea cages. By allowing discrimination between source of trophic interactions, this method could lead to more informed decisions in managing different farming activities.

KEYWORDS

Fish farming, halibut farming, salmon farming, wild fish populations, fatty acid biomarkers, linear discriminant analysis

1. INTRODUCTION

As aquaculture production increases, there is a trend for diversifying the range of species produced, for example cold water marine production of salmonids (principally *Salmo salar*) is being joined by production of high value marine species such as halibut (*Hippoglossus hippoglossus*) and cod (*Gadus morhua*). Different production systems and species have differential impact on the environment. Because of the need for increased aquaculture production and diversification to remain environmentally sustainable (Diana et al., 2013), we require tools for distinguishing the impacts of different production systems on the ecosystem.

Fish production in mesh cages allows the release of organic by-products in the form of particulate matter originating from uneaten food and faeces, dissolved metabolic waste including ammonia and urea excreted from the gills and organic matter resulting from scraping of biofouling on cages in the surrounding environment (reviewed by Holmer, 2010; Uglem, Karlsen, Sánchez-Jerez & Saether, 2014; Price, Black, Hargrave & Morris, 2015). Nutrient emission from fish farms can have a range of ecological impacts on the surrounding aquatic environment such as local

eutrophication, impacts on benthic fauna and local wild fish populations (see Mente, Pierce, Santos & Neofitou, 2006; Holmer, 2010; Uglem et al., 2014). Gaining knowledge on how the environment is affected by aquaculture activities is important for the long term sustainability of the sector (Diana et al., 2013).

Biochemical tracers such as lipids are often used in food web ecology (see reviews by Dalsgaard, St. John, Kattner, Müller-Navarra & Hagen, 2003; Bergé & Barnathan, 2005; Kelly & Scheibling, 2012; Parrish, 2013; White et al. 2019). The main reasoning behind the use of FAs as biomarkers is that groups of primary producers possess unique FAs or ratios of FAs and that this can be conservatively transferred through the aquatic food web (see reviews by Dalsgaard et al., 2003; Bergé & Barnathan, 2005; Kelly & Scheibling, 2012; Parrish, 2013). A number of studies have used terrestrial FA biomarkers to assess whether coastal fish farming influences wild marine fish in the vicinities of the sea cages (reviewed by Fernandez-Jover et al., 2011ab; see also Arechavala-Lopez, Sæther, Marhuenda-Egea, Sanchez-Jerez & Uglem, 2011, 2015; Izquierdo-Gómez et al., 2015).

The farming of species such as Atlantic salmon, Atlantic halibut and cod require a sufficient dietary supply of FAs such as 22:6n-3, 20:5n-3 and 20:4n-6 for optimal growth and health status. The farming industry relies on capture fisheries for the supply of fish oil. However, as the capture fisheries is stagnating the farming industry has explored alternative sources such as vegetable oils (e.g. soybean, rapeseed, linseed, palm oils) (Tacon & Metian, 2008). However, vegetable oils are rich in 18:2n-6 and 18:3n-3 but lack n-3 PUFAs (20:5n-3, 22:6n-3) (Turchini, Torstensen & Ng, 2009). Similar to cultured fish, wild fish incorporate these FAs into their tissues as a result from feeding on waste feed from fish farms. Therefore, influence of fish farming on

71   wild fish populations can be detected using FAs such as 18:2n-6 and low ratio of n-3/n-

72   6 (reviewed by Fernandez-Jover et al., 2011b).

73   As the marine aquaculture sector is rapidly increasing and diversifying it is

74   important to evaluate the impacts of various fish farming activities on the wild fish

75   populations. Knowledge of how wild fish are affected by different forms of aquaculture

76   can guide the site selection of fish farms, management of fish farming activities and

77   wild fish stocks, and conservation of wild fish. The aim of this study was to evaluate the

78   impacts of a halibut and a salmon farm on diet, condition and total lipid and FA profiles

79   of mackerel and whiting sampled near the sea cages. Moreover, comparison between

80   the farmed species and the two impacted fish species was assessed in order to determine

81   how the source of effects (salmon vs. halibut aquaculture) can be distinguished in two

82   distinct target species (mackerel and whiting).

83

84   **2.   MATERIALS AND METHODS**

85   **2.1 Sampling sites**

86   The project was approved by the University of Stirling, Institute of Aquaculture

87   ethics committee (in April 2013), and that fish were sacrificed in accordance with

88   Schedule 1 of the UK Animals (Scientific Procedures) Act 1986.

89   Sampling sites were selected to evaluate the impacts of salmon and halibut

90   farming on wild fish populations around sea cages. Farm and reference sites were

91   selected for each farming activity. All sampling sites (Figure 1) were located on the

92   West Coast of Scotland and selected based on the cooperation of fish farmers and the

93   accessibility to the selected sites.

94   The halibut farm was located in Loch Melfort (Figure 1; 56.2475 N, 5.5145 W)

95   which is a fjordic type small sea-loch that extends about six km in length, maximum

96   depth of 73 metres and a fresh/tidal flow per thousand of 10.2 (Edwards & Sharples,

97   1986). The halibut farm was almost adjacent to the shore in water depth of 14-23

98   metres. The farm was accessed from the shore by a jetty. The farm consisted of six

99   circular cages each having a diameter of 22.3 metres and 7-8 metres depth. The farm

100   produced Atlantic halibut with maximum consented biomass of 250 tonnes/year.

101   The salmon farm was located in Loch Leven (Figure 1; 56.6880 N, 5.1375 W), a

102   sea loch of 13.4 km in length, a maximum depth of 62 metres. The fresh/tidal flow ratio

103   per thousand is 40.5 (Edwards & Sharples, 1986). The selected farm is about 120

104   metres off the shore at an average depth of 25 metres. The farm was accessed from the

105   shore by a boat. The farm comprises of twelve 24 metres^2 steel pens and produces

106   Atlantic salmon (*Salmo salar* L.) with maximum consented biomass of 1450

107   tonnes/year.

108   Loch Melfort and Loch Leven are both relatively small lochs. The catchment

109   area for Loch Leven is larger than for Loch Melfort which indicates a larger freshwater

110   input in Loch Leven. The flushing time (the time it takes for all or some of the water in

111   the loch to be replaced by the tidal currents (Gillibrand, 2001)) in Loch Leven is three

112   days whereas that of Loch Melfort is nine days. The flushing time difference between

113   the two lochs indicates that resident times for phytoplankton and nutrients is higher for

114   Loch Melfort than for Loch Leven.

115   Details on farm management, locations and abbreviations used throughout the

116   studies are given in Table 1. Halibut farming has a limited production as compared to

117   salmon production in Scotland. The maximum allowed biomass for the chosen salmon

118   farm is almost six times more than the halibut farm production (Table 1). The halibut

farm is located in a very sheltered bay whereas the salmon farm is located in a well

flushed area indicating that nutrients from the salmon farm will be more dispersed than

those of the halibut farm. The halibut farm was towards the end of the production cycle

(36-56 months) whereas the salmon farm was in the beginning of the production cycle

(18 months) indicating differences in the diets fed to the cultured fish. At the halibut

farm the feeding frequency was manual whereas at the salmon farm feeding was

automated which may indicate more waste feed at halibut farm (Table 1). However,

halibut farming often has a tarpaulin at the bottom of the cage which allows the halibut

to consume settled feed and therefore less artificial feed would be lost (Gillibrand,

Gubbins, Greathead & Davies, 2002).

2.2 Fish sampling at farm sites

Wild fish were sampled by using baited rod and line fishing gear. Fish collection

using rod and line selects for feeding fish. Mackerel were caught using three hook

feather rig (Shakespeare Mackerel Rig; SP 3240; "J" hooks size 1/0) placed on a

monofilament main line (0.25 mm) on a conventional spinning reel and a 3 metres rod.

Whiting were caught using three hook rig (Shakespeare SP 3280; "J" hooks size 2). The

rig encompassed a 100 g lead at the end of the main line. The rig was placed on a

monofilament main line (0.25 mm) on a conventional spinning reel and a 3 metres rod.

2.3 Fish sampling at reference sites

Three reference sites were chosen for each sampled species (mackerel and

whiting) (Figure 1). Reference sites were chosen based on distance from farm and

accessibility. Majority of the fish were sampled by local fisherman using rod and line.

Whiting caught at a third reference site were bigger in size compared to those caught

143 near the two farms and thus were not included in the study. Fish sampling at the salmon

144 farm took place in July/August 2014.

145

146 **2.4 Fish processing**

147

148   All fish were immediately placed on ice and transported to the Institute of

149 Aquaculture, University of Stirling where they were kept at -20°C until processing. At

150 the time of processing fish were defrosted and individual mass (g) and length (cm) were

151 recorded. Individual fish were dissected. Following dissection fish livers were weighed.

152   Stomachs (from the oesophagus to the pyloric sphincter) were removed and

153 stored in 70% ethanol. Stomachs of mackerel and whiting were analysed between 10-12

154 weeks. Stomach contents were emptied, and prey items were categorized into pellets,

155 invertebrates, fish and unknown. Frequency of occurrence (FO) was calculated using

156 the formula:

157 FO= J_i / P $\times$100

158 where J_i is the number of fish containing prey i and P is the number of fish with food in

159 their stomachs (Hyslop 1980). Fulton's condition index (FCI) was calculated using the

160 formula: FCI= W / L^3 $\times$100

161 where W = mass (g), L = length (cm). The hepatosomatic index (HSI) was calculated

162 with the formula:

163 HSI= Liver mass (g) / Total mass (g) $\times$100.

164

165 **2.5 Lipid extraction and fatty acid methyl esters (FAMEs)**

166

167   Samples of the muscle (flesh) and liver tissues were taken from individual

168   mackerel and whiting. Commercial feed pellets were also collected from the halibut and

169   salmon farms.

170   Total lipids were extracted from feed pellets, muscle and liver tissues of fish

171   according to the method of Folch, Lees & Sloane-Stanley (1957). In brief, total lipids

172   were extracted from samples (~ 0.5 g) by homogenising in 20 volumes of

173   chloroform:methanol (2:1, v/v) using Ultra-Turrax tissue disrupter (Fisher Scientific,

174   Loughborough, UK) in a fume cupboard. Samples were left on ice for one hour

175   followed by addition of 5 ml of 0.88% (w/v) potassium chloride (KCl) to remove non-

176   lipid impurities. Samples were centrifuged at $400 \times g$ (1500 rpm Jouan C 412 bench

177   centrifuge) for 5 minutes and the top layer (aqueous) was removed by aspiration. The

178   percentage of lipids was determined gravimetrically after evaporation of solvent under

179   stream of oxygen-free nitrogen (OFN) and overnight desiccation under vacuum. Lipids

180   were re-dissolved in chloroform:methanol (2:1, v/v) containing 0.01% butylated

181   hydroxytoluene (BHT) at a concentration of 10 mg/ml and stored under nitrogen at -

182   20°C prior to FA analysis. All lipid extractions were done in duplicate. Percent lipid

183   was calculated as follows:

184

185   % Lipid=Mass Lipid (g) / Mass Sample (g) $\times 100$

186

187   FA methyl esters (FAME) were prepared from total lipids by acid-catalysed

188   transesterification according to the method of Christie (1982) and extracted and purified

189   as described by Tocher and Harvie (1988). Total lipids (100 μl) and 17:0 free FA

190   standard (heptadecaenoic acid) at 10% of the total lipid (100 μl) were mixed and the

191   solvent evaporated under nitrogen evaporator. Toluene (1 ml) was added to dissolve

192 neutral lipids followed by addition of 2 ml methylating reagent (1% (v/v) solution of

193 sulphuric acid in methanol). After mixing, the tubes were incubated overnight (16

194 hours) in a hot block at 50°C. Following incubation, tubes were cooled to room

195 temperature and 2 ml of 2% (w/v) $KHCO_3$ and 5 ml of iso-hexane:diethyl ether (1:1,

196 v/v) + 0.01% (w/v) BHT were added, mixed and centrifuged at 400 x g for 2 minutes.

197 The upper organic layer was transferred to another test tube and additional 5 ml of

198 isohexane:diethyl ether (1:1, v/v) (no BHT) was added and same procedure repeated.

199 The solvent was evaporated under nitrogen evaporator and FAMEs re-dissolved in 100

200 μl of iso-hexane.

201 FAMEs were purified by thin layer chromatography (TLC) plates (20 × 20 cm).

202 FAMEs were loaded on the plates using Hamilton syringe (100 μl). Plates were

203 chromatographed in iso-hexane:diethyl ether:acetic acid (90:10:1, v/v/v). To visualise

204 the FAMEs the margins from the edges of the plates were sprayed with 1% (w/v) iodine

205 in chloroform. FAMEs were eluted from the silica with 10 ml of iso-hexane:diethyl

206 ether (1:1, v/v) + 0.01% (w/v) BHT followed by centrifugation. FAMEs were stored

207 under nitrogen at -20°C until further analysis.

208 FAMEs were separated and quantified by gas-liquid chromatography using a

209 Fisons GC-8160 (Thermo Scientific, Milan, Italy) equipped with a 30 m × 0.32 mm i.d.

210 × 0.25 μm ZB-wax column (Phenomenex, Cheshire, UK), on-column injector and a

211 flame ionization detector. Hydrogen was used as a carrier gas with initial oven thermal

212 gradient 50°C to 150°C at 40°C/min to a final temperature of 230°C at 2°C/min.

213 Individual FAME were identified by comparison of their retention times with known

214 standards (heptadecanoic acid (17:0) (internal standard); marinol oil (reference

215 standard); SupelcoTM 37-FAME mix (Sigma-Aldrich Ltd., Poole, UK)) and by

216 reference to published data (Ackman, 1980; Tocher & Harvie, 1988). Data were

collected and processed using Chromcard for Windows (version 2.01; Thermoquest Italia S.p.A., Milan, Italy). Individual FA concentrations were expressed as percentages of the total content. All samples were analysed in duplicates to ensure precision of the method.

Of the 33 identified fatty acids (FAs), 15 fatty acids were selected for statistical analysis based on the abundance and/or importance (14:0, 16:0, 18:0; 16:1n-7; 18:1n-7; 20:1n-9; 22:1n-11, 20:4n-6, 18:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3) and potential aquaculture biomarkers (18:2n-6, 18:3n-3 and 18:1n-9) (Iverson, 2009).

2.6 Statistical Analysis

All analysis were conducted and figures (including maps) plotted using the statistical software R (R Development Core Team 2019) run in RStudio (version 3.6.2, RStudio Team 2019) with libraries rgdal (Bivand, Keitt & Rowlingson, 2016), ggplot2 (Wickham, 2009), rgeos (Bivand & Rundel, 2016), and maptools (Bivand & Lewin-Koh, 2016) and Global Administrative Areas (GADM) database. Confidence intervals for frequency of occurrence were estimated using the function binconf in library Hmisc (Harrell, 2016). The package lsmeans (Lenth, 2016) was used for contrasts between groups. The package plyr was also used for data arrangement (Wickham, 2011). LDA was performed using the package MASS (Venables & Ripley, 2002) with function lda. Packages ggplot2 (Wickham, 2009) and cowplot (Wilke, 2015) were used to plot the data.

Prior to applying any statistical models to the data graphical exploratory tools were used as suggested by Zuur, Elena & Elphick (2010). Boxplots were used to detect outliers or observations that are too far off from most of the observations. Both boxplots and a quantile-quantile (Q-Q) plots were used to get a general impression of the

homogeneity and data distribution. Boxplots for length, weight, condition indices, lipid and fatty acids are provided as supplementary information. Linear regressions were used to check for differences in the length and weight of each species between farm and control sites, as this is a potential confounding variable.

In order to determine the dietary composition of the wild fish frequency of occurrence of each group of items (fish, fish pellets, invertebrates and unidentified) was calculated and plotted for both mackerel and whiting.

In order to detect whether there was any impact of the farming on condition indices and fatty acids, one way analysis of variance (ANOVA) models were applied with single degree contrasts used to evaluate differences between farm and control and the two farms. First, one-way ANOVAs were fitted separately to mackerel and whiting to evaluate differences in length, mass, total lipid and selected individual fatty acid contents of the wild fish, between sites (farms and controls). Single degree of freedom contrasts were then used to detect differences between the combined farm and control sites; and then between the two farms (excluding control sites). This followed the procedure in Mangiafico (2015).

LDA was used to distinguish between mackerel and whiting sampled at the different locations. Linear discriminant analysis (LDA) is a multivariate technique that calculates the combination of FAs that produce the maximum multivariate distance among groups by creating uncorrelated linear equations of the original FAs (Budge et al. 2006). The main assumptions for LDA include that observations are independent, the covariance matrices are homogeneous and the data are multivariate normal (Budge et al. 2006). Budge et al. (2006) notes that these assumptions are rarely met with FA data and one should be aware of the limitations and potential effects on the interpretation of the results.

3. RESULTS

3.1 Stomach contents

Stomach content analysis is presented in Figure 2. Of the mackerel caught near both fish farms 7% had empty stomachs and of reference sites 16% had empty stomachs. Fish (clupeids) was the main item found in most of the stomachs of mackerel sampled near the two fish farms and reference sites (Figure 2A). About 10% of the mackerel sampled near the sea cages had consumed waste pellets and none were found in fish from reference sites. Because of longer transport time and cooling failure, from mackerel collected at Reference Mackerel 3 was difficult to identify because digestion was at its final stages.

Of the whiting caught near both fish farms 17% had empty stomachs and of reference sites 40% had empty stomachs. Invertebrates were the main item found in most of the stomachs of whiting sampled near the sea cages and reference sites (Figure 2B). Of the whiting caught near the sea cages 31% had consumed waste pellets and none were found in whiting caught at reference sites.

3.2 Length, mass and condition

Descriptive statistics for length, mass and condition indices are presented in Table 2. Total length of mackerel sampled near both farms was significantly different than those sampled away from cages. Total length of mackerel sampled near the halibut farm were statistically significant as compared to those sampled near the salmon farm (Table 2). The mass of mackerel near the farms was statistically different than the mass of mackerel sampled away from the cages. The mass of mackerel sampled near the halibut farm was significantly different than the mass of mackerel sampled near the

291 salmon farm (Table 2). The FCI of mackerel sampled near the sea cages was

292 significantly different than the FCI of mackerel sampled at the reference sites and no

293 statistical differences were found in the FCI of mackerel sampled at the two farms

294 (Table 2). The HSI for mackerel sampled near the farms was significantly different than

295 the HSI for mackerel sampled away from the cages. The HSI for mackerel sampled at

296 the halibut farm was significantly different than the HSI for mackerel sampled at the

297 salmon farm (Table 2).

298 The total length of whiting sampled near the fish farms was statistically different

299 than the total length of whiting sampled away from the cages. The total length of

300 whiting sampled at the halibut farm was significantly different than the total length of

301 whiting sampled at the salmon farm (Table 3). The mass of whiting sampled near the

302 fish farms was significantly different than the mass of whiting sampled away from the

303 cages. The mass of whiting sampled at the halibut farm was significantly different than

304 the mass of whiting sampled at the salmon farm. No statistical differences were

305 detected in the FCI of whiting sampled near and away sea cages and between both

306 farms. The HSI of whiting sampled near the farms was statistically different than the

307 HSI of whiting sampled away from the cages (Table 3). No statistical differences were

308 found in HSI of whiting sampled near the halibut and salmon farms.

309 **3.3 Lipid and fatty acid composition**

310 The lipid and FA analysis of the diets fed to farmed fish in both farms can be

311 found in Table 4. Lipid content and levels of selected FAs for mackerel and whiting

312 sampled near the two fish farms and at reference sites can be found in Tables 5 and 6,

313 respectively.

314 **3.4 Commercial diet composition**

315   The proportion of total lipid in commercial fish feeds used in the halibut and

316   salmon farms in 2014 was about 25.6% (Table 4). The diet at the salmon farm was rich

317   in terrestrially based oils such as 18:2n-6, 18:3n-3 whereas the diet at the halibut was

318   rich in marine oils such as 22:6n-3 (Table 4). The halibut diet was also rich in 20:1n-9

319   and 22:1n-11 (Table 4).

320   **3.5 Lipid and fatty acid composition of wild fish**

321   Total lipids of muscle tissues of mackerel sampled near sea cages did not

322   statistically differ from the total lipids in mackerel sampled from reference sites (Table

323   5). No statistical differences were found in the lipid proportions of mackerel sampled

324   near the halibut and salmon farms (Table 5).

325   Fatty acids that differed between mackerel sampled near and away from fish

326   farms included: 14:0, 16:0, 18:0, Total Saturated FAs, 16:1n-7, 18:1n-9, 18:1n-7,

327   20:1n-9, 22:1n-11, Total Monosaturated FAs, 20:4n-6, Total n-6 PUFAs, 18:3n3,

328   18:4n-3, 20:5n-3, 22:5n-3, 22:6n-3, Total n-3 PUFAs, Total PUFAs, n-3/n-6 (Table 5).

329   Fatty acids that differed between mackerel sampled near a halibut and a salmon

330   farm included: 20:4n-6, 20:5n-3 (Table 5).

331   Total lipids of muscle tissues of whiting sampled near sea cages were similar to

332   total lipids of muscle tissues sampled at reference whiting sites (Table 6). Total lipids of

333   whiting sampled near the halibut farm were similar to those of whiting sampled near the

334   salmon farm (Table 6).

335   Fatty acids that were found statistically different between the muscle tissue of

336   whiting sampled near and away from sea cages were: 14:0, 16:0, 18:1n-7, 20:1n-9,

337   22:1n-11, Total Monosaturated FAs, 18:2n-6, 20:4n-6, Total PUFAs, 18:3n-3, 18:4n-3

338   20:5n-3, 22:5n-3, 22:6n-3, n-3 PUFAs, Total PUFAs, n-3/n-6 (Table 6).

339   Fatty acids found statistically different between the muscle tissue of whiting

340   sampled near the halibut farm and the salmon farm were: 14:0, 16:0, 20:1n-9, 22:1n-11,

341   20:5n-3, 22:5n-3, 22:6n-3 (Table 6).

342   **3.6 Linear Discriminant Analysis**

343   Results of LDA for mackerel and whiting sampled near and away from sea

344   cages can be found in Figures 3 and 4. The coefficients of the LDA functions for the

345   fatty acids for mackerel and whiting can be found in Tables 7 and 8, respectively.

346   For mackerel, the linear discriminant function plot showed partial separation

347   between control and farm sites (Figure 3, LD1 axis LD2 partially discriminates the two

348   farms. The FAs that contributed to the most separation between mackerel sampled near

349   and away from sea cages were: 18:3n-3, 18:1n-7, 14:0, and 18:0. The FAs 18:3n-3,

350   18:0, 14:0, 18:1n-7, and 20:5n-3 contributed to the separation between mackerel

351   sampled near sea cages of the salmon and halibut farms (see also Tables 7). Linear

352   discriminant function correctly assigned 52.2% of all samples to their origin (Melfort

353   Farm (50%), Leven Farm (77%), Reference Mackerel 1 (24%), Reference Mackerel 2

354   (65%) and Reference Mackerel 3 (47%)). The reference sites were not separated well

355   indicating dietary similarities.

356   For whiting, the linear discriminant function plot separated the whiting sampled

357   near the sea cages and those caught away from cages more clearly than for mackerel

358   (Figure 4). LD1 separated farm from reference sites, LD2 separated the two reference

359   sites and LD3 separated the salmon and the halibut farms. The FAs that contributed

360   most to the discrimination between whiting sampled near and away from sea cages

361   were: 22:5n-3, 16:1n-7, 22:1n-11 and 18:2n-6. The FAs 18:4n-3, 20:1n-9, 14:0 and

362   18:3n-3 contribute to the discrimination between the two reference sites of whiting (see

363   also Table 8). It is also worth noting that within the whiting sampled at Reference 1 site

15

there appears to be two distinct groups (Figure 4A). The FAs 14:0, 18:3n-3, and 16:1n-7 contributed to the separation between whiting sampled near the halibut and salmon farm (Table 4B). Linear discriminant analysis correctly assigned overall 90.4% of all samples (Melfort Farm (89.5%), Leven Farm (76.5%), Reference Whiting 1 (95%) and Reference Whiting 2 (100%)).

4. DISCUSSION

Both the salmon and halibut farming had an impact on the mackerel and whiting as both species consumed waste feed detected in their stomach and fatty acid profiles. The LDA was able to distinguish between fish sampled near the salmon farming and those sampled near the halibut farming. The overall impacts of both the halibut farm and the salmon farm appear to be more evident in whiting than in mackerel.

4.1 Impacts of fish farming on wild mackerel and whiting

As it has been noted by various studies (see reviews by Sanchez-Jerez et al., 2011; Uglem et al., 2014) sea cages have a large attractive effect which could be because of habitat provision, food availability and/or chemical attraction to the farmed fish. Food availability has been suggested as the strongest attractant of wild fish to fish farms (e.g. Uglem et al., 2014). This has also been termed the "birdfeeder effect" (Eveleigh et al., 2007). The present study provides evidence that both farming activities increased the presence of mackerel and whiting possibly as a response to the presence of food resources.

Some of the feed from both types of fish farming is lost to the environment. More of this waste feed is expected to be lost through salmon cages than the halibut farming. The reason for this is that halibut is a sedentary species and the presence of tarpaulin would allow some of these waste pellets to be consumed by the halibut

389   (Davies & Slaski, 2003). Some of the feed will also be indigested by both the halibut

390   and the salmon. The average feed conversion ratios for halibut are 1.3 and for salmon

391   about 1.1-1.2 (Davies & Slaski, 2003). The rest of the feed is converted in fish biomass

392   and some is excreted as dissolved nutrients that become available for microbial and

393   primary production (Davies & Slaski, 2003).

394   Although the halibut farm was much smaller in scale as compared to the salmon

395   farm both farms appear to impact mackerel and whiting sampled near the sea cages.

396   Both mackerel and whiting sampled near both farming activities were found with

397   aquaculture pellets and other food items in their stomachs. Mackerel sampled near both

398   fish farming activities were overall longer and heavier than mackerel sampled away

399   from the farms, potentially this is a confounding variable that may be driving some of

400   the differences between farm and control sites. Similarly, whiting sampled near the

401   farms were bigger and heavier than those sampled away from the farms. The whiting

402   sampled at the salmon farm were bigger than whiting sampled from all other sites.

403   Both species sampled near the salmon farm were heavier and longer which

404   could be because of the presence of the farm, loch effect and/or age-related differences.

405   The salmon farm is located in Loch Leven which has a higher flushing rate than Loch

406   Melfort indicating potential higher nutrients availability in Loch Melfort. Thus, the wild

407   fish in Loch Leven might benefit more from the additional nutrients released from the

408   salmon farm.

409   The abundance of prey reduces foraging times of an animal which results in

410   improved biological condition (Oro, Genovart, Tavecchia, Fowler & Martínez-Abraín,

411   2013). Some differences in condition indices were noted for mackerel and whiting

412   sampled near and away from the sea cages. However, these indices were not highly

413   reliable to indicate whether the differences were because of the presence of the farms or

414   the loch effect.

415   Results for mackerel differed from whiting. There was both a lower proportion

416   of fish with pellets in the stomach contents, and also a less clear separation between

417   farm and control sites in terms of fatty acid composition (compare Figures 3 and 4).

418   This is likely due to the more mobile behaviour of the mackerel leading to a weaker

419   association between the farm and the fish, with the mackerel visiting the farms for

420   shorter periods and relying less on direct feeding on pellet waste than for the whiting.

421   Mackerel is a species that needs to continuously swim (lack of swimbladder)

422   which raises the energy requirements of the fish (Juell, Holm, Hemre, & Lie, 1998)

423   whereas whiting is a benthopelagic species. A higher portion of the whiting sampled

424   near both farming activities were found with artificial pellets than mackerel sampled

425   near the farms suggesting a strong dependence on the farm by these fish. Other gadoids

426   such as saithe have been found with pellets in their stomachs when caught near cages

427   (Carss, 1990; Skog, Hylland, Torstensen & Berntssen, 2003). Fernandez-Jover et al.

428   (2011a) reported 6-96% of the diet of cod and saithe near fish farms in Norway was

429   composed of waste feed. In contrast, Mente et al. (2008) studied the diets of demersal

430   fish including whiting at four sea lochs that support fish farms on the West Coast of

431   Scotland and did not find any pellets in the diet of whiting. The diet of whiting

432   consisted mainly of *Malacostracan crustacea* (e.g. shrimp) and teleost fish (e.g.

433   clupeids and gadoids) (Mente et al., 2008). Dietary difference between lochs were noted

434   but dietary differences related to the presence of fish farming were less consistent with

435   differences found for individual lochs (Mente et al., 2008). Mente et al. (2008) did not

436   find clear causal relationship between fish farming development and impacts on diet

437   composition. Moreover, Mente et al. (2008) noted lack of clear aquaculture influence

438 on the diets of the sampled fish might be related to the sampling methodology which

439 was using bottom trawlers within 50 m from the nearest sea cages. In the present

440 research, sampling took place at the sea cages using rod and line which selects for

441 feeding fish. The presence of waste pellets in whiting sampled next to the cages

442 indicates direct effect of the halibut and salmon farms. Although this may indicate a

443 local-only effect as Mente et al. (2008) pointed out there may be a wider-scale

444 ecological impact of fish farming on marine fish populations.

445   Although, the weight, length, FCI and HSI were not strong indicators for fish

446 farming influence on the wild fish the FA analysis was better in detecting the impact of

447 farming activities on wild fish. Both mackerel and whiting sampled near both farms had

448 modified FA profiles as compared to those sampled away from the cages. LDA

449 indicated clear separation between fish sampled near the salmon and halibut farms. The

450 difference between fish sampled near the salmon and halibut farms is related to the

451 differences in the aquaculture feeds at both farms. The salmon diet contained higher

452 levels of the FA 18:2n-6, 18:3n-3, 18:1n-9, and lower n-3/n-6 ratios as compared to the

453 halibut diet. The FA 18:2n-6 appears to be a clear causal contributor towards the

454 separation between farm and reference sites. The main contributing FA for the

455 separation between mackerel and whiting sampled near the halibut and salmon farms

456 appears to be 18:3n-3.

457   The impact of both fish farming activities was stronger in whiting than in

458 mackerel. The LDA was able to classify 90.4% of whiting sampled near and away from

459 the sea cages. The classification was much higher than that for mackerel (52.2%)

460 indicating a stronger influence of both the halibut and the salmon farms on whiting than

461 on mackerel.

462   The LDA was also able to classify 89.5% of the whiting sampled near the

463 halibut farm and 76.5% of the whiting sampled near the salmon farm. In mackerel, the

464 LDA correctly differentiated 50% of the mackerel sampled near the halibut farm and

465 77% of the mackerel sampled near the salmon farm. Similar to the LDA results of

466 mackerel, the FA 18:3n-3 appears to be a strong signal for the salmon farm. Fernandez-

467 Jover et al. (2011a) also used LDA to distinguish between cod and saithe sampled near

468 and away from sea cages in Norway. The LDA classified 88.5% and 96.7% of the cod

469 muscle and liver, respectively and 85.7% and 96.7% of the saithe muscle and liver,

470 respectively (Fernandez-Jover et al., 2011a).

471   As indicated by the stomach content and fatty acid results the presence of

472 various farming activities can have an impact on the wild fishes with stronger impacts

473 on more residential species such as whiting. There is limited information on the ecology

474 of whiting in both lochs but it is expected to be similar to other gadoids. In general,

475 gadoids spend their first year in various Lochs on the West Coast of Scotland and could

476 remain inshore for about 2 to 4 years before joining the offshore populations (Hawkins

477 et al. 1985). During the winter months the food availability is scarce in the loch

478 resulting in poor condition and growth of the juvenile gadoid populations (Hawkins et

479 al. 1985). Thus the presence of additional feed resources from the farms could be of

480 benefit for the juvenile gadoid populations. However, it is not clear from this study how

481 changes in their fatty acid profiles would impact the growth and reproduction.

482 **4.2   Study limitations**

483   The study design needs to have lochs without aquaculture activities; however this

484 is very difficult to accomplish as there are almost no lochs without aquaculture

485 activities on the West Coast of Scotland.

486   Both the stomach content and the fatty acid analysis were useful tools for

487   detecting the impacts of the halibut and the salmon farms on migratory and a residential

488   species. However, fatty acids give a better indication of long-term influence of marine

489   farming on the wild fish and other organisms (White et al. 2019).

490   FA analysis was useful in distinguishing between salmon and halibut farming.

491   The use of individual FAs as biomarkers (e.g. 18:2n-6 and 18:3n-3) of terrestrial origin

492   should be taken with caution as some of these FAs are also present in low levels in the

493   marine environment (Fernandez-Jover et al., 2011b). Fish oil and fish meal containing

494   high levels of n-3 PUFAs (20:5n-3 and 22:6n-3) are limited and expensive and

495   therefore there has been increasing research efforts to find alternative replacements

496   such as using plant-based ingredients (Tacon & Metian, 2008). Other potential

497   alternatives for terrestrial based feeds for fish meal and fish oil include microalgae

498   (Sprague, Dick & Tocher, 2016) or genetically modified oilseed crop plants that can

499   synthesize n-3 PUFAs (Betancor et al., 2015). Changes in FA profiles of wild fish

500   feeding waste feed will be minimal as ingredients in the fish feed change towards

501   ingredient that are similar to the natural feed of fish. Thus, to monitor the sustainable

502   growth of marine aquaculture alternative techniques such as stable isotope analysis or a

503   combination of new techniques is needed to detect the environmental impacts.

504   The univariate and multivariate techniques were useful approximation to fit to the

505   data. However, the LDA was a more powerful approach in detecting the differences

506   between fish sampled at the various locations. Although some statistical differences

507   were noted using the univariate approach caution should be taken as not of all these

508   differences were noted using LDA.

509   It is also important to note that although there may be some statistical significance

510   in some of the variables it may not have any ecological relevance (Wilding & Hughes

2010). Any anthropogenic activity will have a localised impact with potential broader impacts (Wilding & Nickell 2007). Thus, it would be of high importance to take a pluralistic approach into detecting broader scale impacts of various farming activities.

5. CONCLUSIONS

Both the salmon and halibut farms provided additional food resources for mackerel and whiting. There is potential for both species to stay longer near this readily available food resource which could have an impact on migration and reproduction. The FA analysis indicated that the feed ingredients of the salmon farm could be detected more easily than those used for the halibut farm. Other methods or a combination of methods would be needed to detect the impact of fish farming on wild fish populations.

As marine aquaculture expands there will be further interactions with the capture fisheries sector and it is of high importance that these two sectors are managed in a sustainable manner. Long-term regional additive effects between both sectors would be of importance to be evaluated. This could be done using various ecosystem-based modelling approaches, spatial planning, stock enhancement and cooperative management of the sectors.

DATA AVAILABILITY

The data that support the findings of this study are openly available in DataSTORRE (Stirling University Online Repository for Research Data) at http://hdl.handle.net/11667/135, reference number 11667/135.

REFERENCES

Ackman, R. G. (1980). Fish lipids. In: Advances in Fish Science and Technology Farnham: Fishing News (ed. by Connell, J. J.), pp. 83–103.

Arechavala-Lopez, P., Sanchez-Jerez, P., Bayle-Sempere, J., Fernandez-Jover, D., Martinez-Rubio, L., Lopez-Jimenez, J. A. & Martinez-Lopez, F.J. (2011). Direct interaction between wild fish aggregations at fish farms and fisheries activity at fishing grounds: a case study with *Boops boops*. *Aquaculture Research, 42,* 996–1010.

Arechavala-Lopez, P., Sæther B.-S., Marhuenda-Egea, F., Sanchez-Jerez, P. & Uglem, I. (2015). Assessing the influence of salmon farming through total lipids, fatty acids, and trace elements in the liver and muscle of wild Saithe *Pollachius virens*. *Marine and Coastal Fisheries: Dynamics, Management, and Ecosystem Science, 7,* 59–67.

Bergé, J. P. & Barnathan, G. (2005). Fatty acids from lipids of marine organisms, Molecular biodiversity, Roles as biomarkers, biologically active compounds and economical aspects. *Advances in Biochemical Engineering/ Biotechnology, 96,* 49–126.

Betancor, M. B., Sprague, M., Sayanova, O., Usher, S., Campbell, P. J., Napier, J. A., Caballero, M. J. & Tocher, D. R. (2015). Evaluation of a high-EPA oil from transgenic Camelina sativa in feeds for Atlantic salmon (*Salmo salar* L.): Effects on tissue fatty acid composition, histology and gene expression. *Aquaculture, 444,* 1–12.

Bivand, R., Keitt, T. & Rowlingson, B. (2016). rgdal: Bindings for the Geospatial Data Abstraction Library. R package version 1.1-10. https://CRAN.R-project.org/package=rgdal

Bivand, R. & Rundel, C. (2016). rgeos: Interface to Geometry Engine - Open Source (GEOS). R package version 0.3-19. https://CRAN.R-project.org/package=rgeos

Bivand, R. & Lewin-Koh, N. (2016). maptools: Tools for Reading and Handling Spatial Objects. R package version 0.8-39. https://CRAN.R-project.org/package=maptools

Budge, S.M., Iverson, S.J. and Koopman, H.N. (2006) Studying trophic ecology in marine ecosystems using fatty acids: a primer on analysis and interpretation. *Marine Mammal Science, 22(4),* 759-801.

574 Carss, D. N. (1990). Concentrations of wild and escaped fishes immediately adjacent to
575   fish farm cages. *Aquaculture, 90,* 29–40.

576 Christie, W. W. (1982). In: Lipid Analysis (ed. by Christie, W. W.), pp. 17-23. Oxford:
577   Pergamon Press.

578 Dalsgaard, J., St.John, M., Kattner, G., Müller-Navarra, D. C. & Hagen, W. (2003).
579   Fatty acid trophic markers in the pelagic marine food environment. *Advances in*
580   *Marine Biology, 46,* 226–340.

581 Davies, I. M. & Slaski, R. J. (2003). Waste production by farmed Atlantic halibut
582   (*Hippoglossus hippoglossus* L.). *Aquaculture, 219,* 495–502.

583 Diana, J. S., Egna, H. S., Chopin, T., Peterson, M. S., Cao, L., Pomeroy, R., Verdegem,
584   M., Slack, W. T., Bondad-Reantaso, M. G. & Cabello, F. (2013). Responsible
585   aquaculture in 2050: valuing local conditions and human innovations will be key
586   to success. *BioScience, 63(4),* 255–262.

588 Edwards, A. & Sharples, F. (1986). Scottish sea lochs: a catalogue. Edinburgh,
589   Scotland: Nature Conservancy Council.

590 Eveleigh, E. S., McCann, K. S., McCarthy, P. C., Pollock, S. J., Lucarotti, C. J., Morin,
591   B., McDougall, G. A., Strongman, D. B., Huber, J. T., Umbanhowar, J. & Faria,
592   L. D. B. (2007). Fluctuations in density of an outbreak species drive diversity
593   cascades in food webs. *Proceedings of the National Academy of Sciences, 104,*
594   16976–16981.

595 Fernandez-Jover, D., Martinez-Rubio, L., Sanchez-Jerez, P., Bayle-Sempere, J. T.,
596   Jimenez, J. A. L., Lopez, F. J. M., Bjørn, P-A., Uglem, I. & Dempster, T.
597   (2011a). Waste feed from coastal fish farms: a trophic subsidy with
598   compositional side-effects for wild gadoids. *Estuarine, Coastal and Shelf*
599   *Science, 91,* 559–568.

600 Fernandez-Jover, D., Arechavala-Lopez, P., Martinez Rubio, L., Tocher, D.R., Bayle-
601   Sempere, J.T., Lopez-Jimenez, J.A., Martinez-Lopez, F.J. and Sanchez-Jerez, P.
602   (2011b) Monitoring the influence of marine aquaculture on wild fish
603   communities: benefits and limitations of fatty acid profiles, Aquaculture
604   Environment Interactions, 2(1), pp. 39-47.

605 Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). A simple method for the isolation
606   and purification of total lipids from animal tissues. *The Journal of Biological*
607   *Chemistry, 226,* 497–509.

608 Gillibrand, P. A. (2001). Calculating exchange times in a Scottish fjord using a two-
609   dimensional, laterally-integrated numerical model. *Estuarine, Coastal and Shelf*
610   *Science, 53 (4),* 437–449.

611 Gillibrand, P., Gubbins, M., Greathead, C. & Davies, I. M. (2002). Scottish Executive
612   Locational Guidelines for Fish Farming: Predicted Levels of Nutrient
613   Enhancement and Benthic Impact. Fisheries Research Service Marine
614   Laboratory, Aberdeen. Scottish Fisheries Research Report number 63.

615 Harrell Jr, F. E. (2016). with contributions from Charles Dupont and many others.
616   (2016). Hmisc: Harrell Miscellaneous. R package version 3.17-4. Available:
617   https://CRAN.R-project.org/package=Hmisc

618 Hawkins, A.D., Soofiani, N.M. and Smith, G.W. (1985) Growth and feeding of juvenile
619   cod (*Gadus morhua* L.). *ICES Journal of Marine Science, 42(1),* 11-32.

620 Holmer, M. (2010). Environmental issues of fish farming in offshore waters:
621   perspectives, concerns and research needs. *Aquaculture Environment*
622   *Interactions, 1,* 57–70.

623 Iverson, S. J. (2009). Tracing aquatic food webs using fatty acids: from qualitative
624   indicators to quantitative determination. In: Lipids in aquatic ecosystems (ed. by
625   Arts, M. T., Brett, M. T., & Kainz, M. eds.), pp. 281–307. New York: Springer,

626 Izquierdo-Gómez, D., González-Silvera, D., Arechavala-López, P., López-Jiménez,
627   J.A., Bayle-Sempere, J. T. & Sánchez-Jerez, P. (2015). Exportation of excess
628   feed from Mediterranean fish farms to local fisheries through different targeted
629   fish species. *ICES Journal of Marine Sciences, 72,* 930–938.

630 Juell, J. E., Holm, J. C., Hemre, G. I. & Lie, Ø. (1998). Growth and feeding behaviour
631   of caged Atlantic mackerel, *Scomber scombrus* L. *Aquaculture research, 29(2),*
632   115–122.

633 Kelly, J. R. & Scheibling, R. E. (2012). Fatty acids as dietary tracers in benthic
634   foodwebs. *Marine Ecology Progress Series, 446,* 1–22.

635 Lenth, R. V. (2016). Least-Squares Means: The R Package lsmeans. Journal of
636   Statistical Software, 69(1), 1–33.

637 Mangiafico, S.S. (2015). An R Companion for the Handbook of Biological Statistics,
638   version 1.3.2. https://rcompanion.org/rcompanion/. (Pdf version:
639   rcompanion.org/documents/RCompanionBioStatistics.pdf.)

640 Mente, E., Pierce, G. J., Santos, M. B. & Neofitou, C. (2006). Effect of feed and
641   feeding in culture of salmonids on the marine aquatic environment: a synthesis
642   for European aquaculture. *Aquaculture International, 14,* 499–522.

643 Mente, E., Pierce, G. J., Spencer, N. J., Martin, J. C., Karapanagiotidis, I., Santos, M.
644   B., Wang, J. & Neofitou, C. (2008). Diet of demersal fish species in relation to
645   aquaculture development in Scottish sea lochs. *Aquaculture, 277,* 263–274.

646 Oro, D., Genovart, M., Tavecchia, G., Fowler, M. S. & Martínez-Abraín, A. (2013).
647   Ecological and evolutionary implications of food subsidies from humans.
648   *Ecology letters, 16(12),* 1501–1514.

649 Parrish, C. C. (2013). Lipids in marine ecosystems. ISRN Oceanography, pp. 1-16.

650 Price, C., Black, K. D., Hargrave, B. T. & Morris, J. A. (2015). Marine cage culture and
651   the environment: effects on water quality and primary productivity. *Aquaculture*
652   *Environment Interactions, 6,* 151–174.

653 R Development Core Team (2019). R: A language and environment for statistical
654   computing. R Foundation for Statistical Computing, Vienna, Austria. Available:
655   https://www.R-project.org/

RStudio Team (2019). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL. Available: http://www.rstudio.com/

Sanchez-Jerez, P., Fernandez-Jover, D., Uglem, I., Arechavala-Lopez, P., Dempster, T., Bayle-Sempere, J.T., Pérez, C.V., Izquierdo, D., Bjørn, P-A. & Nilsen, R. (2011). Coastal fish farms as fish aggregation devices (FADs). In: S.A. Bortone, F. Pereira Brandini, G. Fabi and S. Otake, eds. *Artificial reefs in fisheries management.* Boca Raton, FL: CRC Press.

Skog, T. E., Hylland, K., Torstensen, B. E. & Berntssen, M. H. G. (2003). Salmon farming affects the fatty acid composition and taste of wild saithe *Pollachius virens* L. *Aquaculture Research, 34,* 999–1007.

Sprague, M., Dick, J. R. & Tocher, D. R. (2016). Impact of sustainable feeds on omega-3 long-chain fatty acid levels in farmed Atlantic salmon, 2006–2015. Scientific Reports, 21892.

Tacon, A. G. J. & Metian, M. (2008). Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: trends and future prospects. *Aquaculture, 285,* 146–158.

Tocher, D. R. & Harvie, D. G. (1988). Fatty acid compositions of the major phosphoglycerides from fish neural tissues; (n-3) and (n-6) polyunsaturated fatty acids in rainbow trout (*Salmo gairdneri*) and cod (*Gadus morhua*) brains and retinas. *Fish Physiology and Biochemistry, 5,* 229–239.

Turchini, G. M., Torstensen, B. E. & Ng, W-K. (2009). Fish oil replacement in finfish nutrition. *Reviews in Aquaculture, 1,* 10–57.

Uglem, I., Karlsen, O., Sánchez-Jerez, P. & Saether, B. J. (2014). Impacts of wild fishes attracted to open-cage salmonids farms in Norway. *Aquaculture Environmental Interactions, 6,* 91–103.

Venables, W. N. & Ripley, B. D. (2002). Modern Applied Statistics with S. Fourth Edition. Springer, New York. H. Wickham. ggplot2: elegant graphics for data analysis. Springer New York, 2009.

White, C. A., Woodcock, S. H., Bannister, R. J. & Nichols, P. D. (2019). Terrestrial fatty acids as tracers of finfish aquaculture waste in the marine environment. *Reviews in Aquaculture*, *11*(1), pp.133–148.

Wickham, H. (2009). ggplot2: Elegant Graphics for Data Analysis. New York: Springer-Verlag.

Wickham, H. (2011). The Split-Apply-Combine Strategy for Data Analysis. *Journal of Statistical Software, 40(1),* 1–29.

Wilding, T. & Hughes, D. (2010). A review and assessment of the effects of marine fish farm discharges on Biodiversity Action Plan habitats. ISBN: 978-1-907266-27-0. Available: http://www.sarf.org.uk/cms-assets/documents/28814-36718.sarf036---final-report.pdf

Wilding, T. A. & Nickell, T. D. (2013). Changes in Benthos Associated with Mussel (*Mytilus edulis* L.) Farms on the West-Coast of Scotland. *PLoS ONE,* 8(7): e68313.

698 Wilke, C. O. (2015). cowplot: Streamlined Plot Theme and Plot Annotations for
699   'ggplot2'. R package version 0.4.0. http://CRAN.R-project.org/package=cowplot

700 Zuur, A. F., Elena, N. I. & Elphick, C. S. (2010). A protocol for data exploration to
701   avoid common statistical problems. *Methods in Ecology and Evolution, 1 (1),* 3–
702   14.