

1 **Settling velocity and total ammonia nitrogen leaching from commercial feed and faecal**
2 **pellets of gilthead seabream (*Sparus aurata* L. 1758) and seabass (*Dicentrarchus labrax***
3 **L. 1758).**

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11 **Abstract**

12 The physico-chemical characteristics of particulate wastes of *Sparus aurata* and
13 *Dicentrarchus labrax* were investigated. Changes in dimensions, settling velocity and total
14 ammonia nitrogen (TAN) leached from commercial feed pellets was investigated after
15 soaking. Also, the settling velocity and TAN leached from faecal pellets of these fish were
16 assessed at 15 and 25°C. The settling velocity of feed pellets was influenced positively by
17 pellet weight and negatively by immersion length as a result of changes in pellet dimensions
18 after soaking. The settling velocity of faecal pellets was determined by pellet weight. The
19 experimental design did not allow identifying any consistent effect of water temperature on
20 settling velocity. TAN leaching over time from feed and faecal pellets was successfully
21 explained by means of a first order kinetic equation. For feed pellets, water temperature

22 significantly affected the speed of the process and the time at which the maximum TAN
23 leached was reached, but did not influence the maximum TAN leached. Leaching was related
24 to feed pellet size, so the smaller the pellet, the higher the leaching. TAN leaching from faecal
25 pellets was greater per unit weight than in feed pellets. However neither water temperature
26 nor fish species influenced on TAN leaching from faeces.

27 Keywords: aquaculture; particulate wastes; settling velocity; leaching; *Sparus aurata*;
28 *Dicentrarchus labrax*.

29

30 1. Introduction

31 Marine aquaculture has experienced a rapid development in the Mediterranean since 1970,
32 with gilthead seabream (*Sparus aurata* L. 1758) and seabass (*Dicentrarchus labrax* L. 1758)
33 now intensively cultured in most coastal countries. This expansion has been accompanied by
34 an increasing social sensitivity with respect to the potential short and long term impacts on the
35 marine environment. Fish rearing produces a substantial quantity of particulate organic
36 wastes, mainly faecal pellets and uneaten food that settle in the vicinity of the farms. Several
37 studies have examined the geochemical and biological consequences of this supply of organic
38 matter on the benthos (Aguado-Giménez and García-García, 2004; La Rosa et al., 2004;
39 Hellou et al., 2005). Uneaten food is the main contributor among the particulate wastes loaded
40 by fish farms (Beveridge et al., 1991; Chen et al., 1999b). Most of the information regarding
41 particulate wastes loading refers to Atlantic salmon. In the 1980's, it was estimated that the
42 food loss rate during salmonid on-growing was as high as 200-300 g kg⁻¹ of the supplied food
43 (Gowen and Bradbury, 1987). New diet formulations, and improvements in diet production
44 processes and husbandry operations have lowered the conversion factor and also the wastes
45 loaded, 50-150 g kg⁻¹ now being the food loss rate most often reported (Findlay and Watling,
46 1994; Beveridge et al., 1997; Cho and Bureau, 1997) although Cromey et al. (2002) mentions
47 30 g kg⁻¹. As regards seabream and seabass culture, there is no information available in the
48 scientific literature about uneaten food losses. Producers consulted estimated it around to be
49 50-100 g kg⁻¹ on average in offshore conditions but, in agreement with Reid et al. (2009), this
50 quantity varies widely from operation to operation and even from day to day.

51 Several studies under laboratory conditions reported that 250-300 g kg⁻¹ of ingested food is
52 voided as faeces (Butz and Vens-Cappell, 1982). Just as with food losses, a continuous

53 improvement in diet elaboration has led to a gradual reduction of faecal discharges to about
54 100-250 g kg⁻¹ of ingested food (Cho et al., 1994; Talbot and Hole, 1994). In Mediterranean
55 fish farming, 300-400 g of faeces are released into the environment per kilogram of fish
56 produced (Dosdat, 2001).

57 Both uneaten food and faecal pellets have particular features and undergo a series of physico-
58 chemical changes while dispersing and settling that could influence the spatial range of
59 dispersion of particulate wastes and the net organic load reaching the seabed. According to
60 Gowen and Bradbury (1987), particle settling velocity together with current speed and depth,
61 determine the horizontal distance of particles reaching the bottom, and this obviously depends
62 on particle size (Sutherland et al., 2006). In addition, these wastes release nutrients (leaching)
63 while dispersing and settling. There are several studies that have looked at the settling
64 velocity of feed and faecal pellets and nutrient leaching from faeces in salmonids (Findlay and
65 Watling, 1994; Elberizon and Kelly, 1998; Chen et al., 1999a,b; Chen et al., 2003), but such
66 information is scant for Mediterranean cultured fishes (Vasallo et al., 2006; Magill et al.,
67 2006). Physical changes which relate to the removal or re-distribution of waste have received
68 less attention and only a few studies concerned with salmonids, have investigated leaching
69 from feeds during sinking (Phillips et al., 1993) and the removal of salmonid waste by wild
70 fish (Felsing et al, 2002) and in the Mediterranean for wild fish around seacages (Fernández-
71 Jover et al., 2007) are available. However, increased knowledge of the dynamic of particulate
72 wastes before settling on the seabed could be useful for analysing any environmental impact
73 and for improving the accuracy of waste dispersion models.

74 This study aims to determine under defined laboratory conditions some physico-chemical
75 characteristics of gilthead seabream and seabass solid wastes as they disperse through

76 sedimentation including settling velocity, size and weight changes and total ammonia nitrogen
77 (TAN) leaching of a variety of feed pellets, and settling velocity and TAN leaching of faecal
78 pellets.

79 **2. Materials and methods**

80 2.1. Feed pellets assays

81 The feed pellets used in these assays are part of a range of extruded commercial feedstuffs
82 used for gilthead seabream and seabass ongrowing. Hereafter, we refer to the different feed
83 types according to the nominal diameter of the cylindrical feed pellets: 2mm (FP2), 4mm
84 (FP4a and FP4b), 6mm (FP6) and 8mm (FP8). The proximate composition of the different
85 feed pellets was determined. Dietary moisture was determined by drying samples at 110 °C
86 for 24 h. Crude protein was estimated by Kjeldahl method, with 6.25 as conversion factor.
87 Crude fat was obtained by diethyl ether extraction (SOXTEC System-HTC). Nitrogen free
88 extracted material (NFE) was calculated as $[100 - (\% \text{ protein} + \% \text{ fat} + \% \text{ ash})]$. Total ash
89 was obtained by heating at 550 °C for 18 h. Gross energy was estimated following the
90 Miglavs and Jobling (1989) coefficients: 23.6 kJ g⁻¹ for protein, 38.9 kJ g⁻¹ for fat and 16.7 kJ
91 g⁻¹ for carbohydrate. Protein / Energy ratio was also calculated. Three samples of each pellet
92 type were analyzed. Values of crude protein, crude fat, ash and NFE are expressed as g kg⁻¹
93 dry weight.

94 Diameter, length, weight and density were determined in 15 pellets of each pellet type
95 submerged for 0, 1, 5, 10, 15, 30 and 60 minutes at two different water temperatures
96 representatives of winter and summer conditions in the Mediterranean (15 and 25 °C). After
97 immersion, the pellets were placed on absorbent paper for 60 s to eliminate excess water. The

98 diameter and length were determined using a digital gauge (precision ± 0.01 mm) and the
99 pellets were weighed using an analytical balance (precision ± 0.1 mg). Changes in size or
100 weight were expressed as % of initial size or weight.

101 Settling velocity of dry and immersed (for 1, 5, 10, 15, 30 and 60 minutes) feed pellets was
102 determined using a 1 m long, 0.25 m diameter methacrylate sedimentation column. Feed
103 pellets were carefully placed with forceps just below the water surface, in the centre of the
104 surface avoiding bubbles. The column was marked at 0.05, 0.40 and 0.75 m from the top. The
105 first 0.05m and the final 0.25m of the column length were not considered in order to provide
106 enough reaction time to start the timer manually, and to avoid any bottom shear effect
107 imposed by sedimentation column bottom on pellet velocity (Chen et al., 1999a). Settling
108 velocity was determined in 30 pellets of each type by timing the descent between two marks
109 0.35m along its length, at 15 and 25°C.

110 For TAN leaching determination, feed samples of each type were weighted and assigned at
111 random to one of six leaching periods (1, 5, 10, 15, 30 and 60 minutes). This wide range of
112 immersion lengths was chosen in order to assure that settling velocity and TAN leaching
113 estimations and the dynamic of the processes could be revealed for long settling periods that
114 even exceeded the time needed for deposition. Feed pellets were individually dropped in
115 different 50 ml beakers filled with filtered seawater (glass microfibre filter GF/C 0.45 μ m), at
116 15 and 25°C. To simulate turbulence while settling, samples were gently shaken with an
117 automatic shaker approximately at their previously determined settling velocity. Each
118 incubation time was replicated five times. Immediately after incubation, the samples were
119 fixed with 0.1ml of 0.5N HCl to displace the ionic balance to the soluble form NH_4^+ . TAN
120 was measured with an ion-selective electrode (ORION 9512 BN) as described in APHA

121 (1995). The accuracy of this method has been favourably compared with the autoanalyzer
122 (indophenol blue method), which is the most widely used technique for ammonia
123 determination in seawater, no significant differences being observed between them (Arango-
124 Pulgarín and Pérez-Navarro, 2005, and references therein). Before measuring, 1ml of Ionic
125 Strength Adjustor solution (ISA: 5M NaOH, 0.5M disodium EDTA and 10% methanol with
126 blue colour indicator) was added to displace the ionic balance to the gaseous form NH_3 , at
127 which the electrode membrane is permeable. TAN leaching is expressed as $\mu\text{g g}^{-1}$ dry weight
128 of TAN released from the samples.

129 2.2. Faecal pellets assays

130 Gilthead seabream (0.528 ± 0.122 g) and seabass (0.636 ± 0.218 g) were stocked in circular
131 2000L tanks supplied with running seawater (salinity 37 g L^{-1}). The tanks were part of
132 recirculating system fitted with biological filtration and ultraviolet lamp; and the fish were
133 allowed to acclimate to the test diet for at least 10 days. Fish were fed to satiation twice per
134 day at 9:00 and 12:00 a.m. with FP 6 feedstuff. Fish were killed by immersion in iced
135 seawater, and fresh faeces were collected by dissection of the distal 4 cm of the gut according
136 to Chen et al. (1999a) just before the assays. The proximate composition of faecal pellets was
137 determined as described in the previous section.

138 Once obtained, faecal pellets were partially dried on blotting paper for 10 seconds (Chen et
139 al., 2003) and weighed prior to assays. The settling velocity and TAN leaching from faecal
140 pellets was determined as explained in the previous section, at 15 and 25°C. The mean faecal
141 (\pm s.e.m.) pellets weight for TAN leaching assays was 0.15 ± 0.01 g.

142 2.3. Statistical treatment of data

143 The proximate compositions of feed and faecal pellets were tested by one-way ANOVA, and
144 differences between pellet types or fish species by means of the *post hoc* Student-Newman-
145 Keuls (SNK) test. Multiple regression analyses (MRA) were performed: i) for the settling
146 velocity of feed pellets as dependent variable, and pellet size and density, water temperature
147 and immersion time as independent variables; ii) for weight and volume increase of feed
148 pellets after immersion as dependent variables, pellet size, water temperature and time of
149 immersion as independent variables; iii) for pellet density as dependent variable, water
150 temperature and time of immersion as independent variables; iv) for settling velocity of faecal
151 pellets as dependent variable, faecal pellet weight, water temperature and fish specie were
152 tested as independent variables. The significance of the coefficients of the independent
153 variables and their correlation indicated the influence on dependent variables.

154 TAN leaching from feed and faecal pellets was fitted by non-linear regression to the first
155 order kinetic equation (Fernández-Jover et al., 2007):

$$156 \quad y = a \cdot (1 - e^{-k \cdot t})$$

157 where y is the TAN leaching ($\mu\text{g g}^{-1} \text{ d.w.}$), a and k are fit parameters that represent the
158 maximum leached TAN ($\mu\text{g g}^{-1} \text{ d.w.}$) and the velocity of the process (min^{-1}) respectively and t
159 is the immersion length (min).

160 To test the influence of feed pellet size and water temperature on the leaching process, MRA
161 were performed for a , k and t_a (immersion time at which a is reached: estimated from the
162 equations) as dependent variables.

163 Differences in TAN leaching of faecal pellets between fish species and water temperature
164 were tested using the Chow test (Fernández-Jover et al., 2007):

165

$$F = \frac{[\sum S_{pool}^2 - (\sum S_A^2 + \sum S_B^2)] / K}{(\sum S_A^2 + \sum S_B^2) / (n_A + n_B - 2K)}$$

166 where $\sum S_{pool}^2$ is the residual sum of squares of the pooled samples $\sum S_A^2$ and $\sum S_B^2$ that
 167 represent the residual sums of squares for the samples A and B, respectively. K is the number
 168 of regression parameters (here K =2, slope and intercept), while n_A and n_B are the sample
 169 sizes of A and B. If the F-value exceeds the tabulated value for the F-distribution for P=0.05,
 170 K degrees of freedom for the numerator, and $n_A + n_B - 2K$ degrees of freedom for the
 171 denominator, the regressions lines are significantly different.

172

173 3. Results

174 3.1. Proximate composition of feed and faecal pellets

175 The results of the proximate composition analyses of feed and faecal pellets are shown in
176 Tables 1 and 2 respectively. Feed pellet densities were very similar, with no statistical
177 differences between them (SNK $P > 0.05$), although smaller pellets showed a slightly higher
178 density. Differences between feed types with respect to macronutrient composition were not
179 very outstanding. FP8 showed the lowest protein content (435.28 g kg⁻¹) and FP2 the highest
180 content (506.84 g kg⁻¹) (SNK $P < 0.05$). The feedstuff with the lowest lipid content (187.71 g
181 kg⁻¹) was FP2 which also showed the lowest moisture content (55.74 g kg⁻¹) (SNK $P < 0.05$).
182 FP8 showed the highest NFE values (285.29 g kg⁻¹) and FP4b the lowest (198.62 g kg⁻¹)
183 (SNK $P < 0.05$). Gross energy was very similar for all the feedstuffs. The P/E ratio in FP8
184 was also the lowest (18.56g MJ⁻¹) (SNK $P < 0.05$). Faecal pellet density was significantly
185 lower than for feed pellets, but there were no differences between fish species, and only minor
186 differences were observed with regard to the ash and crude protein content (SNK $P < 0.05$).

187 3.2. Physical changes after soaking of feed pellets and settling velocities of feed and faecal 188 pellets

189 MRAs for feed pellets (Table 3) showed that water temperature and pellet density had no
190 influence on changes in the physical characteristics of the pellets or the settling velocity ($P >$
191 0.05). Pellet size had a positive influence on settling velocity ($P < 0.001$), while immersion
192 time had a negative influence ($P < 0.001$). Thus, the larger the pellets the faster the settling,
193 and the longer the submergence time for any pellet type, the slower the settling velocity
194 (Figure 1A). Immersion time caused significant increases in weight and volume (mainly

195 diameter) ($P < 0.001$) and a decreases in density of feed pellets ($P < 0.001$). Smaller feed
196 pellets, with larger surface/volume ratios, had greater weight and volume increases after
197 soaking ($P < 0.001$), which became greater with soaking time (Figures 1B and 1C). Smaller
198 pellets underwent a weight and volume increase of 8.18% and 9.02%, respectively after 1
199 minute of immersion, and 64.21% and 59.92% after 60 minutes respectively, while larger
200 pellets underwent a weight and volume increase of 5.19% and 3.46%, respectively, after 1
201 minute of immersion, and 27.63% and 23.90% after 60 minutes.

202 The settling velocity of faecal pellets (Figure 2) was not influenced by water temperature ($P >$
203 0.05), and was statistically similar for gilthead seabream and seabass ($P > 0.05$). Faecal pellet
204 weight showed a positive correlation with the settling velocity ($P < 0.001$) (Table 4), being
205 fastest in the largest faecal pellets. The settling velocities of faecal pellets were approximately
206 60% slower than of feed pellets for all pellet sizes and species assessed.

207 3.3. TAN leaching from feed and faecal pellets

208 TAN leaching from feed pellets were successfully described by mean of first order kinetic
209 equations (Table 5, Figure 3A-E). Table 6 shows the results of MRAs for the parameters of
210 the fitting equations and water temperature and pellet size. The constant a was not
211 significantly influenced by water temperature ($P > 0.05$) but it was by pellet size ($P < 0.01$),
212 so the smaller the pellets, the higher the maximum TAN leached (a). Respect total nitrogen in
213 samples, % of TAN leached from larger feed pellets was 2-3 times lower than from smaller
214 pellets, and % TAN leached from faecal pellets was 10-20 times greater than from feed pellets
215 (Table 5). Constant k was significantly higher at 25 °C ($P < 0.05$) and in smaller feed pellets
216 ($P < 0.05$), while t_a was significantly larger at 15 °C ($P < 0.05$) but also for smaller pellets (P
217 < 0.05) (Table 6), so that the largest pellets at low temperature reached a later. On average, a

218 was reached after 60 and 45 minutes at 15 and 25 °C respectively.

219 TAN leaching from gilthead seabream and seabass faecal pellets was also described by means
220 of first order kinetic equations (Table 6 Figure 4A-B). A Chow F-test comparison of the
221 regression parameters (Table 7) showed that there were no significant differences in leaching
222 for gilthead seabream and seabass faecal pellets ($P > 0.05$). Water temperature only had a
223 significant effect on leaching from seabass faeces at 15 *versus* 25 °C ($P < 0.05$) as shown by
224 the pairwise comparison. The maximum leached TAN (a) from faecal pellets was around 3-
225 fold higher per unit weight than from feed pellets. Also, leaching velocity (k) and time to
226 reach a level (t_a) were faster for faecal pellets per unit weight than for feed pellets.

227

228 4. Discussion

229 4.1. Physical changes after soaking of feed pellets and settling velocities of feed and faecal
230 pellets

231 The settling velocity of feed pellets was between 0.068 and 0.136 m s⁻¹ for the diameters of 2–
232 8 mm in our assays. These values are largely similar to the range of settling velocities of
233 0.087–0.144 m s⁻¹ reported by Vassallo et al. (2006) for 3-5 mm seabream and seabass feed
234 pellets, although in this case the pellets were larger non-extruded pellets, which have a greater
235 propensity to sink than the extruded pellets used during this study. There is considerably more
236 information on settling velocity available for salmonid feedstuffs, which are normally
237 extruded pellets. In any case, settling velocity of salmon feed pellets is similar to that found in
238 this study. Findlay and Watling (1994) reported settling velocities ranging from 0.055 – 0.155
239 m s⁻¹ for 3–10 mm pellets; Elberizon and Kelly (1998) indicated settling velocities of 0.05–
240 0.12 m s⁻¹ for 2 and 8 mm pellets; and Chen et al. (1999b) recorded settling velocities of
241 0.058–0.109 m s⁻¹ for 2-8 mm pellets. The settling velocity of an object depends on many
242 factors relating to the object itself and to the medium in which it is settling, such as pellet
243 weight, shape, floating or porosity, and temperature, salinity, density, viscosity or pressure in
244 the case of seawater, although Elberizon and Kelly (1998) and Chen et al. (1999b) suggested
245 that this influence does not comply with the Stokes' Law. Vassallo et al. (2006) revealed that
246 pellet size and its floating time prior to sinking were key factors to explain settling velocity.
247 The influence of pellet weight was also identified by other authors (Elberizon and Kelly,
248 1998; Chen et al. 1999b, Sutherland et al., 2006). In this study, we not only found that initial
249 pellet size determined settling velocity but also, unlike Chen et al. (1999b), that velocity
250 changed as the pellets sank due to physical transformations that the pellets underwent. As

251 immersion time increases, pellet weight also increased, but contrary to expectations, settling
252 velocity did not increase. Pellet volume, especially diameter, and density also increased with
253 time of immersion. It was therefore hypothesized that pellet weight increase was due to
254 hydration and this caused a volume increase and shape change, causing a greater influence on
255 settling velocity than weight because of greater friction produced, and a higher resistance to
256 fall. Weight increment after soaking was higher in smaller pellets, as Chen et al. (1999b) and
257 Vasallo et al. (2006) noticed, but these authors did not observe the dimension and shape
258 changes that we saw, probably because their immersion periods were shorter or because they
259 simply did not measure the pellets after soaking (a diameter increase of 10% in a 6mm
260 diameter pellet is negligible to the naked eye). Elberizon and Kelly (1998) also mentioned the
261 increased density of trout feed pellets after immersion in fresh water, but they did not provide
262 data on weight and dimension increases nor on the settling velocity after immersion. In the
263 present study, floating time was not considered (unlike Vasallo et al., 2006) because
264 observations show that under industrial rearing conditions, water motion is dynamic, feed is
265 not supplied slowly and methodically, while large number of fish moving and eating
266 voraciously, the result being that pellets tend to sink immediately. Seawater density depends
267 on both temperature and salinity. No clear effect of seawater temperature or salinity was
268 found on the settling velocity of feed pellets in our experiment. Nor was it in the recent
269 literature (Elberizon and Kelly, 1998; Vasallo et al. 2006), probably because, as the above
270 authors state, the range of parameters studied was not so critical to the settling velocity.
271 Despite this, we observed greater but non significant settling velocities at low temperature, as
272 Chen et al. (1999b) noted. These authors suggested that this could be due to the influence of
273 temperature on pellet density, although in our experiments we found that seawater
274 temperature did not affect pellet density.

275 Regardless of pellet weight and in agreement with Chen et al. (2003), the settling velocities of
276 faecal pellets were much lower than that observed for of feed pellets, due to the lower density
277 of the faecal pellets. Water temperature (with the exception of seabass for 15 and 25 °C)
278 showed no effect on settling velocity, as in the case of feed pellets. In this study, the settling
279 velocity of faecal pellets ranged from 0.022 to 0.075 m s⁻¹ in faeces of 0.02–0.74 g wet
280 weight, there being no differences between gilthead seabream and seabass in this respect. For
281 salmon faeces, Chen et al. (1999a, 2003) showed a great variability in settling velocity:
282 0.053–0.066 m s⁻¹ in faeces of 0.04–0.09 g wet weight, and 0.051–0.064 m s⁻¹ in faeces of
283 0.13–0.22 g wet weight. These authors suggested that faecal pellet mass is not a good
284 predictor of settling velocity. Our results (Figure 2) also showed noticeable variability, but
285 they were significantly influenced by faeces wet weight, so the heavier the faecal pellet, the
286 faster the settling. Magill et al. (2006) reported much slower settling velocities for a wide
287 weight range of gilthead seabream and seabass faecal pellets (average 0.005 and 0.007 m s⁻¹
288 respectively), but they studied almost the total fractionating particles (macro and micro-
289 particles at maximum pixel resolution) by means of computer image analysis. Fish faecal
290 pellets have high water content so their nature in seawater is very close to liquid (Vita et al.,
291 2004). Their shape is very variable and not correlated with fish size (Magill et al., 2006).
292 While feed pellets are stable in seawater for long time, faecal pellets tend to fractionate into
293 smaller particles and even become disaggregated pieces which positive buoyancy (Chen et al.,
294 1999a; Magill et al., 2006). Such disaggregating can be caused by the turbulence created by
295 fish swimming under high density rearing conditions. In short, establishing predictions for the
296 settling velocity of faecal pellet is complicated since these friable particles can settle as fast as
297 some medium-size feeding pellets (this study), while micro-particles may show a very slow
298 settling rate or even remain suspended. This erratic behaviour has been successfully integrated

299 into a deposition model by Magill et al. (2006), the most accurate model available at the
300 moment. If large intact or semi-intact faecal pellets and feed pellets are able to reach the
301 seabed, then the settling velocities measured in this study and the study mentioned above,
302 along with the influence of variables such as feed and faecal pellet weight and changes in
303 dimensions of feed pellets while sinking, should be taken into account for waste dispersal
304 modelling purposes. These settling velocities and models using them suggest that uneaten
305 food is dispersed and settles closer to the farms, while faecal particles are more widely
306 dispersed (Doglioli et al., 2004). Fractionated particles from feed pellets and feed pellet dust
307 have still not been studied in terms of buoyancy, flocculation, settling velocity and dispersion,
308 but should be included in deposition models.

309 4.2. TAN leaching from feed and faecal pellets

310 TAN leaching from feed and faecal pellets were successfully explained by fitting data to a
311 first order kinetic equation, which permitted us to derive the dynamic of the TAN leaching
312 process. Maximum leached TAN (a) proved to be independent of water temperature, for feed
313 and faecal pellets. Smaller feed pellets leached more TAN than expected since their
314 surface/volume relationship, and hence their contact with seawater, is greater than larger
315 pellets. However, in feed pellets the speed of the process (k) and time in which a value was
316 reached (t_a) were significantly influenced by temperature the higher the temperature, the faster
317 the process and the shorter the t_a . That is to say, water temperature affected the speed of TAN
318 leaching and the immersion length until reaching the maximum was reached, but did not
319 influence the maximum level reached, demonstrating the influence of temperature in accelerating
320 some biochemical processes. In every feed and faecal pellet type, TAN leaching was very fast
321 during the first few minutes, and the smaller the feed pellets, the faster the process. The only

322 reference found in the literature about leaching from feed pellets is that of Fernández-Jover et
323 al. (2007), who also showed that leaching was faster during the initial stages, but who
324 obtained lower a and higher k values than ours in feed pellets of non-specified size. They also
325 found that water temperature significantly and positively influenced both a and k , but this
326 comparison is not entirely valid since both methods for measuring TAN and the degree of
327 replication differed between the respective studies.

328 In agreement with Chen et al. (2003) who postulated that leaching from faeces is a rapid
329 process, we found that TAN leaching from faeces was three times faster/greater per unit
330 weight than feed pellets. These results agree with Fernández-Jover et al. (2006). For modeling
331 purposes Chen et al. (2003) proposed that leaching values over ten minutes are sufficient for
332 faeces produced from extruded salmon feeds. Fernández-Jover et al. (2006) suggested that ten
333 to twenty minutes is more suitable for seabream and seabass faeces and their feeds. According
334 to our results, maximum leached TAN from gilthead seabream or seabass faecal pellets is not
335 reached until fifteen to thirty minutes, while forty five to sixty minutes is necessary for feed
336 pellets. In any case, it is expected that feed and faecal pellets settled on the seafloor before the
337 maximum leachable TAN (a) is reached, although this, obviously depends on water depth and
338 current velocity. Fernández-Jover et al. (2006) ascertained that leaching from faeces was a
339 temperature and species-dependent variable, showing that leaching was faster at low
340 temperatures. Our experimental design did not allow us to demonstrate that TAN leaching
341 from faeces was temperature-dependent, although, in our case, t_a was always reached more
342 quickly at 25°C. The fact that fish faeces were so labile (Tlustý et al., 2000; Vita et al., 2004)
343 and leach so fast may have obscured the effect of temperature. As regards to species-
344 dependence, the faecal pellets from gilthead seabream and seabass were qualitatively similar

345 and, as both species ate the same food, it is not to be unexpected that TAN leaching from their
346 faeces was similar.

347

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441 Effects of wild fishes on waste exportation from a Mediterranean fish farm. Mar. Ecol. Prog.
442 Ser. 277: 253-261.
- 443

444 Table 1: Physical characteristics and proximate composition of the feed pellets used in the experiments (mean \pm
 445 s.e.m.). Different superscript in the same row indicates statistical differences between pellet types (SNK, $P <$
 446 0.05). Macronutrients and energetic indices are referred as dry weight. NFE: nitrogen-free extracted material.
 447 P/E: crude protein / gross energy ratio.

| Pellet types | FP 2 | FP 4a | FP 4b | FP 6 | FP 8 |
|-------------------------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| Diameter (mm) | 2.50 \pm 0.11 | 4.22 \pm 0.09 | 4.07 \pm 0.46 | 5.42 \pm 0.17 | 8.07 \pm 0.02 |
| Length (mm) | 2.43 \pm 0.05 | 4.59 \pm 0.09 | 4.21 \pm 0.16 | 7.07 \pm 0.16 | 8.19 \pm 0.11 |
| Weight (mg) | 14.10 \pm 0.50 | 72.10 \pm 1.90 | 68.20 \pm 1.90 | 187.30 \pm 5.60 | 360.10 \pm 7.60 |
| Density (kg m ⁻³) | 1071.85 \pm 29.65 ^a | 1119.42 \pm 15.06 ^a | 1125.36 \pm 16.49 ^a | 1069.85 \pm 25.36 ^a | 1069.77 \pm 15.06 ^a |
| Ash (g kg ⁻¹) | 66.03 \pm 0.77 ^a | 78.29 \pm 0.30 ^b | 85.17 \pm 0.81 ^c | 78.74 \pm 0.36 ^b | 58.35 \pm 0.34 ^d |
| Moisture (g kg ⁻¹) | 55.74 \pm 0.66 ^a | 69.75 \pm 0.39 ^b | 63.40 \pm 0.81 ^c | 73.55 \pm 0.72 ^d | 75.11 \pm 0.47 ^d |
| Crude protein (g kg ⁻¹) | 506.84 \pm 2.65 ^a | 489.98 \pm 0.91 ^a | 494.87 \pm 2.52 ^a | 485.75 \pm 2.36 ^a | 435.28 \pm 17.75 ^b |
| Crude fat (g kg ⁻¹) | 187.71 \pm 6.25 ^a | 218.04 \pm 3.18 ^b | 215.57 \pm 0.24 ^b | 214.82 \pm 1.19 ^b | 216.33 \pm 0.74 ^b |
| NFE (g kg ⁻¹) | 235.51 \pm 3.16 ^a | 207.80 \pm 2.06 ^{ab} | 198.62 \pm 1.91 ^b | 214.43 \pm 3.15 ^{ab} | 285.29 \pm 17.01 ^c |
| Gross energy (MJ kg ⁻¹) | 23.19 \pm 0.13 ^a | 23.51 \pm 0.07 ^a | 23.38 \pm 0.03 ^a | 23.40 \pm 0.05 ^a | 23.45 \pm 0.11 ^a |
| P / E (g protein MJ ⁻¹) | 21.85 \pm 0.13 ^a | 20.84 \pm 0.04 ^a | 21.16 \pm 0.14 ^a | 20.76 \pm 0.14 ^a | 18.56 \pm 0.94 ^b |

448 Table 2: Physical characteristics and proximate composition of faecal pellets used in the
 449 experiments (mean \pm s.e.m.). Different superscript in the same row indicates statistical
 450 differences between species (SNK, $P < 0.05$). Percentages are referred as dry weight. NFE:
 451 nitrogen free extracted material.

| Faecal pellets | <i>Sparus aurata</i> | <i>Dicentrarchus labrax</i> |
|-------------------------------------|----------------------------------|----------------------------------|
| Diameter range (mm) | 3.01 – 7.50 | 3.19 – 7.92 |
| Length range (mm) | 3.20 – 14.50 | 3.60 – 14.90 |
| Weight range (mg) | 23.10 – 648.00 | 29.12 – 740.00 |
| Density (kg m ⁻³) | 1021.43 \pm 12.28 ^a | 1018.29 \pm 12.97 ^a |
| Ash (g kg ⁻¹) | 24.10 \pm 1.06 ^a | 36.85 \pm 1.38 ^b |
| Moisture (g kg ⁻¹) | 892.91 \pm 0.98 ^a | 892.25 \pm 1.46 ^a |
| Crude protein (g kg ⁻¹) | 215.91 \pm 1.01 ^a | 207.10 \pm 1.44 ^b |
| Crude fat (g kg ⁻¹) | 43.47 \pm 5.88 ^a | 43.97 \pm 3.99 ^a |
| NFE (g kg ⁻¹) | 714.61 \pm 5.76 ^a | 709.16 \pm 6.92 ^a |

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454 Table 3: Results of the multiple regression analyses for settling velocity, weight and volume increase and density changes of feed
 455 pellets after immersion.

| Independent Variables | Dependent Variables: y | | | | | | | |
|---|---|-------|------------------------|-------|-----------------------|-------|------------------------|-------|
| | Settling Velocity (m s ⁻¹) | | Weight Increase (%) | | Density (%) | | Volume Increase (%) | |
| | Coefficients | S.E. | Coefficients | S.E. | Coefficients | S.E. | Coefficients | S.E. |
| Intercept: <i>a</i> | 5.749** | 1.749 | 22.616*** | 2.019 | 1.085* | 0.019 | 24.632*** | 2.878 |
| Immersion time (<i>It</i>): <i>b</i> | -0.035*** | 0.004 | 0.514*** | 0.034 | -0.001*** | 0.000 | 0.653*** | 0.063 |
| Temperature (<i>T</i>): <i>c</i> | -0.135 ^{n.s.} | 0.154 | 0.164 ^{n.s.} | 1.381 | 0.010 ^{n.s.} | 0.013 | 1.727 ^{n.s.} | 1.969 |
| Pellet size (<i>Ps</i>): <i>d</i> | 0.906*** | 0.039 | -2.722*** | 0.338 | 0.008*** | 0.003 | -3.420*** | 0.483 |
| Density (<i>D</i>): <i>e</i> | 0.866 ^{n.s.} | 1.598 | - | - | - | - | - | - |
| R | 0.95 | | 0.91 | | 0.54 | | 0.90 | |
| R ² adj | 0.91 | | 0.83 | | 0.25 | | 0.79 | |
| F-ANOVA | 226.61*** | | 95.73*** | | 7.65*** | | 75.90*** | |
| n | 70 | | 60 | | 60 | | 60 | |
| Fitting equation: $y = a + b \cdot (It) + c \cdot (T) + d \cdot (Ps) + e \cdot (D)$ | | | | | | | | |
| *P < 0.05; **P < 0.01; ***P < 0.001; n.s. non-significant. | | | | | | | | |

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 460 Table 4: Results of multiple regression analysis for settling velocity of faecal pellets as a function of fish species, water temperature and
 461 faecal pellet wet weight.

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| Dependent Variable y : Settling Velocity (m s^{-1}) | | |
|--|------------------------|-------|
| Independent Variables | Coefficients | S.E. |
| Intercept: b | 3.439 ^{***} | 0.211 |
| Species (S): c | 0.037 ^{n.s.} | 0.159 |
| Temperature (T): d | -0.245 ^{n.s.} | 0.184 |
| Faecal pellet weight (Fp): e | 4.642 ^{***} | 0.656 |
| R | 0.67 | |
| R ² | 0.44 | |
| F-ANOVA | 31.03 ^{***} | |
| n | 116 | |
| Fitting equation: $y = b + c \cdot (S) + d \cdot (T) + e \cdot (Fp)$ | | |
| *P < 0.05; **P < 0.01; ***P < 0.001; n.s. non-significant. | | |

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468 Table 5: Results of the non-linear regression (1st order kinetic equation) for TAN leaching (y) of feed and faecal pellets as a function
 469 of immersion length (t). t_a is estimated from the equation as the immersion time at which a was reached.

| Feed pellet | 15 °C | | | | | 25 °C | | | | |
|--|------------------------------|---------------------------|----------------------------------|-------------|--|------------------------------|---------------------------|----------------------------------|-------------|--|
| | a ($\mu\text{g g}^{-1}$) | k (min^{-1}) | R ² ESS F-ANOVA | t_a (min) | a respect total nitrogen in the pellets (%) | a ($\mu\text{g g}^{-1}$) | k (min^{-1}) | R ² ESS F-ANOVA | t_a (min) | a respect total nitrogen in the pellets (%) |
| FP 2 | 296.31** | 0.0437* | 0.95 22.72 74.43*** | 52.69 | 0.365 | 278.06*** | 0.0917** | 0.96 24.38 86.61*** | 25.11 | 0.343 |
| FP 4a | 249.34*** | 0.0394*** | 0.99 7.41 581.23*** | 58.44 | 0.318 | 234.33* | 0.0664* | 0.83 33.86 19.65* | 34.68 | 0.299 |
| FP 4b | 292.28** | 0.0315* | 0.97 16.45 124.80*** | 73.10 | 0.369 | 114.93*** | 0.0526** | 0.97 7.09 135.35*** | 43.77 | 0.145 |
| FP 6 | 78.13** | 0.0399* | 0.94 6.24 68.32** | 57.71 | 0.101 | 135.75** | 0.0455* | 0.91 14.40 38.33** | 50.81 | 0.175 |
| FP 8 | 72.15** | 0.0361* | 0.93 6.02 55.39** | 63.78 | 0.104 | 139.86* | 0.0304* | 0.92 12.43 45.27** | 75.74 | 0.201 |
| Faecal pellets | | | | | | | | | | |
| <i>Sparus aurata</i> | 921.15*** | 0.1063* | 0.92 142.14 21.78** | 23.26 | 2.666 | 834.36*** | 0.1463* | 0.90 99.56 36.95** | 15.53 | 2.415 |
| <i>Dicentrarchus labrax</i> | 791.09** | 0.0758* | 0.84 125.92 20.93** | 28.57 | 2.387 | 888.42*** | 0.1452* | 0.85 140.33 22.47** | 15.87 | 2.681 |
| Fitting equation: $y = a \cdot (1 - e^{-k \cdot t})$ *P < 0.05; **P < 0.01; ***P < 0.001; n.s. non-significant | | | | | | | | | | |

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472 Table 6: .Results of multiple regression analyses for the maximum TAN leached (a), speed of
 473 the leaching process (k) and time in which a is reached (t_a) as a function of water temperature
 474 and feed pellet size. t_a is estimated from the equation as the immersion time at which a was
 475 reached.

| Independent Variables | Dependent Variables y | | | | | |
|---------------------------|------------------------------|--------|---------------------------|-------|----------------------|-------|
| | a ($\mu\text{g g}^{-1}$) | | k (min^{-1}) | | t_a (min) | |
| | Coefficients | S.E. | Coefficients | S.E. | Coefficients | S.E. |
| Intercept: b | 357.547 ^{***} | 52.503 | 0.063 [*] | 0.011 | 2.639 ^{***} | 0.239 |
| Temperature (T): c | -17.056 ^{n.s.} | 38.245 | 0.019 [*] | 0.008 | -0.501 [*] | 0.200 |
| Pellet size (Ps): d | -33.314 ^{**} | 9.375 | -0.005 [*] | 0.002 | 0.432 [*] | 0.148 |
| R | 0.80 | | 0.80 | | 0.82 | |
| R ² adj | 0.55 | | 0.54 | | 0.59 | |
| F-ANOVA | * | | * | | * | |
| n | 10 | | 10 | | 10 | |

Fitting equation: $y = b + c \cdot (T) + d \cdot (Ps)$
 *P<0.05; **P<0.01; ***P<0.001; n.s. non-significance.

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Table 7: Chow test F-values for the pairwise comparisons of TAN leaching from faecal pellets of *Sparus aurata* (S) and *Dicentrarchus labrax* (D) at 15 and 25 °C.

| Pairwise combinations | Tabulated F _(2, 56) = 3.15 |
|-----------------------------------|---------------------------------------|
| S ₁₅ - S ₂₅ | 0.317 ^{n.s.} |
| S ₁₅ - D ₂₅ | 0.635 ^{n.s.} |
| S ₁₅ - D ₁₅ | 2.426 ^{n.s.} |
| S ₂₅ - D ₂₅ | 0.204 ^{n.s.} |
| S ₂₅ - D ₁₅ | 1.378 ^{n.s.} |
| D ₁₅ - D ₂₅ | 3.848 [*] |

*P < 0.05; **P < 0.01; ***P < 0.001; n.s. non-significant

Figure captions

Figure 1(A-C): (A) Settling velocities of the different feed pellets after increasing immersion length; (B) mean weight increase (%) and (C) volume increase of feed pellets after immersion at 15 and 25°C. Mean \pm s.e.m.

Figure 2: Settling velocities of faecal pellets of different weight from *Sparus aurata* and *Dicentrarchus labrax* at 15 and 25°C, and predicted values from the equation $y = b + c \cdot (S) + d \cdot (T) + e \cdot (Fp)$.

Figure 3(A-E): TAN leaching (mean \pm s.e.m.) from the different food pellets types after immersion at 15 and 25°C, and predicted values from the equation $y = a \cdot (1 - e^{-k \cdot t})$. (A): FP 2; (B): FP 4a; (C) FP 4b; (D) FP 6; (E): FP 8.

Figure 4(A-B): TAN leaching (mean \pm s.e.m.) from the faeces of (A) *Sparus aurata* and (B) *Dicentrarchus labrax* after immersion at 15 and 25°C, and predicted values from the equation $y = a \cdot (1 - e^{-k \cdot t})$.

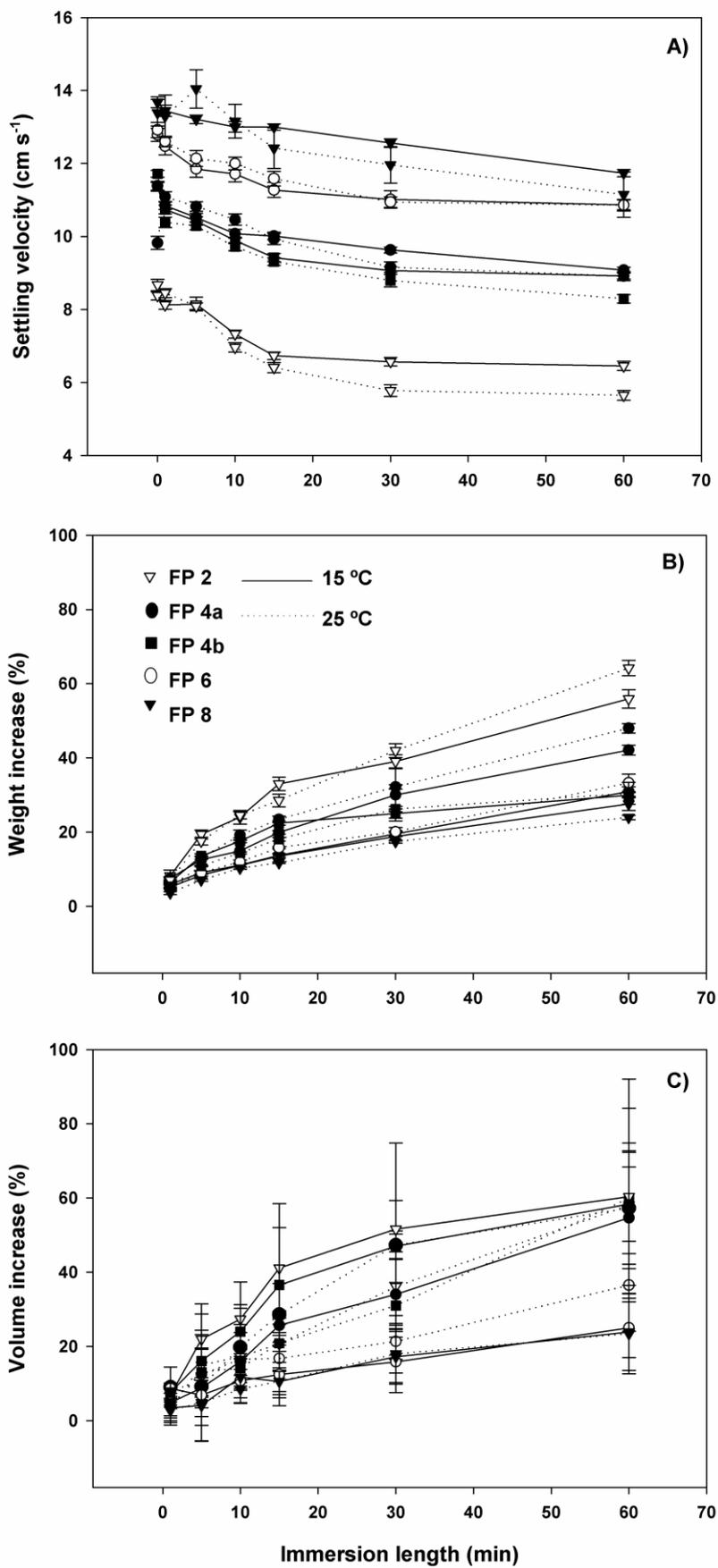


Fig1

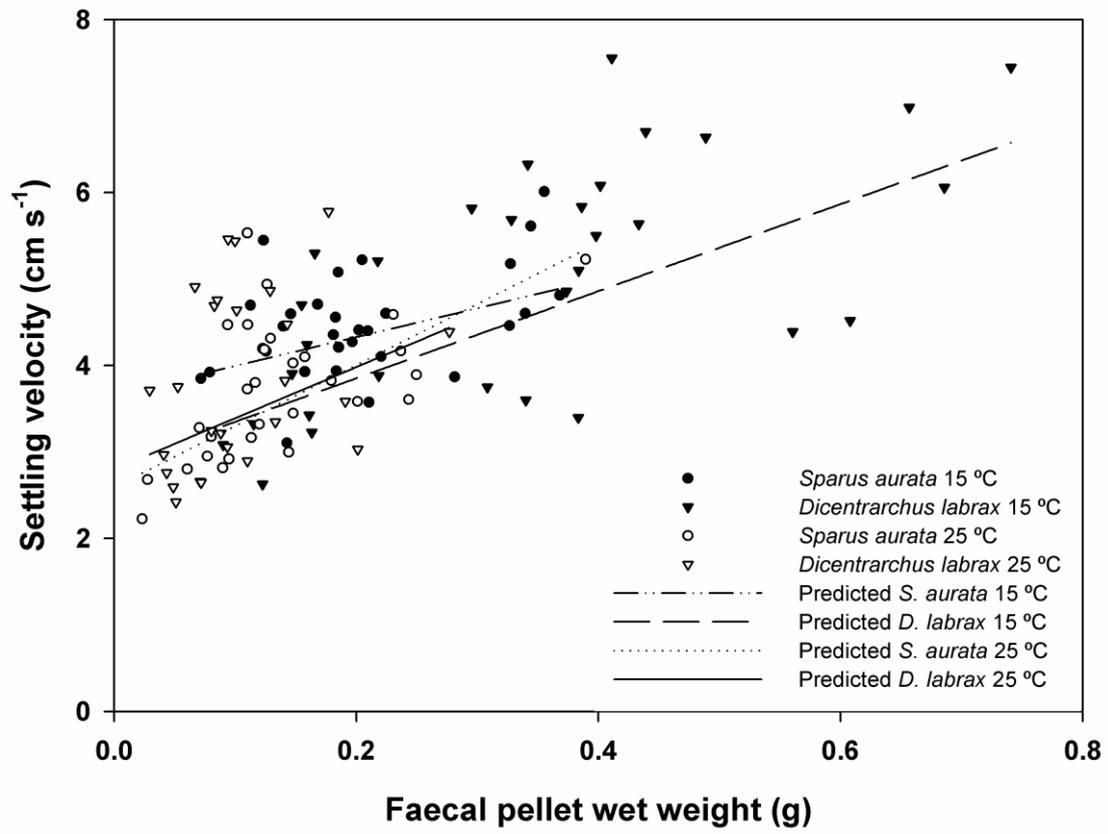


Fig 2

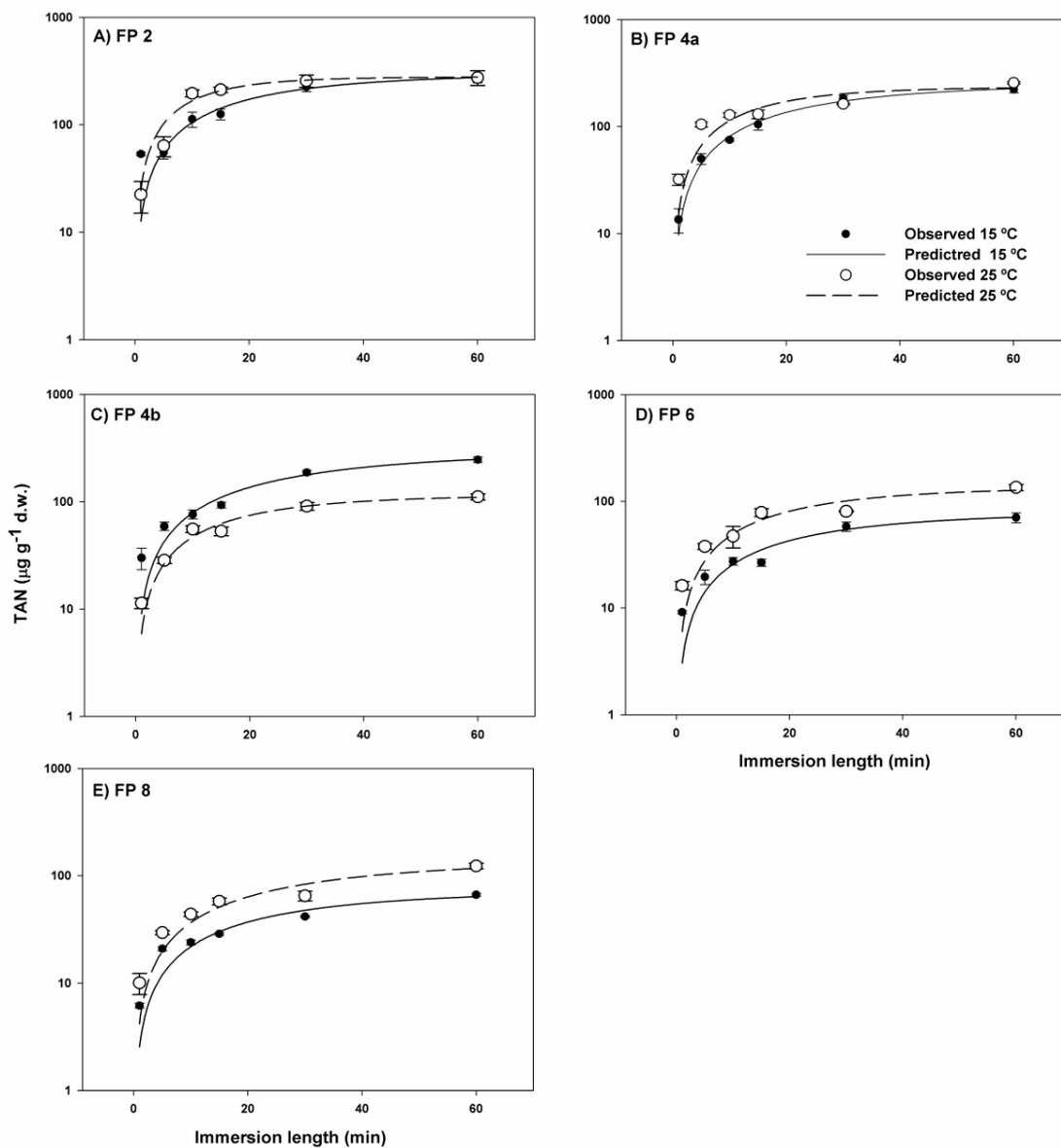


Fig 3

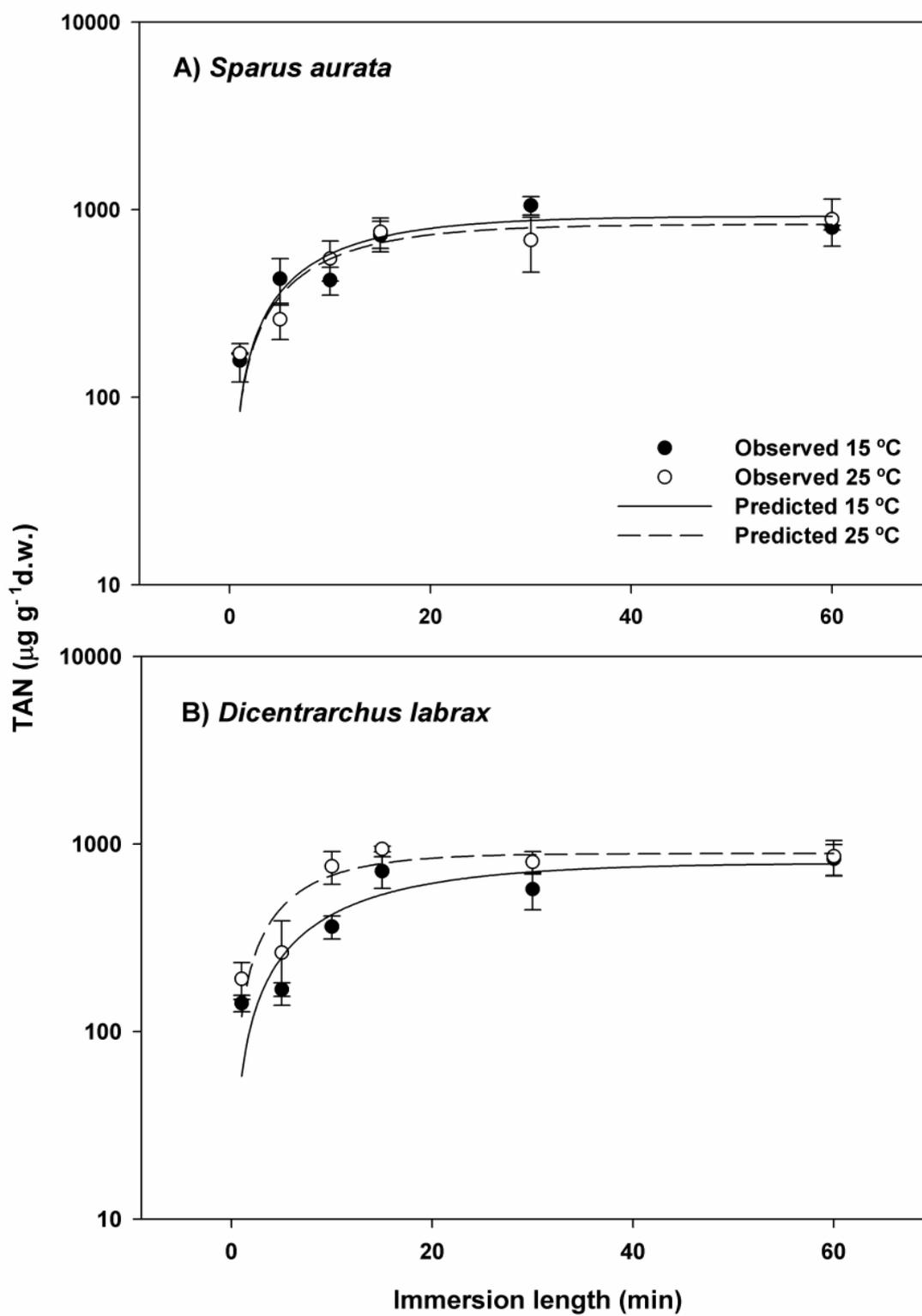


Fig 4