

1 **Occurrence and potential transfer of mycotoxins in gilthead sea bream and**
2 **Atlantic salmon by use of novel alternative feed ingredients.**

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28 **Abstract**

29 Plant ingredients and processed animal proteins (PAP) are suitable alternative feedstuffs for
30 fish feeds in aquaculture practice, although their use can introduce contaminants that are not
31 previously associated with marine salmon and gilthead sea bream farming. Mycotoxins are
32 well known natural contaminants in plant feed material, although they also could be present
33 on PAPs after fungi growth during storage. The present study surveyed commercially
34 available plant ingredients (19) and PAP (19) for a wide range of mycotoxins (18) according
35 to the EU regulations. PAP showed only minor levels of ochratoxin A and fumonisin B1 and
36 the mycotoxin carry-over from feeds to fillets of farmed Atlantic salmon and gilthead sea
37 bream (two main species of European aquaculture) was performed with plant ingredient
38 based diets. Deoxynivalenol was the most prevalent mycotoxin in wheat, wheat gluten and
39 corn gluten cereals with levels ranging from 17 to 814 and $\mu\text{g kg}^{-1}$, followed by fumonisins
40 in corn products (range 11.1-4901 $\mu\text{g kg}^{-1}$ for fumonisin B1+B2+B3). Overall mycotoxin
41 levels in fish feeds reflected the feed ingredient composition and the level of contaminant in
42 each feed ingredient. In all cases the studied ingredients and feeds showed levels of
43 mycotoxins below maximum residue limits established by the Commission Recommendation
44 2006/576/EC, and following these guidelines no mycotoxin carry-over was found from feeds
45 to edible fillets of salmonids and a typically marine fish, such as gilthead sea bream. As far
46 we know, this is the first report of mycotoxin surveillance in farmed fish species.

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48 **Keywords:** Mycotoxins, marine aquaculture, plant ingredients, processed animal
49 proteins, fish feed, fish

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53 **1. Introduction**

54 Serious concern on fish meal and fish oil availability to support the rapidly growing
55 aquaculture industry has led to extensive search of alternative raw materials for
56 aquafeeds (Tacon and Metian, 2008; Torrissen et al., 2011). The most obvious
57 alternatives are plant oils and proteins, and the long-term consequences of high
58 inclusion levels of these feedstuff have been addressed in past and ongoing large EU
59 projects, such as AQUAMAX (www.aquamaxip.eu) and ARRAINA (www.arraina.eu),
60 where main results highly support the feasibility of a high level of replacement of
61 marine feed ingredients in both Atlantic salmon (*Salmo salar*) and gilthead sea bream
62 (*Sparus aurata*) (Benedito-Palos et al., 2008; Torstensen et al., 2008). Processed animal
63 protein (PAP) from the rendering industry is another valuable alternative feed ingredient
64 (Davies et al., 2009; Burr et al., 2012; Toldra et al., 2012), and recently the EU has set
65 out a working plan for the re-authorization of the use of non-ruminant PAPs in
66 aquafeeds after previous bans following outbreaks of transmissible spongiform
67 encephalopathies (EC, 2013a).

68 The use of these alternative feed ingredients can introduce contaminants that
69 were previously not associated with marine salmon and sea bream farming. One
70 example of this are mycotoxins, which are world-wide found in cereal grains and animal
71 feed (Binder, 2007 a,b; Streit et al., 2013). Mycotoxins are produced by fungi that pre-
72 harvest infect agricultural crops (field mycotoxins) or post-harvest agricultural
73 commodities stored under certain temperature and humidity conditions (storage
74 mycotoxins) (Magan et al., 2010; Bryden, 2012). Meat products can also be
75 contaminated with mycotoxins (Mizáková et al., 2002; Sorensen et al., 2010; Ostry et
76 al., 2013), and animal by-products could hence be a potential source for these
77 mycotoxins in animal feeds (Caruso et al., 2013). The mycotoxin aflatoxin B1 (AFB1)

78 is under EU feed regulation (EU, 2002), while guidance values have been set for animal
79 feed ingredients and animal feed for several mycotoxins, including deoxynivalenol
80 (DON), zearalenone (ZEN), ochratoxin A (OTA), and fumonisin B1 + B2
81 (FB1+FB2)(EC, 2006). For other mycotoxins, such as T-2 and HT-2 toxins, indicative
82 levels for cereal products, including those intended for animal feed have been set (EC,
83 2013b; Cheli et al., 2014). In fact, many surveillance studies have reported mycotoxin
84 levels on a wide range of randomly sampled feed ingredients and finished feeds from
85 terrestrial animals (Binder, 2007 a,b; Rodrigues and Naehrer, 2012; Streit et al., 2012;
86 Streit et al., 2013), but only few studies recent studies are done in fish feeds or farmed
87 fish (Pietsch et al., 2013; Wozny et al., 2013). Besides, most fish studies on mycotoxins
88 are focused on the hazards for fish health in experimental trials with fortified feeds
89 (Poston et al., 1982; Arukwe et al., 1999; Manning et al., 2003; EFSA, 2005; Manning
90 et al., 2005; Wozny et al., 2008; EFSA, 2011; Hoofst et al., 2011; Caruso et al., 2013)
91 with little information on the carry-over to the edible parts of the fish.

92 Multi occurrence of mycotoxins requires, however, the need for the application
93 of multi-mycotoxin methods in order to get a more accurate picture of the extent of the
94 wide range of mycotoxin contamination (Monbaliu et al., 2010; Streit et al., 2012;
95 Aberg et al., 2013). Earlier studies established feasible analytical approaches for
96 mycotoxins in feed ingredients, aquafeeds and fish fillets (Malachová et al., 2014;
97 Beltran et al., 2013; Nacher-Mestre et al., 2013). Based on this previous experience, the
98 present work aims to quantify a wide range of mycotoxins in commercially available
99 plant and PAP feed ingredients, fish feeds based on these ingredients, and their transfer
100 to the edible part of farmed Atlantic salmon and gilthead sea bream, two main species of
101 the European aquaculture. In addition to the 8 mycotoxin under EU regulation/guidance
102 in feed and feed ingredients (AFB1, DON, ZEN, OTA, FB1+FB2, T-2 and HT-2), 10

103 additional mycotoxins of potential relevance for food safety are included (AFB2, AFG1,
104 AFG2, FB3, nivalenol (NIV), 3-acetyldeoxynivalenol (3-AcDON), 15-
105 acetyldeoxynivalenol (15-AcDON), diacetoxyscirpenol (DIA), fusarenon-X (Fus X) and
106 neosolaniol (NEO)) in the study.

107

108 **2. Material and methods**

109 *2.1. Feed ingredients*

110 A total of 19 commercially available plant feed ingredients were provided by Biomar
111 (Grangemouth, UK) feed producer: wheat (n=3, Germany and Denmark), wheat gluten
112 (n=4, UK, Germany, and China), pea (n=1, Denmark), pea protein (n=2, Norway),
113 rapeseed meal (n=1, Denmark), corn gluten (n=3, China and Germany), soya protein
114 (n=4, Brazil) and sunflower meal (n=1, Russia). Nineteen commercially available PAPs
115 from non-ruminants were provided by the European Fat Processors and Renderers
116 Association (EFPPRA). All PAPs were produced according the EU regulation for PAP
117 intended for use as feed-ingredients in animal feed (EC, 2001, 2009). These PAPs are
118 category 3 products that are fit for human consumption at the point of slaughter (EC,
119 2009). The PAPs sourced are all produced in central Europe and included poultry bone
120 and meat meal (n=4), poultry blood meal (n=4), pork meal (n=3), pork blood meal
121 (n=3), pork greaves (n=2) and feather meal (n=3). All feed ingredients were stored at -
122 18° C until analyses.

123

124 *2.2. Experimental diets and feeding trials*

125 Fish feeds for feeding trials were based on plant feed ingredients, and not PAPs, as only
126 noticeable mycotoxin levels were found on the former feedstuffs (see results section).
127 The feeds were produced by Biomar under commercial aquafeed production techniques

128 based on high-temperature extrusion processes, which potentially could affect
129 mycotoxin residue levels. For gilthead sea bream, two diets were formulated with the
130 same feed ingredients varying the replacement of fish meal and fish oil by plant
131 ingredients. Salmon feeds were production triplicates of high plant ingredient diets
132 based on the same feed ingredients (Table 1).

133 Sea bream trial. Juvenile gilthead sea bream of Atlantic origin were fed with the
134 respective diet (triplicate tanks of 2500 l in groups of 150 fish each) for 8 months (May-
135 December) in the indoor experimental facilities of the Institute of Aquaculture of Torre
136 la Sal (CSIC, Spain) under natural light and temperature conditions at our latitude
137 (40°5'N; 0°10'E). Fish grew from an initial body weight of 15 g until 296-320 g with a
138 feed:gain ratio (feed/weight gain) of 1-1.05 regardless of diet composition. Over the
139 course of the trial, fish were fed daily (5-6 days per week) at visual satiety. At harvest
140 (week 31), 6 fish per dietary treatment were killed by a blood to the head and deboned
141 fillets were stored at -80 °C until analyses.

142 Salmon trial. Post-smolts were randomly distributed among 6 sea cages (5m x 5m x 5m;
143 125 m³; 150 fish per cage) at Gildesskål Research Station, GIFAS, Gildeskål kommune,
144 Norway. Prior to the start of the trial, fish were acclimated to the environmental
145 conditions for two weeks. At the start, the average fish weight was 228 ± 5 g and during
146 the 6th month feeding period (duplicate cages per diet) the weight fish is more than
147 doubled. Over the course of the trial, fish were hand-fed until satiation two times daily
148 and feed intake was recorded for each sea cage. At harvest (week 27), 3 fish per dietary
149 treatment were killed by a blood to the head and deboned fillets were stored at -80 °C
150 until analyses.

151

152 2.3. Analytical procedure

153 Up to 18 mycotoxins, AFB1, AFB2, AFG1, AFG2, OTA, NEO, FB1, FB2, FB3, T-2,
154 DIA, ZEN, NIV, DON, 3-AcDON, 15-AcDON, Fus X, and HT-2 were analyzed
155 according to the methodology of Beltran et al. (2013), adapted to the aquaculture
156 matrices (Nacher-Mestre et al., 2013). Briefly, 2.5 g homogenized samples were
157 extracted with acetonitrile:water 80:20 (1% HCOOH) using an automatic mechanical
158 shaker for 90 min. Then, the extract was centrifuged followed by a 4-fold dilution with
159 water and finally centrifuged prior analysis. Analyses were performed by ultra-high
160 performance liquid chromatography (UHPLC, BEH C18 analytical column, 1.7 μm
161 particle size, 2.1 mm \times 50 mm; Acquity, Waters, Milford, MA, USA,) coupled to
162 tandem mass spectrometry (MS/MS) with a triple quadrupole analyser (QqQ; TQ-S,
163 Waters Micromass, Manchester, UK) using an orthogonal Z-spray-electrospray
164 interface (ESI). The LC-MS/MS conditions are given in more detail in supplementary
165 data (Table 1 supplementary data). Matrix-matched calibration was used for a correct
166 quantification in order to compensate for matrix effects (details in reagents,
167 supplementary information). In every sequence of analysis (for each sample matrix),
168 two quality control samples (QCs), i.e. “blank” samples (previously analyzed) fortified
169 at the two different concentration levels were also analyzed together with the samples to
170 assure reliability of data reported. QC recovery experiments were performed at 40 and
171 400 $\mu\text{g kg}^{-1}$ for DON, HT-2, NIV, FUS X, 3-AcDON, 15-AcDON and OTA; at 4 and
172 40 $\mu\text{g kg}^{-1}$ for fumonisins, T-2, ZEN, DIA and NEO; and at 0.4 and 4 $\mu\text{g kg}^{-1}$ aflatoxins
173 (details in reagents, supplementary information). Figure 1 shows a general overview for
174 the QC recoveries in every matrix (ingredients, feeds and fish) for the different groups
175 of mycotoxins. In general, recoveries (%) were satisfactory in the range between 60 and
176 110 %. Linearity of the quantification was evaluated taking seven matrix-matched
177 standard solutions which were analyzed in duplicate in the following ranges: 0.01–1

178 ng/mL (aflatoxins), 0.1–10 ng/mL (fumonisins, T-2, ZEN, DIA, NEO) and 1–100
179 ng/mL (DON, HT-2 Toxin, NIV, Fus X, 3-AcDON, 15-AcDON and OTA). It was
180 considered satisfactory when correlation coefficients were higher than 0.99 with
181 residuals lower than 20%. The acquisition of three SRM (Selected Reaction
182 Monitoring) transitions per compound allowed the unequivocal confirmation of positive
183 samples, supported by the accomplishment of ion intensity ratios and retention time
184 when compared with reference standards. TargetLynx (MassLynx v. 4.1, Waters,
185 Manchester, UK) software was used to process the quantitative data obtained from
186 calibration standards and from samples. Limits of quantification (LOQ) as well as limits
187 of detection (LOD) were estimated in the matrices studied for a signal-to-noise ratio
188 (S/N) equal to 10 and 3, respectively, from the SRM chromatograms of samples spiked
189 at the lowest concentration level (Table 2).

190

191 **3. Results and Discussion**

192 The multi mycotoxin LC-ESI-MS/MS method was applied to the analysis of 18
193 mycotoxins in plant and animal ingredients used in the elaboration of fish feed, in
194 different experimental feeds and in cultured fish tissues from marine aquaculture trials.
195 Results of the QC recoveries included in each batch were satisfactory in the range
196 between 60 and 110% with some exceptions for fish fillet matrices. Figure 1 shows a
197 general overview for the QC recoveries in every matrix (ingredients, feeds and fish) for
198 the different groups of mycotoxins. Regarding matrix effect, fumonisins, DON, OTA
199 and ZEN were the compounds which showed higher matrix suppression in all matrices
200 studied. Table 2 shows the LOQ obtained for the matrices analyzed. LOQs at
201 concentrations around the level of $\mu\text{g kg}^{-1}$ were obtained for almost all studied
202 mycotoxins. For some mycotoxins no proper quantification could be obtained for some

203 matrices (NIV in rapeseed, corn, pea, poultry feather and blood meal and ZEN in
204 poultry feather and blood meal and pork meal) due to the presence of coeluted matrix
205 interference peaks. The LOQs for the different ingredients, feeds and fish muscle from
206 the feeding experiments were in all cases below the maximum permitted levels (EU,
207 2002; EC, 2006, 2013b).

208

209 3.1. Feed ingredients of plant origin

210 Table 3 gives the level of mycotoxins in plant feed ingredients that are
211 commonly used in commercial aquafeeds for Atlantic salmon and gilthead sea bream.
212 Fumonisin (sum FB1+FB2+FB3) in corn was the most prevalent mycotoxin
213 contamination (min.-max. 11.1-4901 $\mu\text{g kg}^{-1}$) followed by DON in wheat and corn
214 products (min-max. 17-504 and 139-814 $\mu\text{g kg}^{-1}$, respectively). Fumonisin was also
215 present in one wheat gluten sample, but with lower levels (13.2 $\mu\text{g kg}^{-1}$) than observed
216 in corn. ZEN as well as T-2 and HT-2 were found in some of the wheat and corn feed
217 ingredients (min-max. 8-17 and 2.8-67 $\mu\text{g kg}^{-1}$, respectively). OTA was found in wheat,
218 corn and pea protein products (min-max. 0.4-5.2 $\mu\text{g kg}^{-1}$ for all products). All levels
219 were under the EU regulation or guidance levels for mycotoxins in plant material
220 intended for animal feeds (Cheli et al., 2014). Plant feed ingredients for aquafeeds are
221 sourced from the global market and in the present study ingredients were obtained from
222 Asia, South-America and central and Northern Europe. The current study included only
223 a limited number of possible plant feed ingredients used for aquafeeds, not providing a
224 basis for global mycotoxin contamination assessment. Other studies on plant feed
225 ingredients used for terrestrial animal feeds, however, have performed a far more
226 extensive global surveillance showing regional and plant specific differences in
227 mycotoxin contamination (Binder et al., 2007b; Monbaliu et al., 2012; Njobeh et al.,

228 2012; Rodrigues and Naehrer, 2012; Afsah-Hejri et al., 2013; Schatzmayr and Streit,
229 2013; Streit et al., 2013).

230 In Northern world-wide regions such as North-America, North-Asia and central
231 Europe the main corn contaminants are DON (average levels ranging 1085-1421 $\mu\text{g kg}^{-1}$
232 ¹) and fumonisins (average levels ranging 1357-2861, 2180 $\mu\text{g kg}^{-1}$). In contrast, in
233 Southern regions such as South-America, South-East Asia and Southern Europe the
234 corn has far lower DON than fumonisins levels (average levels ranging 214-985 and
235 1568-3226, $\mu\text{g kg}^{-1}$, respectively) (Rodrigues and Naehrer, 2012). Similarly in the
236 present study, one corn sample from South-China had a lower DON than fumonisins
237 level (815 versus 4901, $\mu\text{g kg}^{-1}$ respectively) while the other two corn samples from
238 Europe (Germany) had lower and more equal DON and fumonisin levels. Both central-
239 European corn samples also had relatively high trichothecenes levels such as HT-2
240 toxin (67 $\mu\text{g kg}^{-1}$) followed by ZEN (8 $\mu\text{g kg}^{-1}$), as could be expected for *fusarium* fungi
241 producing toxicants in moderate climates (Binder et al. 2007b). The *fusarium* fungi
242 species are the most common source for corn fumonisins contamination but also
243 *Aspergillus niger* produces fumonisins on corn, mainly as FB2 (Soares et al., 2013).
244 Corn is a plant feed ingredient that is most affected by co-contamination of several
245 mycotoxins (Scudamore and Livesey, 1998) and similarly in the present study the corn
246 samples had co-occurrence of fumonisins B1, B2 and B3, DON, 15-AcDON, HT-2, T-
247 2, ZEN, and OTA.

248 Earlier global surveillance showed that DON was the main wheat contaminant
249 independently from region of origin (Rodrigues and Naehrer, 2012). Similarly, for
250 wheat products in the present trial which were sourced from central Europe and Asia,
251 DON was the main contaminant followed by ZEN and to a lesser degree T-2, HT-2
252 toxin and fumonisins (Table 3). Soybean meal products are widely used feed ingredient

253 in Atlantic salmon and sea bream farming, and only GMO-free soy products are used
254 which are mostly source from Brasil. Global surveys showed DON and fumonisins
255 equally present in soy from South-America, but at far lower levels than wheat and corn
256 (Rodrigues and Naehrer, 2012). In present study soy had only low mycotoxin
257 contaminations compared to wheat and especially corn (Table 3).

258 The mycotoxin OTA is mostly produced by *penicillium* species under storage
259 conditions and was mainly found in the present study in wheat and pea proteins (Table
260 3). The fungi *P. verrucosum* is typically primarily found on cereals and is therefore
261 responsible for the major contributor to OTA contamination of cereal products (Lund
262 and Frisvad, 2003). OTA can also be produced by several *Aspergillus* species which are
263 adapted to grown on various leguminous seeds (Bayman et al., 2006) which could
264 explain the low OTA contamination of peas. Clearly, as for terrestrial animal farming,
265 sourcing of plant feed ingredients based on product type and regions of origin is a first
266 step in control of mycotoxin aquafeed contamination.

267

268 3.2. Feed ingredients of animal origin

269 Table 4 gives the mycotoxin levels in the different processed animal proteins that can be
270 used for future aquafeeds after the lift of the ban on these products in the EU food
271 supply chain. As expected, only the typical storage mycotoxins, OTA but also FB1 were
272 detected in poultry feather and bone and meat meal (fumonisin) and pork blood (OTA).
273 The levels were, however, around detection limit and are by far under the EU guidelines
274 for plant products intended for animal feeds (60 mg kg⁻¹ for FB1+FB2 in maize and 250
275 µg kg⁻¹ for OTA in cereals (EC, 2006)). Fumonisin are mainly produced by a small
276 number of *Fusarium* species, which have specific crops (corn) as habitat (Pitt and
277 Hocking, 2009). However, other fumonisin producing fungi such as *Aspergillus niger*

278 (Mogensen et al., 2009) has been isolated from warm air-dried meat products
279 (Mizáková et al., 2002; Sorensen et al., 2010). The most common fumonisin produced
280 by *Aspergillus niger* is FB2 at high amounts of carbohydrate or NaCl (Frisvad et al.,
281 2007), although additional FB4 can be produced in agar cultures (Noonim et al., 2009)
282 and other fumonisins forms (FB1-4) are found on *A. niger* contaminated dried raisins
283 (Varga et al., 2010). In the present study, FB1 was the only fumonisin form detected on
284 PAP material. The *Aspergillus niger* strains are also known to produce OTA (Accensi et
285 al., 2004), which could be a source for the detected OTA in one of the PAP samples.
286 The fungi *Penicillium nordicum* is the most known OTA producer (Larsen et al., 2001;
287 Lund and Frisvad, 2003) and grows well at low temperatures on meat products but
288 mostly only at increased salinity (Schmidt-Heydt et al., 2012). Storage OTA
289 contamination by *P. nordicum* is, therefore, often limited to salted meat food products
290 such as cured ham and sausage (Sonjak et al., 2011; Schmidt-Heydt et al., 2012).
291 Products of animal origin such as pork and poultry raw meat or blood products can be
292 also indirectly contaminated by OTA when monogastric animals are fed with
293 contaminated feed stuffs (EFSA, 2004a) as dietary OTA can be transferred from the
294 feed to animal meat (Malagutti et al., 2005). From the present study, however, the risk
295 of OTA or FB1+B2+B3 contamination of EU produced PAP products intended for
296 aquafeeds seems low. Similarly, from surveillance studies on foodstuffs of both plant
297 and animal origin it was concluded that plant products rather than cured animal products
298 could be contaminated with OTA (Bertuzzi et al., 2013; Ostry et al., 2013)

299

300 3.3. Feed and fish muscle

301 The present study assess the transfer of mycotoxins throughout the sea bream
302 and Atlantic salmon food production chain by assessing mycotoxin levels in feed

303 ingredients and follow their transfer to commercially produced aquafeeds and
304 eventually carry-over to the edible parts of the fish fed on these feeds. Commercially
305 produced aquafeeds were made based on the same analysed plant feed ingredients given
306 in table 3. Table 5 gives the mycotoxin levels of sea bream feed with either a low or
307 high overall plant protein content as well as three Atlantic salmon feed production
308 repeats with a similar high-plant feed composition based on the same batch of feed
309 ingredients. For the sea bream feeds, the low plant-protein feeds had an unexpected
310 higher DON level than high-plant protein based feeds. One of the main sources for
311 DON in sea bream diets was contaminated wheat ($371 \mu\text{g kg}^{-1}$), which inclusion levels
312 in low plant feed was slightly higher than high plant feed (11 versus 7 %, respectively),
313 thus explaining the slightly higher levels in low-plant diets. The wheat gluten used in
314 the sea bream diets had only minor DON levels ($17 \mu\text{g kg}^{-1}$). For the sea bream feeds,
315 the main plant-protein increase in high plant protein diets came from corn and soya
316 (from 31% to 50 % : low plant feed with 15% and 16% and high plant feed with 25%
317 and 25% for corn and soya, respectively). The soy protein concentrate (SPC) feed
318 ingredient had only detectable levels of FB1 and FB2, while corn was the main source
319 for fumonisin ($139 \mu\text{g kg}^{-1}$ sum FB1+2+3) and 15 Ac-DON ($53 \mu\text{g kg}^{-1}$), causing an
320 increase in these mycotoxins in high plant-protein feeds. The differences in mycotoxin
321 contamination of traditional marine feeds and high plant-protein substitution feeds in
322 the present study, exemplifies that mycotoxin levels in plant-protein based feeds are
323 more dependent on the individual contamination level of each plant-protein ingredient
324 rather than the overall higher inclusion level of plant protein. In addition to the
325 substitution of fish-meal with plant-proteins, an extra sea bream feed was produced in
326 which fish oil was substituted with plant oils. This substitution had no effect on feed

327 mycotoxin level supporting the notion that the plant proteins and not the plant oils are
328 the main source for mycotoxin contamination.

329 For the Atlantic salmon high plant-protein feed production repeats, mycotoxin
330 levels were as expected from the contamination level of the feed ingredients and with
331 similar levels among the repeats with the exception of fumonisins. Higher feed
332 fumonisin levels were found than could be expected from the low inclusion level (4%)
333 of the sole fumonisin feed ingredient source (corn, 403 $\mu\text{g kg}^{-1}$ sum FB1+2+3), and with
334 a large variation (112-754 $\mu\text{g kg}^{-1}$ sum FB1+2+3) among the production repeats. The
335 large variation in fumonisin levels suggest the present of storage fungi that can grow
336 heterogeneously within and among feed batches. The main source for fumonisins in
337 corn are *Fusarium* species which normally grow very little under storage conditions and
338 storage is not expected to increase *furasium* derived fumonisin contamination (Pitt et
339 al., 2013). *Aspergilles niger* fungi species can also produce fumonisins (Baker, 2006)
340 but they are also the source for the typical storage mycotoxin OTA, which were only
341 present at detectable levels in the salmon feeds as could be excepted from the inclusion
342 of OTA contaminated pea proteins (1.8 $\mu\text{g kg}^{-1}$ at inclusion level of 13%). Surveillance
343 of finished feed for terrestrial animals in Europe and the Mediterranean area gave
344 average fumonisin levels of 638 $\mu\text{g kg}^{-1}$ in 3 out of 10 analysed samples (Binder,
345 2007b). Slovenian poultry feed had fumonisin levels ranging from 36-1160 $\mu\text{g kg}^{-1}$
346 (Streit et al., 2012). Surveillance of feed ingredients and finished feeds in Europe and
347 the Mediterranean showed maximum OTA level in feed ingredients to be 33 $\mu\text{g kg}^{-1}$
348 while in finished feeds the mean levels were 305 $\mu\text{g kg}^{-1}$ with maximum of 530 $\mu\text{g kg}^{-1}$,
349 thus suggesting OTA contamination during storage of finished feeds. Studies on
350 rainbow trout feeds in Poland showed ZEN contamination up to 82 $\mu\text{g kg}^{-1}$, in the
351 present study however ZEN was not detected in any of the feeds.

352 Information on carry-over of contaminants from feed ingredients and feed to animal
353 food products is essential for appropriate human risk assessment of feed contaminants
354 (Leeman et al., 2007). Expert opinions by the European Food Safety Authorities (EFSA)
355 have evaluated the carry-over of several mycotoxins in terrestrial animals such as poultry,
356 swine and cow (EFSA, 2004a,b, 2005, 2007), while no information exists on the carry-over
357 in farmed fish species. In the present study, neither gilthead sea bream nor Atlantic salmon
358 had any detectable levels of mycotoxins in their fillet (data not shown) after respectively 8
359 and 7 months of feeding with the diets presented in table 5. In general, the carry-over of
360 mycotoxins in terrestrial animals is limited (EC, 2006) which is partly the basis for the use of
361 only guidance limits and not regulation limits for mycotoxins in feeds (with the exception of
362 the aflatoxins) as contaminated feed does not directly or indirectly impact the human health
363 (Siegel and Babuscio, 2011). Similarly in the present study, for marine farmed sea bream and
364 Atlantic salmon the potential carry-over of mycotoxin residue levels in commercial relevant
365 feeds was limited. It should be noted though, that the present study only assessed the parent
366 compounds of mycotoxins in limited feeding trials with ambient feed contaminations. More
367 detailed studies on the toxico-kinetics of dietary mycotoxins and their metabolites in the
368 main EU farmed fish species are needed to provide an appropriate risk assessment of food
369 safety from mycotoxin contaminated aquafeeds. More importantly, assessment on the
370 adverse effects of dietary mycotoxins on fish health and welfare is needed for the main EU
371 farmed fish species in order to establish acceptable feed mycotoxin levels for farmed fish
372 (Manning et al., 2005; Bernhoft et al., 2013).

373

374 **Acknowledgements**

375 This work has been (partly) funded under the EU Seventh Framework Programme by
376 ARRINA Project 288925: Advanced Research Initiatives for Nutrition and

377 Aquaculture. The views expressed in this work are the sole responsibility of the authors
378 and do not necessarily reflect the views of the European Commission. The authors also
379 acknowledge the financial support (partly) of the project SAFE-PAP, Research Council
380 Research and Development Project (227387) National Institute of Nutrition and
381 Seafood Research (NIFES, Norway). The authors acknowledge the financial support of
382 Generalitat Valenciana, as research group of excellence PROMETEO II/2014/023 and
383 PROMETEO II/2014/085. The authors are grateful with Serveis Centrals
384 d'Instrumentació Científica (SCIC) of University Jaume I for using the UPLC-TQS
385 Mass Spectrometer. This work has been developed in the framework of the Research
386 Unit of Marine Ecotoxicology (IATS (CSIC)-IUPA (UJI)).

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400 **References**

- 401 Aberg, A.T., Solyakov, A., Bondesson, U., 2013. Development and in-house validation
402 of an LC-MS/MS method for the quantification of the mycotoxins deoxynivalenol,
403 zearalenone, T-2 and HT-2 toxin, ochratoxin A and fumonisin B1 and B2 in
404 vegetable animal feed. *Food Addit. Contam. Part A-Chem.* 30, 541-549.
- 405 Accensi, F., Abarca, M.L., Cabanes, F.J., 2004. Occurrence of *Aspergillus* species in
406 mixed feeds and component raw materials and their ability to produce ochratoxin A.
407 *Food Microbiol.* 21, 623-627.
- 408 Afsah-Hejri, L., Jinap, S., Hajeb, P., Radu, S., Shakibazadeh, S., 2013. A Review on
409 Mycotoxins in Food and Feed: Malaysia Case Study. *Compr. Rev. Food. Sci. Food*
410 *Saf.* 12, 629-651.
- 411 Arukwe, A., Grotmol, T., Haugen, T.B., Knudsen, F.R., Goksoyr, A., 1999. Fish model
412 for assessing the in vivo estrogenic potency of the mycotoxin zearalenone and its
413 metabolites. *Sci. Total Environ.* 236, 153-161.
- 414 Baker, S.E., 2006. *Aspergillus niger* genomics: Past, present and into the future. *Med.*
415 *Mycol.* 44, S17-S21.
- 416 Bayman, P., Baker, J.L., 2006. Ochratoxins: A global perspective. *Mycopathologia* 162,
417 215-223.
- 418 Beltran, E., Ibanez, M., Portoles, T., Ripolles, C., Sancho, J.V., Yusa, V., Marin, S.,
419 Hernandez, F., 2013. Development of sensitive and rapid analytical methodology for
420 food analysis of 18 mycotoxins included in a total diet study. *Anal. Chim. Acta* 783,
421 39-48.
- 422 Benedito-Palos, L., Navarro, J.C., Sitja-Bobadilla, A., Bell, J.G., Kaushik, S., Perez-
423 Sanchez, J., 2008. High levels of vegetable oils in plant protein-rich diets fed to

424 gilthead sea bream (*Sparus aurata* L.): growth performance, muscle fatty acid profiles
425 and histological alterations of target tissues. *Br. J. Nutr.* 100, 992-1003.

426 Bernhoft, A., Sundstøl Eriksen, G., Sundheim, L., Berntssen, M.H.G., Brantsæter, A.L.,
427 Brodal, G., Kruse Fæste, C., Skow Hofgaard, I., Rafoss, T., Sivertsen, T., Tronsmo,
428 A.M., 2013. Risk assessment of mycotoxins in cereal grain in Norway. Norwegian
429 Scientific Committee for Food Safety (VKM) 109-211,
430 <http://www.vkm.no/dav/eee04d10c04.pdf>.

431 Bertuzzi, T., Gualla, A., Morlacchini, M., Pietri, A., 2013. Direct and indirect
432 contamination with ochratoxin A of ripened pork products. *Food Control* 34, 79-83.

433 Binder, E.M., 2007a. Managing the risk of mycotoxins in modern feed production.
434 *Anim. Feed Sci. Technol.* 133, 149-166.

435 Binder, E.M., Tan, L.M., Chin, L.J., Handl, J., Richard, J., 2007b. Worldwide
436 occurrence of mycotoxins in commodities, feeds and feed ingredients. *Anim. Feed*
437 *Sci. Technol.* 137, 265-282.

438 Bryden, W.L., 2012. Mycotoxin contamination of the feed supply chain: Implications
439 for animal productivity and feed security. *Anim. Feed Sci. Technol.* 173, 134-158.

440 Burr, G.S., Wolters, W.R., Barrows, F.T., Hardy, R.W., 2012. Replacing fishmeal with
441 blends of alternative proteins on growth performance of rainbow trout
442 (*Oncorhynchus mykiss*), and early or late stage juvenile Atlantic salmon (*Salmo*
443 *salar*). *Aquaculture* 334, 110-116.

444 Caruso, D., Talamond, P., Moreau, Y., 2013. Mycotoxins and fish farming: A risk left
445 behind? *Cah. Agric.* 22, 165-173.

446 Cheli, F., Battaglia, D., Gallo, R., Dell'Orto, V., 2014. EU legislation on cereal safety:
447 An update with a focus on mycotoxins. *Food Control* 37, 315-325.

448 da Rocha, M.E.B., Freire, F.D.O., Maia, F.B.F., Guedes, M.I.F., Rondina, D., 2014.
449 Mycotoxins and their effects on human and animal health. Food Control 36, 159-165.
450 Davies, S.J., Gouveia, A., Laporte, J., Woodgate, S.L., Nates, S., 2009. Nutrient
451 digestibility profile of premium (category III grade) animal protein by-products for
452 temperate marine fish species (European sea bass, gilthead sea bream and turbot).
453 Aquac. Res. 40, 1759-1769.

454 EC, 2001. Regulation (EC) no 999/2001 of the european parliament and of the council
455 of 22 May 2001 laying down rules for the prevention, control and eradication of
456 certain transmissible spongiform encephalopathies. Official Journal of the European
457 Union L 147, 1-38.

458 EC, 2006. Commission Recommendation No 2006/576 of 17 August 2006 on the
459 presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and
460 fumonisins in products intended for animal feeding. Official Journal of the European
461 Union L 229, 7-9.

462 EC, 2009. Regulation (EC) No 1069/2009 of the european parliament and of the council
463 of of 21 October 2009 laying down health rules as regards animal by-products and
464 derived products not intended for human consumption and repealing Regulation (EC)
465 No 1774/2002 (Animal by-products Regulation). Official Journal of the European
466 Union L300, 1-33.

467 EC, 2013a. Commission regulation (EU) No 56/2013 of 16 January 2013 amending
468 Annexes I and IV to Regulation (EC) No 999/2001 of the European Parliament and
469 of the Council laying down rules for the prevention, control and eradication of
470 certain transmissible spongiform encephalopathies. Official Journal of the European
471 Union 21, 3-16.

472 EC, 2013b. Commission Recommendation No 2013/165/EU of 27 March 2013 on the
473 presence of T-2 and HT-2 toxin in cereals and cereal products. Official Journal of the
474 European Union L 91, 12–15.

475 EFSA, 2004a. Opinion of the Scientific Panel on Contaminants in Food Chain on a
476 request from the Commission related to ochratoxin A (OTA) as undesirable
477 substance in animal feed Request No EFSA-Q-2003-039 Adopted on 22 September
478 2004. The EFSA Journal 101, , 1-36.

479 EFSA, 2004b. Opinion of the Scientific Panel on Contaminants in the Food Chain on a
480 request from the Commission related to Zearalenone as undesirable substance in
481 animal feed (Question N° EFSA-Q-2003-037 Adopted on 28 July 2004. The EFSA
482 journal 89, 1-35.

483 EFSA, 2005. Opinion of the Scientific Panel on Contaminants in Food Chain on a
484 request from the Commission related to fumonisins as undesirable substances in
485 animal feed Request No. EFSA-Q-2003-040. The EFSA Journal 235.

486 EFSA, 2007. Opinion of the Scientific Panel on Contaminants in the Food Chain on a
487 request from the Commission related to Deoxynivalenol (DON) as undesirable
488 substance in animal feed (Question N° EFSA-Q-2003-036) Adopted on 2 June 2004
489 adapted 2007. The EFSA Journal (2004) 73, 1-42, 1-42.

490 EFSA, 2011. Scientific Opinion on the risks for animal and public health related to the
491 presence of T-2 and HT-2 toxin in food and feed¹. The EFSA journal 9, 2481.

492 EU, 2002. Directive 2002/32/EC of the European parliament and of the council of 7
493 May 2002 on undesirable substances in animal feed. Off. J. Eur. Commun L140, 10-
494 22.

495 Frisvad, J.C., Smedsgaard, J., Samson, R.A., Larsen, T.O., Thrane, U., 2007. Fumonisin
496 B(2) production by *Aspergillus niger*. J. Agric. Food Chem. 55, 9727-9732.

497 Hooft, J.M., Elmor, H.I., Encarnacao, P., Bureau, D.P., 2011. Rainbow trout
498 (*Oncorhynchus mykiss*) is extremely sensitive to the feed-borne *Fusarium* mycotoxin
499 deoxynivalenol (DON). *Aquaculture* 311, 224-232.

500 Larsen, T.O., Svendsen, A., Smedsgaard, J., 2001. Biochemical characterization of
501 ochratoxin A-producing strains of the genus *Penicillium*. *Appl. Environ. Microbiol.*
502 67, 3630-3635.

503 Leeman, W.R., Van den Berg, K.J., Houben, G.F., 2007. Transfer of chemicals from
504 feed to animal products: The use of transfer factors in risk assessment. *Food Addit.*
505 *Contam.* 24, 1-13.

506 Lund, F., Frisvad, J.C., 2003. *Penicillium verrucosum* in wheat and barley indicates
507 presence of ochratoxin A. *J. Appl. Microbiol.* 95, 1117-1123.

508 Magan, N., Aldred, D., Mylona, K., Lambert, R.J.W., 2010. Limiting mycotoxins in
509 stored wheat. *Food Addit. Contam. Part A-Chem.* 27, 644-650.

510 Malachová, A., Sulyok, M., Beltrán, E., Berthiller, F., Krska, R., 2014. Optimization
511 and validation of a quantitative liquid chromatography–tandem mass spectrometric
512 method covering 295 bacterial and fungal metabolites including all regulated
513 mycotoxins in four model food matrices. *J. Chromatogr. A.*
514 DOI:10.1016/j.chroma.2014.08.037

515 Malagutti, L., Zannotti, M., Scampini, A., Sciaraffia, F., 2005. Effects of Ochratoxin A
516 on heavy pig production. *Anim. Res.* 54, 179-184.

517 Manning, B.B., Li, M.H., Robinson, E.H., Gaunt, P.S., Camus, A.C., Rottinghaus, G.E.,
518 2003. Response of channel catfish to diets containing T-2 toxin. *J. Aquat. Anim.*
519 *Health* 15, 229-238.

520 Manning, B.B., Li, M.H., Robinson, E.H., 2005. Feedborne mycotoxins in aquaculture
521 feeds: impact and management of aflatoxin, fumonisin, and moniliformin.

522 Mizáková, A., Pipová, M., Turek, P., 2002. The occurrence of moulds in fermented raw
523 meat products. Czech Journal of Food Sciences 20 89-94.

524 Mogensen, J.M., Nielsen, K.F., Samson, R.A., Frisvad, J.C., Thrane, U., 2009. Effect of
525 temperature and water activity on the production of fumonisins by *Aspergillus niger*
526 and different *Fusarium* species. BMC Microbiol. 9.

527 Monbaliu, S., Van Peteghem, C., De Saeger, S., 2012. Detection and determination of
528 natural toxins (mycotoxins and plant toxins) in feed. in: FinkGremmels, J. (Ed.).
529 Animal Feed Contamination: Effects on Livestock and Food Safety. Woodhead Publ
530 Ltd, Cambridge, pp. 286-325.

531 Monbaliu, S., Van Poucke, C., Detavernier, C., Dumoulin, F., Van De Velde, M.,
532 Schoeters, E., Van Dyck, S., Averkieva, O., Van Peteghem, C., De Saeger, S., 2010.
533 Occurrence of Mycotoxins in Feed as Analyzed by a Multi-Mycotoxin LC-MS/MS
534 Method. J. Agric. Food Chem. 58, 66-71.

535 Nacher-Mestre, J., Ibanez, M., Serrano, R., Perez-Sanchez, J., Hernandez, F., 2013.
536 Qualitative Screening of Undesirable Compounds from Feeds to Fish by Liquid
537 Chromatography Coupled to Mass Spectrometry. J. Agric. Food Chem. 61, 2077-
538 2087.

539 Njobeh, P.B., Dutton, M.F., Aberg, A.T., Haggblom, P., 2012. Estimation of Multi-
540 Mycotoxin Contamination in South African Compound Feeds. Toxins 4, 836-848.

541 Noonim, P., Mahakarnchanakul, W., Nielsen, K.F., Frisvad, J.C., Samson, R.A., 2009.
542 Fumonisin B2 production by *Aspergillus niger* in Thai coffee beans. Food Addit.
543 Contam. Part A-Chem. 26, 94-100.

544 Ostry, V., Malir, F., Ruprich, J., 2013. Producers and Important Dietary Sources of
545 Ochratoxin A and Citrinin. Toxins 5, 1574-1586.

546 Pietsch, C., Kersten, S., Burkhardt-Holm, P., Valenta, H., Danicke, S., 2013.
547 Occurrence of Deoxynivalenol and Zearalenone in Commercial Fish Feed: An Initial
548 Study. *Toxins* 5, 184-192.

549 Pitt, J.I., Hocking, A.D., 2009. *Fungi and Food Spoilage*, Third Edition. Springer, New
550 York.

551 Pitt, J.I., Taniwaki, M.H., Cole, M.B., 2013. Mycotoxin production in major crops as
552 influenced by growing, harvesting, storage and processing, with emphasis on the
553 achievement of Food Safety Objectives. *Food Control* 32, 205-215.

554 Poston, H., Coffin, J., Combs, G., 1982. Biological effects of dietary T-2 toxin on
555 rainbow-trout, *Salmo-gairdneri*. *Aquat. Toxicol.* 2, 79-88.

556 Rodrigues, I., Naehrer, K., 2012. A Three-Year Survey on the Worldwide Occurrence
557 of Mycotoxins in Feedstuffs and Feed. *Toxins* 4, 663-675.

558 Schatzmayr, G., Streit, E., 2013. Global occurrence of mycotoxins in the food and feed
559 chain: facts and figures. *World Mycotoxin J.* 6, 213-222.

560 Schmidt-Heydt, M., Graf, E., Stoll, D., Geisen, R., 2012. The biosynthesis of ochratoxin
561 A by *Penicillium* as one mechanism for adaptation to NaCl rich foods. *Food*
562 *Microbiol.* 29, 233-241.

563 Scudamore, K.A., Livesey, C.T., 1998. Occurrence and significance of mycotoxins in
564 forage crops and silage: a review. *J. Sci. Food Agric.* 77, 1-17.

565 Siegel, D., Babuscio, T., 2011. Mycotoxin management in the European cereal trading
566 sector. *Food Control* 22, 1145-1153.

567 Soares, C., Calado, T., Venancio, A., 2013. Mycotoxin production by *Aspergillus niger*
568 aggregate strains isolated from harvested maize in three Portuguese regions. *Revista*
569 *Iberoamericana De Micologia* 30, 9-13.

570 Sonjak, S., Licen, M., Frisvad, J.C., Gunde-Cimerman, N., 2011. Salting of dry-cured
571 meat - A potential cause of contamination with the ochratoxin A-producing species
572 *Penicillium nordicum*. Food Microbiol. 28, 1111-1116.

573 Sorensen, L.M., Mogensen, J., Nielsen, K.F., 2010. Simultaneous determination of
574 ochratoxin A, mycophenolic acid and fumonisin B-2 in meat products. Anal.
575 Bioanal. Chem. 398, 1535-1542.

576 Streit, E., Schatzmayr, G., Tassis, P., Tzika, E., Marin, D., Taranu, I., Tabuc, C.,
577 Nicolau, A., Aprodu, I., Puel, O., Oswald, I.P., 2012. Current Situation of Mycotoxin
578 Contamination and Co-occurrence in Animal Feed-Focus on Europe. Toxins 4, 788-
579 809.

580 Streit, E., Naehrer, K., Rodrigues, I., Schatzmayr, G., 2013. Mycotoxin occurrence in
581 feed and feed raw materials worldwide: long-term analysis with special focus on
582 Europe and Asia. J. Sci. Food Agric. 93, 2892-2899.

583 Tacon, A.G.J., Metian, M., 2008. Global overview on the use of fish meal and fish oil in
584 industrially compounded aquafeeds: Trends and future prospects. Aquaculture 285,
585 146-158.

586 Toldra, F., Aristoy, M.C., Mora, L., Reig, M., 2012. Innovations in value-addition of
587 edible meat by-products. Meat Sci. 92, 290-296.

588 Torrissen, O., Olsen, R.E., Toresen, R., Hemre, G.I., Tacon, A.G.J., Asche, F., Hardy,
589 R.W., Lall, S., 2011. Atlantic Salmon (*Salmo salar*): The "Super-Chicken" of the
590 Sea? Rev. Fish. Sci. 19, 257-278.

591 Torstensen, B.E., Espe, M., Sanden, M., Stubhaug, I., Waagbo, R., Hemre, G.I.,
592 Fontanillas, R., Nordgarden, U., Hevroy, E.M., Olsvik, P., Berntssen, M.H.G., 2008.
593 Novel production of Atlantic salmon (*Salmo salar*) protein based on combined

594 replacement of fish meal and fish oil with plant meal and vegetable oil blends.
595 Aquaculture 285, 193-200.

596 Varga, J., Kocsube, S., Suri, K., Szigeti, G., Szekeres, A., Varga, M., Toth, B., Bartok,
597 T., 2010. Fumonisin contamination and fumonisin producing black *Aspergilli* in
598 dried vine fruits of different origin. *Int. J. Food Microbiol.* 143, 143-149.

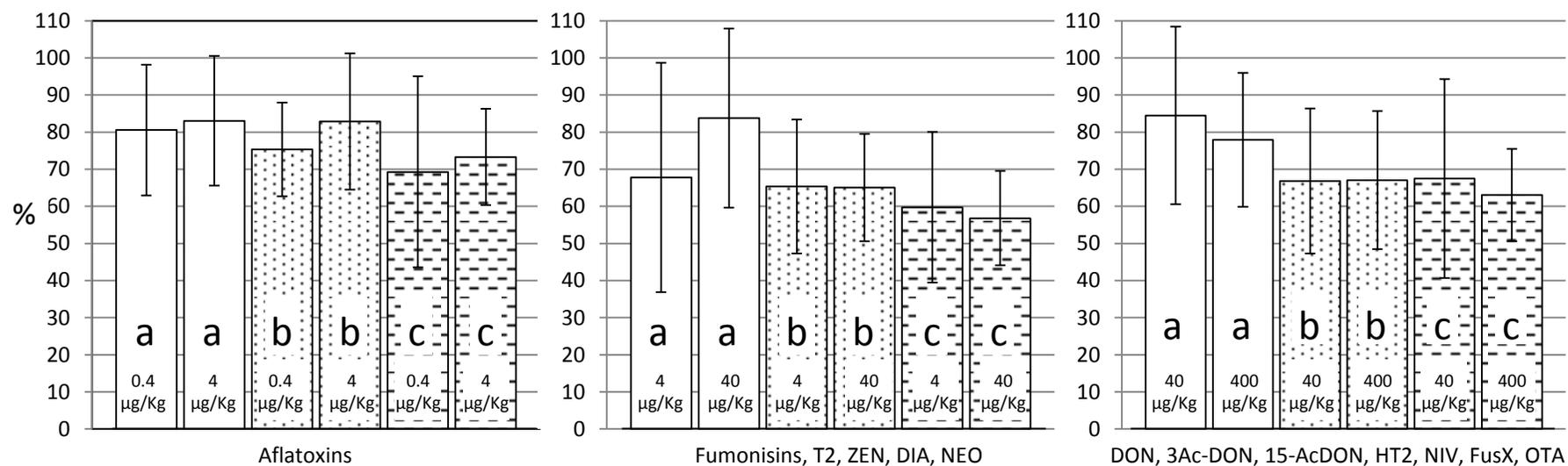
599 Wozny, M., Brzuzan, P., Luczynski, M.K., Gora, M., Bidzinska, J., Jurkiewicz, P.,
600 2008. Effects of cyclopenta[c]phenanthrene and its derivatives on zona radiata
601 protein, ER alpha, and CYP1A mRNA expression in liver of rainbow trout
602 (*Oncorhynchus mykiss* Walbaurn). *Chem.-Biol. Interact.* 174, 60-68.

603 Wozny, M., Obremski, K., Jakimiuk, E., Gusiatin, M., Brzuzan, P., 2013. Zearalenone
604 contamination in rainbow trout farms in north-eastern Poland. *Aquaculture* 416, 209-
605 211.

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Figure 1. General overview about the QC recoveries in every matrix (a=ingredients, b=feeds and c=fish fillets) for the different groups of mycotoxins.

616 **Table 1.** Feed formulation (as % inclusion level) of two gilthead sea bream diets (GSB-D) with low or high inclusion levels of plant material
 617 (GSB-D1 and GSB-D2, respectively), and three production replicates for Atlantic salmon diets with similar high plant ingredient inclusion levels
 618 with feed ingredients from the same batch (AS-D1-3).
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Ingredient (%)	Diets		
	GSB-D1	GSB-D2	AS-D1-3
Fish meal	23.00	3.00	8.00
Krill meal	-	-	2.00
SPC 60%	-	-	18.00
SPC 90 %	2.00	2.00	-
Soya protein	16.00	25.00	-
Corn gluten	15.00	25.00	4.00
Wheat gluten	4.00	7.30	15.00
Rapeseed cake	12.00	9.70	-
Wheat	11.08	6.80	6.00
Pea protein	-	-	13.00
Field peas	-	-	9.00
Fish oil	15.60	6.56	4.40
Rapeseed oil	0	4.40	8.80
Palm olein	0	4.40	4.80
Linseed oil	-	-	2.20
Mineral and vitamin mixtures	1.32	5.84	4.80

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Table 2. LOQs obtained for mycotoxins in the matrices studied. For some mycotoxins (nivalenol and zearalenone) no proper quantification could be obtained for some matrices due to high interference.

Mycotoxins	LOQs (µg/Kg)																	
	Plant ingredients								Animal protein						Feed		Fish	
	Wheat	Wheat Gluten	Pea	Pea Protein	Rapesed Cake	Corn Gluten	Soya Protein	Sunflower Meal	Poultry Meal	Poultry Blood Meal	Pork Meal	Pork Blood Meal	Pork greaves	Feather Meal	Salmon Feed	Sea bream Feed	Salmon	Sea bream
NIV	44,4	200					182		400	400	190	364		364				
DON	0,9	4,5	1,7	3,7	222	18,3	3,5	22,2	26,0	3,4	11,8	4,0	13,0	23,0	2,7	22,3	4,7	4,5
Fus X	11,9	9,3	12,2	10,5	222	30,8	5,4	41,2	36,4	11,4	13,8	7,0	11,1	19,0	19,0	61,5	9,5	11,8
NEO	1,1	0,9	0,4	0,2	1,8	1,6	0,2	1,2	3,1	1,8	1,8	0,9	1,5	2,9	0,4	1,6	0,6	1,0
3-Ac DON	3,9	1,9	6,7	3,6	12,1	3,4	1,8	6,9	13,8	1,3	4,3	1,7	5,3	3,7	8,0	6,3	8,5	5,1
15-AcDON	3,4	1,3	6,2	1,5	11,1	3,3	0,8	11,1	5,9	8,3	5,5	0,8	2,4	7,4	7,2	5,3	3,2	4,9
AFG2	0,3	0,2	0,2	0,3	4,0	1,1	0,1	1,5	2,9	0,2	1,1	0,1	0,2	2,2	0,2	3,6	0,1	0,2
AFG1	0,2	0,1	0,1	0,2	2,7	0,9	0,1	1,1	1,8	0,2	0,5	0,1	0,2	1,7	0,2	2,2	0,1	0,1
AFB2	0,1	0,04	0,1	0,4	1,6	1,0	0,1	0,4	2,1	0,2	0,4	0,1	0,2	2,2	0,1	1,4	0,2	0,3
AFB1	0,2	0,1	0,1	0,4	1,7	3,6	0,1	2,7	1,3	0,3	0,7	0,1	0,4	4,0	0,4	3,1	0,1	0,1
DIA	0,03	0,01	0,01	0,02	0,08	0,05	0,01	0,05	0,12	0,04	0,13	0,01	0,02	0,21	0,03	0,11	0,04	0,03
HT-2	2,4	2,1	4,8	3,7	10,9	6,9	1,6	27	14,3	5,6	11,1	2,4	4,4	30,3	4,8	30,8	3,5	5,8
FB1	0,4	0,6	0,6	1,9	1,4	0,5	0,9	0,9	1,8	1,6	2,1	4,2	3,8	2,9	0,3	2,4	1,4	0,7
T-2	0,1	0,03	0,1	0,1	0,1	0,2	0,02	0,2	0,5	0,3	0,5	0,1	0,2	0,6	0,1	0,6	0,2	0,3
ZEN	0,3	14,3	1,4	0,1	4,4	0,7	4,4	8,2		6,2	5,5	5,7			36,4			
OTA	0,4	0,3	0,4	0,7	0,7	1,4	0,2	0,5	1,6	0,9	1,4	0,6	0,8	1,7	0,6	1,5	0,8	0,9
FB2	0,5	1,0	0,5	1,3	0,9	0,1	0,4	0,4	3,6	1,1	1,1	2,2	1,9	1,8	0,2	0,6	0,5	0,5
FB3	0,8	1,5	0,8	1,8	3,0	0,6	1,3	0,9	7,3	2,4	1,8	4,3	4,0	6,7	1,2	3,0	1,0	1,3

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Limit of detection (LOD) could be obtained from LOQ: $LOD=3 \cdot LOQ/10$

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Table 3. Levels of mycotoxins ($\mu\text{g kg}^{-1}\text{ww}$, minimum-maximum (number of positive samples)) in commercially available plant feed ingredients used in aquafeeds (n=number of different samples). - = not detectable at given matrix limit in table 1.

	Sunflower meal (n=1)	Rapeseed meal (n=1)	wheat (n=3)	wheat gluten (n=4)	corn gluten (n=3)	Pea protein (n=3)	soy protein concentrate (n=4)
AFG2	-	-	-	-	-	-	-
AFG1	-	-	-	-	-	-	-
AFB2	-	-	-	-	-	-	-
AFB1	-	-	-	-	-	-	-
NIV	-	-	-	-	-	-	-
Fus X	-	-	-	-	-	-	-
DON	-	-	53-371 (3)	17-504 (4)	139-814 (3)	-	-
3-AcDON	-	-	-	-	-	-	-
15-AcDON	-	-	-	-	53-452	-	-
NEO	-	-	-	-	-	-	-
DIA	-	-	-	-	-	-	-
HT-2	-	-	4-8.1 (2)	4 (2)	67 (1)	-	-
T-2	-	-	4 (1)	4 (2)	2.8 (1)	-	-
ZEN	-	-	-	14-17 (2)	8-13 (3)	-	-
OTA	0.4	0.4	0.4 (1)	2.0-5.2 (4)	0.4 (3)	1.8 (1)	-
FB1	-	-	-	0.4-8.2 (2)	0.4-2319 (3)	-	0.4 (2)
FB2	-	-	-	2.9 (1)	2.9-1943 (3)	-	0.5 (1)
FB3	-	-	-	2.1 (1)	7.8-638 (3)	-	-
Sum FB1+FB2+FB3	-	-	-	13.2	11.1-4901	-	-

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628 **Table 4.** Levels of mycotoxins ($\mu\text{g kg}^{-1}\text{ww}$, minimum-maximum (number of positive samples)) in commercially available processed animal
 629 proteins used in aquafeeds (n=number of different PAP samples), - = not detectable at given matrix limit in table 2.

	Poultry Blood (n=4)	Poultry Meal (n=4)	Poultry Feather Meal (n=3)	Pork Blood Meal (n=3)	Pork Meal (n=3)	Pork Greaves (n=2)
AFG2	-	-	-	-	-	-
AFG1	-	-	-	-	-	-
AFB2	-	-	-	-	-	-
AFB1	-	-	-	-	-	-
NIV	-	-	-	-	-	-
Fus X	-	-	-	-	-	-
DON	-	-	-	-	-	-
3-AcDON	-	-	-	-	-	-
15-AcDON	-	-	-	-	-	-
NEO	-	-	-	-	-	-
DIA	-	-	-	-	-	-
HT-2	-	-	-	-	-	-
T-2	-	-	-	-	-	-
ZEN	-	-	-	-	-	-
OTA	-	-	-	0.4 (2)	-	-
FB1	-	0.4-2.6 (2)	0.4 (1)	-	-	-
FB2	-	-	-	-	-	-
FB3	-	-	-	-	-	-

630

631 **Table 5.** Levels of mycotoxins ($\mu\text{g kg}^{-1}\text{ww}$) of two gilthead sea bream diets (GSB-D) with low or high inclusion levels of plant material (GSB-
632 D1 and GSB-D2, respectively), and three production replicates for Atlantic salmon diets with high plant ingredient inclusions levels (AS-D1-3). -
633 = not detectable at given matrix limit in table 1. None of the dietary mycotoxins were detected in the fillets of sea bream or Atlantic salmon fed
634 for respectively 8 or 7 months on these diets.

Diets	GSB-D1	GSB-D2	AS-D1	AS-D2	AS-D3
AFG2	-	-	-	-	-
AFG1	-	-	-	-	-
AFB2	-	-	-	-	-
AFB1	-	-	-	detected	detected
NIV	-	-	-	-	-
Fus X	-	-	-	-	-
NEO	-	-	-	-	-
DON	79,2	53,5	22,4	19,4	23,1
3-AcDON	-	-	-	-	-
15-AcDON	8,1	13,6	detected	detected	detected
DIA	-	-	-	-	-
HT-2	-	-	detected	5	-
T-2	-	-	0,1	0,1	0,1
ZEN	-	-	-	-	-
OTA	-	-	detected	detected	detected
FB1	-	4,5	66,9	335	50,6
FB2	-	1,9	62,2	324	43,9
FB3	-	detected	18,9	95,3	18
Sum FB1+FB2+FB3	-	6,4	148	754	112

636 **Table 1.** Experimental conditions of the optimized UHPLC–(ESI)-MS/MS method for
637 the selected mycotoxins.

Compound	Retention	Precursor ion	Cone	Product	Collision	
Nivalenol (NIV)	0.70	[M+H] ⁺	313.1	10	175.1	20
					159.1	20
					91.0 (q2)	40
Deoxynivalenol (DON)	0.96	[M+H] ⁺	297.0	20	249.1	10
					231.1	10
					203.1	10
Fusarenon X (FusX)	1.49	[M+H] ⁺	355.1	30	175.1	20
					229.1	20
					247.1	20
Neosolaniol (NEO)	1.90	[M+NH ₄] ⁺	400.2	20	185.1	20
					305.1	10
					215.1	10
3-Acetyl Deoxynivalenol (3-AcDON)	2.70	[M+H] ⁺	339.1	20	231.1	10
					213.1	20
					279.1	10
15-Acetyl Deoxynivalenol (15-AcDON)	2.78	[M+H] ⁺	339.1	20	137.0	10
					261.1	10
					297.1	10
Aflatoxin G2 (AFG2)	3.42	[M+H] ⁺	331.1	30	245.1	30
					189.1	40
					257.1	30
Aflatoxin G1 (AFG1)	3.49	[M+H] ⁺	329.1	30	243.1	30
					200.1	40
					215.1	30
Aflatoxin B2 (AFB2)	3.56	[M+H] ⁺	315.1	30	259.1	30
					287.1	30
					243.1	30
Aflatoxin B1 (AFB1)	3.62	[M+H] ⁺	313.1	30	285.1	20
					269.1	30
					241.1	30
Diacetoxyscirpenol (DIA)	3.67	[M+NH ₄] ⁺	384.2	10	307.1	10
					247.1	10
					349.2	10
Fumonisin B1 (FB1)	3.85	[M+H] ⁺	722.2	30	334.2	40
					352.2	30
					686.2	30
HT-2 toxin (HT-2)	3.88	[M+NH ₄] ⁺	442.2	20	263.1	10
					215.1	15
					197.0	15
T-2 toxin (T-2)	3.99	[M+NH ₄] ⁺	484.2	20	185.1	20
					305.1	10
					245.1	10
Fumonisin B3 (FB3)	4.00	[M+H] ⁺	706.2	30	336.2	40
					336.2	40
					354.2	30
Fumonisin B2 (FB2)	4.04	[M+H] ⁺	706.2	30	336.2	40
					318.2	40
					354.2	30
Zearalenone (ZEN)	4.07	[M-H] ⁻	317.1	20	175.1	25
					273.1	20
					131.1	35
Ochratoxin A (OTA)	4.08	[M+H] ⁺	404.2	30	239.1	30
					221.1	35
					102.0	60

638 **SUPPLEMENTARY MATERIAL**

639 **Reagents**

640
641 All mycotoxin standards (>99% purity) were supplied by Sigma Aldrich (Madrid,
642 Spain). HPLC-grade water was obtained from water passed through a MilliQ water
643 purification system (Millipore Ltd., Bedford, MA, USA). HPLC-grade methanol
644 (MeOH), HPLC-grade acetonitrile (ACN), acetic acid (>99.8%) and ammonium acetate
645 (NH₄Ac) (>99%) were purchased from ScharLab (Barcelona, Spain). Formic acid
646 (HCOOH) (>98%) was obtained from Fluka (Buchs, Switzerland).
647 Mycotoxins were divided into three different groups depending on their intensity
648 response. Thus, individual stock solutions, MIX A (10 mg L⁻¹, containing DON, HT-2,
649 NIV, FUS X, 3AcDON, 15AcDON and OTA), MIX B (1 mgL⁻¹, containing FB1, FB2,
650 FB3, T-2, ZEN, DIA and NEO) and MIX C (0.1 mgL⁻¹, containing AFG1, AFG2,
651 AFB1, AFB2), respectively, were prepared by diluting the reference standards solutions
652 in acetonitrile (abbreviations used for mycotoxins are specified in Table 1,
653 supplementary data). The total mixed standard solution was prepared by adding 1 mL of
654 MIX A, 1 mL of MIX B, and 1 mL of MIX C, and diluting to 10 mL with water.
655 Working standard solutions for LC-MS/MS analysis and for fortification of samples
656 were prepared by dilution of the total mixed standard solution with water. Stock
657 standard solutions were stored in a freezer at -20 °C, whereas working solutions were
658 stored in a fridge. Matrix-matched standard calibration was used for quantification
659 purposes. Standards in matrix were prepared by adding 100 µL from the corresponding
660 standard solution plus 900 µL of the 4-fold diluted blank extract.
661