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1 **Development of an exposure protocol for toxicity test (FEET) for a marine species –**
2 **the European sea bass (*Dicentrarchus labrax*)**

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24

25 **Abstract**

26

27 Regulatory assessment of the effects of chemicals requires the availability of validated tests
28 representing different environments and organisms. In this context, developing new tests is
29 particularly needed for marine species from temperate environments. It is also important to
30 evaluate effects that are generally poorly characterized and seldom included in regulatory
31 tests. In this study, we designed an exposure protocol using European sea bass
32 (*Dicentrarchus labrax*) larvae. We examined classical toxicological values (LC_x) as well as
33 behavioral responses. By comparing different hatching and breeding strategies, we defined
34 the optimal conditions of exposure as non-agitated conditions in 24- or 48-well microplates.
35 Our exposure protocol was then tested with 3,4-dichloroaniline (3,4-DCA), a recommended
36 reference molecule. Based on our results, the 96h LC₅₀ for 3,4-DCA corresponded to 2.04
37 mg/L while the 168h LC₅₀ to 0.79 mg/L. Behavioral analyses showed no effect of 3,4-DCA
38 at low concentration (0.25 mg/L). In conclusion, the present work established the basis for a
39 new test which include comportemental analysis and shows that the use of sea bass is
40 suitable to early life stage toxicity tests.

41

42 *Keywords:* early life stage toxicity test, temperate conditions, sea bass larvae, 3,4-
43 dichloroaniline, behavioral test, 96h LC₅₀

44

45 **Introduction**

46

47 In the last decade, the global production of chemicals has almost doubled and projections
48 indicate a continual growth in the coming years (SEI et al. 2019). As a consequence,
49 concern about the environmental and health effects of these substances are strongly
50 expressed (Nellemann et al. 2008; Magulova and Priceputu 2016; Van den Berg et al. 2017;
51 SEI et al. 2019; Fiedler et al. 2020). At each step of a chemical's life cycle (synthesis,
52 incorporation in products, use and end of life of the products), wastes are produced. If
53 released into the environment, they are distributed based on their chemo-physical properties
54 in different environmental compartments including air, soil, water, or biota (Koumanova
55 2006; Mackay et al. 2006; Bonmatin et al. 2015) and may exert detrimental effects.
56 Chemicals release may also arise from various sources once produced, including domestic,
57 industrial (via wastewater disposals) and agricultural (discharge from the field to surface
58 waters or percolate to groundwaters) uses (Olsson et al. 2013; Keller et al. 2014; SEI et al.
59 2019). Many substances may then contaminate surface or groundwater, eventually reaching
60 oceans (Roose et al. 2011). Moreover, during their transport, chemicals can undergo
61 physical (e.g., volatilization) or chemical transformations (e.g. photodegradation, microbial
62 degradation, hydrolysis) generating new by-products (Koumanova 2006; Olsson et al.
63 2013). The complex mixture thus occurring in aquatic ecosystems gives rise to considerable
64 concern due to the potential adverse effects it may induce on ecosystems (Roose et al. 2011;
65 Potter 2013; Bashir et al. 2020).

66 In 2006, the EU adopted a regulation named REACH (Registration, Evaluation,
67 Authorisation and Restriction of Chemicals) to collect information on the properties and
68 hazards of all chemicals produced and traded in the continent (REACH 2006). The aim is to
69 request industries/producers to assess and manage environmental and health risks posed by
70 produced chemicals (REACH 2006). To reach this scope, animal experimentation remains
71 essential. For instance, the assessment of toxic effects and thresholds of most chemicals
72 requires a step of testing on model fish species (Balzano et al. 2015). To help the users, test
73 guidelines have been published by the OECD (OECD 2019).

74 Nevertheless, driven by the 3 Rs approach (*Reduce, Reuse, Recycle*), EU authorities
75 encourage the development and the use of alternative tests as (Q)SAR (Quantitative
76 Structure-Activity Relationship), tests on algae, on invertebrates, or embryo-larval test

77 (ECHA 2014). Among them, the fish embryo toxicity test (FET) and the fish
78 eleutheroembryo toxicity test (FEET) are two examples of alternative methods encouraged
79 (Embry et al. 2010). The FET is a short-term test (96 h) designed to determine the acute
80 toxicity of chemicals on embryonic stages of fish (OECD 2013; Embry et al. 2010). The
81 FEET instead involved the use of larvae during the eleutheroembryonic stage which extends
82 from hatching until resorption of the yolk-sack (Balon 1975; Embry et al. 2010). In most
83 species, the eleutheroembryonic stage is more sensitive to chemical exposure than the
84 embryonic stage, most likely due to the absence of a protective chorion (Woltering 1984;
85 Léonard et al. 2005). Developing tests on early-life stages is hence advisable as they are
86 simultaneously precious alternatives to animal testing (as intended by the 3 R's rule) and
87 sensitive tools to analyze toxicity and sub-lethal endpoints such as growth and behavior.

88

89 For toxicological studies, the most commonly used model organisms are zebrafish (*Danio*
90 *rerio*), fathead minnow (*Pimephales promelas*) and Japanese medaka (*Oryzias latipes*)
91 (OECD 2013). Despite the advantages provided by these species for laboratory testing (easy
92 to maintain and breed, very short generation time *etc.*), they are not representative of the
93 various ecosystems encountered in Europe (OECD 2013, 2014). The European chemical
94 agency (ECHA) has already underlined the need to include European native species into
95 routine toxicology tests (Balzano et al. 2015). The European sea bass (*Dicentrarchus*
96 *labrax*) is a pelagic teleost native to European and North Africa coastlines (Kaushik 2009;
97 Sánchez Vázquez and Muñoz-Cueto 2014). Due to its high commercial value, it has been
98 largely studied and is intensively produced in Mediterranean aquaculture industries (Pickett
99 and Pawson 1994; Bagni 2020). In addition, the sea bass has already been successfully used
100 in several short- and long-term toxicological studies evaluating the effects of exposure to
101 heavy metals, surfactants, insecticides or oil dispersants (Athanasopoulou et al. 2002;
102 ICRAM Taxa 2005; Spaggiari et al. 2005; Almeida et al. 2012; Balzano et al. 2015; Della
103 Torre et al. 2015) and it has been added to the list of recommended species for test on
104 juveniles and adult (OECD 1992, 2014). It appears therefore as a good candidate for a
105 model species representing European pelagic and temperate environments.

106

107 The aim of the present study was to develop an exposure protocol using a native European
108 pelagic species, the European sea bass. More specifically, the objectives were 1) to design

109 an optimized procedure for incubation/rearing, 2) to assess the repeatability and define the
110 toxicological parameters (LC_{50} , LC_{10}) after exposure to a reference molecule, 3,4-
111 dichloroaniline (3,4-DCA), and 3) to evaluate the effects of sub-lethal concentrations of 3,4-
112 DCA exposure on behavioral endpoints (Annex I). 3,4-DCA is an intermediate in the
113 chemical industry for the synthesis of the herbicide propanil and a biodegradation product
114 of several phenylcarbamates and acylanilide herbicides. It is classified as “very toxic to
115 aquatic organisms” by the ECHA (Munn et al. 2006). Its use as a reference molecule in
116 toxicology has been suggested due to its high acute and chronic toxicity to aquatic
117 organisms (Crossland 1990; Munn et al. 2006; Schäfers and Nagel 1993). Standard
118 toxicological values for 3,4-DCA, as the LC_x , have been already determined using different
119 model fish species, allowing assessment of the sensitivity of our test (Adema and Vink
120 1981; Hodson 1985; Call et al. 1987; Becker et al. 1990; Schäfers and Nagel 1993; Busquet
121 et al. 2014).

122

123 **Material and Methods**

124

125 *1. Test species*

126 Fertilized eggs of European sea bass were purchased from a local hatchery (Ecloserie
127 Marine de Gravelines Ichtus, Gravelines, France). After their arrival to the laboratory,
128 fertilized eggs (~ 2 dpf, stage 10-18S as described by Cucchi et al. (2012)) were gradually
129 acclimate to filtered and aerated natural seawater with a salinity corresponding to 33 psu
130 and transferred into a cylindroconical aquarium ($d \sim 10\text{-}20\text{ L}^{-1}$). Eggs were kept at a
131 constant temperature of 15 °C in darkness. All procedures were performed in accordance
132 with the French and European legislation concerning the protection of animals used in
133 experimentation. Procedures undertaken were approved (#10263-2017061911009684v3) by
134 the regional ethical committee (Comité d’Ethique Normandie en Matière d’Expérimentation
135 Animale, CENOMEXA; agreement number 54).

136

137 *2. Preliminary experiment 1: Definition of the experimental conditions*

138 The experiment was performed at the CREC (Centre de Recherche en Environnement
139 Côtier, University of Caen). Different incubations and rearing conditions were evaluated by

140 comparing in parallel: 1) incubation volume; 2) agitation conditions; 3) frequencies of
141 medium change (Annex II).

142 *Incubation volume* Eggs (stage18-22 S) were individually transferred in either 48- or 24-well
143 culture plates (Thermo Fisher Scientific, Denmark) containing 1 and 2 mL of natural
144 seawater respectively. A single egg was placed in each well.

145 *Agitation conditions* Culture plates were submitted to three different conditions: non-
146 agitated, agitation before hatching, and agitation during the whole experiment. For the
147 agitated conditions, plates were disposed on a moving benchmark (BenchRocker™ 2D,
148 Benchmark Scientific) with an average speed of 90 rotations per minute.

149 *Frequency of medium changes* Culture plates were submitted to three different conditions: no
150 change, daily change, changes every 3.5 days. One third of each plate (24-wells and 48-
151 wells) was dedicated to one of the medium change conditions leading to a different number
152 of replicates per kind of plate (n=16 or 32 according to microplate type). For changing
153 medium, half of the well volume was slowly pipet out and replaced by the same volume of
154 new medium. Plates were incubated in the dark at 15°C in an incubator (R Biopharm) for 10
155 days.

156

157 Hatching rate, calculated as cumulative percentage of hatched eggs per total eggs, and
158 subsequent larval survival rate, percentage of alive larvae per total larvae, were recorded
159 daily. Embryonic and larval death were determined by coagulation and absence of
160 movement/response to stimuli, respectively.

161 Concentrations of oxygen (HANNA, HI 9828) and nitrites (visocolorECO, Macherey-
162 Nagel, France) were measured every 48h. At the end of the experiment, larvae were
163 sacrificed with an overdose (250 mg/L) of buffered tricaine methanesulfonate (MS-222;
164 Sigma-Aldrich, France).

165

166 3. Preliminary experiment 2: 3,4-DCA range-finding

167 The experiment was performed at the CREC (Centre de Recherche en Environnement
168 Côtier, University of Caen). To identify a relevant window of exposure for LCx
169 determination, the first assay was performed using wide-range of 3,4-dichloroaniline (3,4-
170 DCA, purity: ≥ 98 %, Sigma-Aldrich, France) concentrations. In 24-well plates, eggs were
171 individually allocated in wells filled with 2 ml of natural seawater (33 psu). Plates were then

172 placed in an incubator at 15°C, in the dark. The experiment included one control group in
173 seawater, plus four groups exposed to increasing concentrations of 3,4-DCA. Three
174 replicates were performed for each group (number of eggs per group = 72). A stock solution
175 of 3,4-DCA (100 mg/L) was prepared in distilled water. Then, test solutions were obtained
176 by serial dilutions of the stock solution in natural and filtered seawater at concentrations of:
177 10, 1, 0.1 and 0.01 mg/L. For each test solution, aliquots were prepared and stored at -20°C
178 until the day of exposure (*i.e.* day of medium change). 3,4-DCA is readily soluble in water
179 (water solubility = 580 mg/L at 20 °C) and is characterized by a low Kow (log Pow = 2.7)
180 which ensure a complete dissolution in water. As no significant degradation of 3,4-DCA
181 occurs in surface waters (estimated half-lives of 18 days) (Munn et al. 2006), no degradation
182 is assumed during the test duration.

183

184 At 15 °C, sea bass eggs hatching occurs at ca. 92-93 hpf (Cucchi et al., 2012). The
185 experiment started (t0) when hatching rate reached 80 %; non-hatched eggs were replaced
186 with newly hatched larvae from a stock population. At t0, half of each well content was
187 replaced by 1 ml of the corresponding test solution to reach the final desired concentrations
188 (1X), namely 5, 0.5, 0.05 and 0.005 mg/L 3,4-DCA. Control group underwent the same
189 procedure using seawater. Test solutions were renewed every 48h to allow water
190 oxygenation and avoid 3,4-DCA degradation. Sea bass larvae were not fed as exogenous
191 feeding start at ca. 8 dph, age corresponding to mouth opening (Sánchez Vázquez &
192 Muñoz-Cueto, 2014). The duration of the exposure was 10 days and survival rate was
193 recorded daily. At the end of the experiment, larvae were sacrificed with an overdose of MS-
194 222.

195

196 4. Main Experiments

197

198 4.1 LCx determination

199 A first experiment for LCx determination was performed at the CREC (experiment 1, May
200 2018) and repeated twice at IFREMER in Port-en-Bessin (experiment 2, July 2018;
201 experiment 3, November 2018). Seawater used by both laboratories was pumped at the
202 same location (Luc-Sur-Mer, France; Platform: 6200310). Characteristics of natural sea
203 water at the sampling location is described in details in Annex III.

204 Fertilized eggs were individually placed in 24-well plates filled with 2 ml of oxygenated sea
205 water (33 psu) at 15°C, in a dark incubator. The experiment included one control group in
206 seawater, plus five exposed groups to 3,4-DCA at the following concentrations 0.25, 0.50, 1,
207 2 and 4 mg/L. The experiment (start and renewal of test solutions) was performed following
208 the protocol described above (see: Preliminary experiment 2). The number of replicates was
209 five in experiment 1 (n=120 per experimental group) and three in experiment 2 and 3 (n=72
210 per experimental group). Survival rate was daily recorded over 8 days and at the end of the
211 experiment, larvae were sacrificed with an overdose of MS-222.

212

213 *4.2 Behavioral test*

214 At the end of the exposure period of experiment 3, a behavioral test was performed with
215 alive individuals from control (n=28) and 0.25 mg/L 3,4-DCA conditions (n=14). Plates
216 were placed in a light-controlled chamber with infrared backlight, with a camera (Sony FDR-
217 AX53) placed on the top. After 5 minutes of acclimation, larvae were recorded directly in
218 their wells for 10 minutes.

219

220 Recordings were analyzed using a video tracking software (Ethovision XT 10, Noldus).
221 Each well was virtually divided into a central ($\frac{1}{2}$ well diameter) and a peripheral zone. An
222 increased time spent in the inner or outer zone (*i.e.* thigmotaxis) would indicate an increased
223 exploratory behavior or anxiety-like behavior, respectively. Behavioral endpoints included
224 distance traveled and time spent in each well-zone.

225

226 *5. Statistics*

227 Data were computed using R software (version 3.5.1).

228

229 *Preliminary experiment 1:* To determine the best rearing conditions the effects incubation
230 volume, agitation condition, and frequency of medium change were analyzed using
231 generalized linear models fitted with a binomial distribution (GLM: glm function). Removal
232 of non-significant effects and model selection for the GLM analyses were based on Akaike
233 information criterion.

234

235 *Preliminary experiment 2:* Effects of time (i.e. number of exposure days) and 3,4-DCA
236 concentration on larvae survival were analyzed using generalized linear models fitted with a
237 binomial distribution (GLM: glm function). Removal of non-significant effects and model
238 selection for the GLM analyses were based on Akaike information criterion. Survival rates
239 were compared daily using the Fisher's exact test for count data with Bonferroni correction
240 for multiple pairwise comparisons.

241

242 *Main experiments:* Effects of time and 3,4-DCA exposure on survival rate were evaluated as
243 described above (see: Preliminary experiment 2). LC_{50} and LC_{10} values at 96 h and 168 h
244 were calculated using MOSAIC survivalguts-fit (Modeling and Statistical tools for
245 ecotoxicology; <https://mosaic.univ-lyon1.fr/guts>).

246

247 *Behavioral analysis:* Comparison of the distance traveled and the time spent in each well-
248 zone between exposed and control groups were performed using non-parametric analyses
249 (exact permutation tests; wilcox_test function in coin package).

250

251 **Results**

252

253 1. *Preliminary experiment 1:* Definition of the experimental conditions

254 For each experimental conditions, the hatching rate was analyzed at day 1 and day 2.
255 Hatching rate was dependent on agitation condition ($p < 0.001$) and day ($p < 0.001$) (Fig.
256 1), with a significant interaction between these factors ($p < 0.001$). The hatching rate was
257 higher at day 1 in agitated condition (86 %) in comparison with non-agitated condition (44
258 %), but this difference was no longer observed at day 2 (93 % and 94 % in agitated and non-
259 agitated conditions, respectively) revealing no differences in the overall hatching success
260 (Fig.1). Concerning the other conditions, incubation volume or frequency of medium
261 change, no significant effect was observed on hatching rate.

262

263 The larval survival rate was measured for the different experimental conditions between day
264 2 and day 9. The survival rate was statically dependent on incubation volume ($p = 0.011$),
265 and day ($p < 0.001$) and decreased from 100 % (at day 2) to 88 % and 84 % (at day 9) in the
266 48- and 24-well plates, respectively. A significant interaction between incubation volume

267 and frequency of medium change was also found ($p < 0.001$). In 48-well plates the best
268 frequency of medium change was 3.5 days while for the 24-well plates it corresponded to
269 24h. No effect of agitation or medium change conditions was observed on survival rates.
270 Overall, the survival rates were thus very similar at the end of the experiments, with a mean
271 mortality rate of 1.5-2% per day (Fig. 2). A follow-up of larvae morphology during the 8
272 days test is shown in Annex IV.

273 Dissolved oxygen and nitrite levels were measured every 48 h. Average concentrations
274 corresponded to 7.38 mg/L for O₂ and 0.03 mg/L for NO₂⁻ and no evidence of any
275 detrimental condition was observed.

276 These results led to the following exposure conditions for the experiments: larvae were
277 exposed in 24-well plates under non-agitated conditions. Regarding the frequency of
278 medium change, the choice was made in order to keep the balance between water quality,
279 handling stress and chemical degradation.

280

281 2. Preliminary experiment 2: 3,4-DCA range-finding

282 In order to define a relevant range of exposure for determining lethal concentrations (LC_x),
283 sea bass larvae were exposed to a wide range of nominal 3,4-DCA concentrations (0.005,
284 0.05, 0.5 and 5 mg/L) (Fig 3). Survival rate was dependent on treatment ($p < 0.001$) and day
285 ($p < 0.001$) with a significant interaction between these factors ($p < 0.001$). Comparison of
286 the daily survival rates showed a significant decrease of larval survival in the 5 mg/L group
287 ($p < 0.001$; survival= 74 %) as compared to control (survival= 98 %) from day 3. In this 5
288 mg/L group, all the larvae were dead at day 4.

289 In the 0.5 mg/L group, a significant decrease in survival rate was observed from day 6 ($p <$
290 0.001 ; survival= 34 %) as compared to control group (survival= 96 %). From day 9, no
291 larvae from 0.5 mg/L group was still alive.

292 Regarding the groups exposed to the lowest concentrations of 3,4-DCA (0.005 mg/L and
293 0.05 mg/L groups), no significant differences in survival rates were detected when
294 compared to the control group (at day 10: control survival= 95 %; 0.005 mg/L survival= 92
295 %; and 0.05 mg/L DCA survival= 92 %; $p = 1$).

296 Based on these results, the following experiments were performed using a narrow
297 concentration range.

298

299

3. Main Experiments

300 To determine LC_x values for 3,4-DCA in sea bass larvae, three independent experiments
301 were performed using the following concentrations: 0, 0.25, 0.50, 1, 2 and 4 mg/L.

302

3.1 Experiment 1

304 Survival was found to be dependent on both time ($p < 0.001$) and treatment ($p < 0.001$)
305 (Fig. 4). In this experiment, from day 4, a significant decrease in survival was observed in
306 the groups 1 mg/L (survival= 80 %, $p = 0.03$) and 4 mg/L (survival= 77 %, $p = 0.005$) as
307 compared to controls (survival= 95 %). In addition, in the group 4 mg/L the survival rate
308 continued to decline until day 8, thereafter all the larvae were dead. In the 2 mg/L group, a
309 significant difference in survival rate was detected from day 6 (survival= 60 %; $p = 0.02$ as
310 compared to control survival= 91 %) and from day 8 for the 0.5 mg/L group (survival= 76
311 %; $p < 0.001$ as compared to control survival= 90 %).

312

313 In this experiment, at 96 h, the LC₅₀ and the LC₁₀ values obtained for 3,4-DCA were 3.87
314 mg/L and 3.09 mg/L, respectively (Table 1). At 168 h, the values obtained for LC were:
315 LC₅₀=2.08 mg/L and LC₁₀ =1.61 mg/L.

316

3.2 Experiment 2

318 Survival was dependent on both time ($p < 0.001$) and treatment ($p < 0.001$), with a
319 significant interaction between the two factors ($p < 0.001$). From day 2, a significant
320 decreased in survival rate was detected in the 4 mg/L (survival= 83 %; $p < 0.001$) and 2
321 mg/L (survival= 90 %; $p = 0.02$) groups in comparison to control group (survival= 100 %).
322 Mortality reached 100 % for the 4 mg/L group at day 5 and for the 2 mg/L group at day 6
323 (Fig. 4). Regarding the 1 mg/L group, a significant effect in survival rate was observed from
324 day 5 (survival= 60 %; $p < 0.001$ as compared to control group survival= 93 %). Then, it
325 continued to decline until day 7 where the mortality reached 100 %. Concerning the 0.25
326 mg/L and 0.5 mg/L groups, no significant differences in survival rates were observed in
327 comparison to the control group (survival 0.25 mg/L = 81%, survival 0.5 mg/L =76 %,
328 survival control = 79 %; $p = 1$).

329

330 In this experiment, LC_x values obtained for 3,4-DCA were: LC₅₀ = 1.57 mg/L and LC₁₀ =
331 0.65 mg/L at 96 h; LC₅₀ = 0.73 mg/L and LC₁₀ = 0.46 mg/L at 168 h (Table 1).

332

333 3.3 Experiment 3

334 Survival was dependent on both time ($p < 0.001$) and treatment ($p < 0.001$) with a
335 significant interaction between the two factors ($p < 0.001$). From day 4, a significant effect
336 of treatment was detected on survival in the 1 mg/L (survival = 56 %), 2 mg/L (survival =
337 65 %) and 4 mg/L (survival = 78 %; $p < 0.001$) groups in comparison to control condition
338 (survival = 97 %). On day 5, all the larvae were dead in the 4 mg/L and 2 mg/L groups,
339 while in the 1 mg/L group, the survival declined to 3 % (Fig.4). Regarding the 0.5 mg/L
340 group, a significant effect was detected at day 5 (survival = 81 %; $p = 0.006$ as compared to
341 control survival = 96 %); and at day 6 for the 0.25 mg/L group (survival = 81 %; $p = 0.006$
342 as compared to control survival = 92 %).

343

344 LC_x values obtained for 3,4-DCA were LC₅₀ = 1.35 mg/L and LC₁₀ = 0.39 mg/L at 96 h;
345 LC₅₀ = 0.41 mg/L and LC₁₀ = 0.15 mg/L at 168 h (Table 1).

346

347 3.4 Behavioral test

348 In the experiment 3, behavioral measurements were undertaken on larvae at day 8 (control
349 $n=28$ and 0.25 mg/L $n=14$). The results obtained indicated no significant difference ($p =$
350 0.99) in the total distance traveled between the larvae from the control (median \pm IQR: 76 \pm
351 66 cm) and 0.25 mg/L groups (median \pm IQR: 107 \pm 71 cm) (Table 2). A comparison of the
352 distance traveled in the central or the peripheral part between the two groups was also
353 performed and no significant differences were observed (central part: $p= 0.27$; peripheral
354 part $p= 0.86$). Finally, the zone preference of larvae was analyzed. In both groups, the time
355 spent in the periphery was similar ($p= 0.21$) and constituted more than 90 % of total time
356 (Table 2).

357

358 Discussion

359

360 1. *Development of an exposure protocol*

361 The set of preliminary experiments aimed at determining optimal conditions of exposure for
362 early life stage sea bass. They revealed that the use of both cell-culture plates (48- and 24-
363 well) is a convenient method providing good results in terms of hatching and survival rates.
364 The OECD guidelines on chemical testing give specific instruction regarding hatching
365 (from 70 to 80 %) and post-hatching success (from 60 to 80 %) for model organisms
366 (OECD 2013). In our condition, the results obtained were largely above the accepted
367 survival and hatching rates, which is in accordance with a previous study performed by
368 Panini et al. (2001), where sea bass hatching success reached 95 % and mean larval survival
369 90 %.

370

371 Culture medium agitation, was shown to be essential for hatching in several organisms as
372 mosquitoes, crabs or fish (Diamond et al. 1995; Griem and Martin 2000; Roberts 2001;
373 Ehlinger and Tankersley 2003; Ebrahimi et al. 2014). In the present study, the effects of
374 agitation did not change the final results with hatching rates >80% under both agitated and
375 non-agitated conditions after 2 days. Agitation was therefore not considered as an essential
376 parameter for the protocol.

377

378 Finally, the frequencies of medium change tested (none, every 24h, every 3.5 days) had no
379 effect on larvae survival. Medium renewal is needed to maintain good environmental
380 conditions, i.e. low concentrations of NO_2^- and an optimal concentration of dissolved
381 oxygen. High levels of NO_2^- can compromise blood oxygen transport and cause hyperplasia,
382 vacuolisation or influence the potassium balance (Kroupova et al. 2005; Yildiz et al. 2006).
383 NO_2^- concentrations as low as 0.5 mg/L were shown to impair fish fitness (Kroupova et al.
384 2005). Regarding oxygen, its depletion can have detrimental effects on growth, behavior,
385 physiological and immune responses (Pichavant et al. 2001; Abdel-Tawwab et al. 2019). For
386 optimal breeding conditions, dissolved O_2 should be maintain near the saturation level
387 (Abdel-Tawwab et al. 2019), which in our conditions (15 °C, 33 psu) corresponds to 7.5
388 mg/L. In our study, O_2 and NO_2^- concentrations were measured each 48 h and average
389 values correspond to 7.38 mg/L and 0.03 mg/L, respectively. These results confirm that 48 h
390 is a suitable time range for renewal of test solutions.

391 Nevertheless, this should be adapted to the chemo-physical properties of the molecule tested
392 in order to prevent fluctuation of test concentrations. Regarding 3,4-DCA, the dominant loss

393 process is photo-transformation, while evaporation, hydrolysis and biodegradation are of
394 minor importance (Wolff and Crossland 1985). Its estimated half-life in surface water under
395 photolysis is of 18 days (Munn et al. 2006). More recently, Philippe et al (2019) measured
396 the degradation of 3,4-DCA throughout a week in freshwater tanks hosting killifish. Results
397 showed a slow degradation, with 3,4-DCA concentrations at day 7 corresponding to half of
398 their initial values (Philippe et al. 2019). In our protocol, a frequency of medium change of
399 48h was chosen to ensure high water quality, limit handling stress and prevent fluctuation of
400 test concentrations.

401

402 To conclude, our results endorse a semi-static experimental design, allowing the use of both
403 24- or 48-well plates. In our protocol, 24-well plates were preferred to increase the number
404 of group replicates and simultaneously reduce the total number of individuals devoted to the
405 tests in accordance with 3R advices.

406

407 *1.1 3,4-DCA exposure and LC₅₀*

408 Once the conditions designed, experiments with the reference molecule 3,4-DCA were
409 performed to define LC_x values. When pooling all three experiments together, the average
410 96 h LC₅₀ obtained corresponds to 2.04 mg/L (95 % interval of confidence: 1.89 - 2.22).

411

412 LC₅₀ experiments were carried out respectively in May, July and November. Even if the
413 management of the reproductive cycle by hormonal induction or manipulation of
414 environmental factors is well established in sea bass (Mylonas and Zohar 2000), offspring
415 fitness may vary between spontaneous and induced spawns. For example, Mañanós et al.
416 (1997) showed a significant reduction in hatching rate and larval survival between spawns
417 from fish maintained under natural conditions and fish maintained under manipulated
418 temperature and photoperiods. In sea bass, the natural spawning season starts in January and
419 ends up in June for the coldest climates (Haffray et al. 2006). In our FEET assays,
420 experiment 1 (i.e. performed in May) showed a higher survival rate if compared to
421 experiments 2 and 3 (performed outside the natural breeding season). Differences in egg's
422 fitness might also arise from genitors' choice in hatcheries. Nevertheless, the confidence
423 interval of the overall 96h LC₅₀ (2.04 mg/L) remains narrow (1.89-2.22), showing good
424 repeatability of our assay despite possible seasonal or genetic influence on larvae fitness.

425

426 96h LC₅₀ in sea bass larvae for 3,4-DCA, appears similar to values obtained in other fish
427 species such as rainbow trout larvae (96 h LC₅₀: 1.94 mg/L; Hodson 1985), perch larvae (96
428 h LC₅₀: 3.1 mg/L; Schäfers and Nagel 1991) and zebrafish larvae (96 h LC₅₀: 2.7 mg/L;
429 Busquet et al. 2014). In a developmental study on rare minnow, 72 h LC₅₀ corresponded to
430 4.1 mg/L for embryos and 1.1 mg/L for larvae (Zhu et al. 2013). These values suggest that
431 larvae are more sensitive to 3,4-DCA exposure than older fishes. Indeed, in juvenile and
432 adult organisms, the reported 96 h LC₅₀ ranged from 6.99 mg/L in juvenile fathead minnow
433 (Call et al. 1987) or 8.5 mg/L in adult zebrafish (Becker et al. 1990) and 2.7 mg/L in adult
434 rainbow trout (Crossland 1988). Adema and Vink (1981) reported 96 h LC₅₀ values for
435 several species including to freshwater young guppy (8.5 to 9 mg/L), seawater young guppy
436 (5 mg/L), adult seawater guppy (3.5 mg/L), adult gobi (2.4 mg/L) and adult European plaice
437 (4.6 mg/L).

438

439 At 168 h (7 days), the average LC₅₀ measured in sea bass larvae dropped down to 0.79
440 mg/L. This is in accordance with the description of 3,4-DCA made by Schäfers and Nagel
441 (1993), who defined it as a molecule of great interest due to its toxicity at low concentration
442 in extended exposures. In perch larvae (96 h LC₅₀: 3.1 mg/L, 6 days LC₅₀: 1.5 mg/L;
443 Schäfers and Nagel 1993), European plaice (96 h LC₅₀: 4.6 mg/L, 7 days LC₅₀: 1.7 mg/L;
444 Adema and Vink 1981), as well as in juvenile guppy (96h LC₅₀: 8.5 mg/L, 14 days LC₅₀: 6.8
445 mg/L; Adema and Vink 1981), a decrease in LC₅₀ was measured after extended exposure.
446 On the other hand, guppy (adult: 96 h LC₅₀: 4.6 mg/L, 7 days LC₅₀: 1.7 mg/L; freshwater
447 young: 96 h LC₅₀: 8.7-9.0 mg/L, 7 days LC₅₀: 8.5-8.2 mg/L; seawater young: 96 h LC₅₀: 5.0
448 mg/L, 7 days LC₅₀: 4.6 mg/L; Adema and Vink 1981), and gobi (96 h LC₅₀: 2.4 mg/L, 7
449 days LC₅₀: 2.2 mg/L; Adema and Vink 1981) showed quite similar LC₅₀ after 96h or 7 days
450 of exposure. Considering the LC₅₀ at day 7, sea bass appears more sensitive to 3,4-DCA
451 exposure than the organisms reported above.

452

453 LC_x values are precious toxicological parameters. Values reported here (96h LC₅₀: 2.04
454 mg/L; 168h LC₅₀: 0.79 mg/L) can be used to assess the success and repeatability of our test
455 protocol.

456

457

1.2 Behavior

458 Quantitative structure-activity analysis by Arnold et al. (1990) suggested that 3,4-DCA
459 might follow a polar narcosis mode of action. Indeed, reduction in locomotor activity was
460 detected in zebrafish exposed at concentrations as low as 0.50 mg/L (Scheil et al. 2009).
461 Nevertheless, our results in sea bass larvae showed that 3,4-DCA exposure did not
462 significantly modulated the behavioral endpoints investigated, such as distance traveled
463 (index of locomotor activity) or zone preference (index of thigmotaxis). This could be a
464 consequence of i) the high individual variation in terms of behavioral responses (Table 2) or
465 ii) the low exposure concentration (0.25 mg/L).

466

467 Only limited literature is available describing the effects of 3,4-DCA on animal behavior. In
468 the freshwater rotifer *Brachionus calyciflorus*, a dramatic decrease in locomotion (as
469 movement cumulative duration) is observed after exposure to high concentrations of 3,4-
470 DCA (80 mg/L) while no effect was detected at lower concentrations (Charoy et al. 1995).
471 In fish, Scheil et al. (2009) observed a decrease in locomotor activity in zebrafish larvae
472 after exposure to 3,4-DCA (0.5 mg/L). The authors concluded that the decrease might be the
473 consequence of the heavy body deformations registered. In another study, using an infrared
474 fast behavioral assay, Bichara et al. (2014) also detected a reduction in swimming activity in
475 zebrafish larvae but in this case, the effect was observed in larvae exposed to high
476 concentrations (8 mg/L) while no effect was detected in fishes exposed at 5 mg/L. Finally, a
477 recent study performed in adult Nile tilapia showed a significant decrease of aggressive
478 behavior in fishes exposed to 80 ng/L of 3,4-DCA (Boscolo et al. 2018). In this study,
479 exposed tilapias also showed variations in testosterone and cortisol plasma levels leading
480 the authors to suggest potential endocrine disrupting actions of the 3,4-DCA (Boscolo et al.
481 2018).

482

483 Regardless the specific results obtained in our study, behavioral tests remain precious tools
484 to investigate sub-lethal effects after chronic exposures (Clotfelter et al. 2004; Murphy et al.
485 2008; Sobanska et al. 2018). The protocol described is rather feasible and easy to run and
486 may elucidate subtle mechanisms of toxicity. In fact, behavioral analyses are suggested by
487 the ECHA as valuable additional endpoints in FET or FEET tests to screen for neurotoxicity
488 or endocrine disruption (Clotfelter et al. 2004; Sobanska et al. 2018). The locomotor

489 activities recorded in this study could serve as a basis for future explorations and
490 identification of specific effects of chemical compounds.

491

492 2. *General consideration for the adoption of a new test*

493 In this paper, we provide a successful example of the use of *Dicentrarchus labrax* in a
494 simple and reliable toxicological test. Future studies including additional molecules should
495 be carried out in order to validate and standardize our test protocol. It is important to
496 consider that the proposed test has many advantages over its inclusion in a battery of tests to
497 assess environmental risks.

498 Firstly, it is obvious that some environments are ignored by the tests currently available.
499 OECD approved species for standardized early life stages tests are rainbow trout, fathead
500 minnow, zebrafish, and medaka for freshwater fish, and sheephead minnow and silverside
501 for estuary and marine fish (OECD 2013). Zebrafish and medaka are both native to warm
502 waters in South Asia, while fathead minnow is native to shallow, weedy lakes in North
503 America (OECD 2013; Parichy 2015). Sheephead minnow and silverside are also warm
504 water fishes native to Central and North America, more representatives of warm swamps
505 and lagoons than open oceans. The only European species endorsed in standardized test is a
506 fresh water fish, the rainbow trout (OECD 2013). None of these species can be an accurate
507 representative of European marine environments.

508

509 Furthermore, it is advisable to include species tolerating different conditions of salinity and
510 temperature, since these two parameters are known to affect pollutants fate in the
511 environment. Salinity is known to potentially modulate the bioavailability of several
512 contaminants such as methylmercury, copper and PAH (Barkay et al. 1997; Ramachandran
513 et al. 2006), while temperature alters degradation rates of pollutants (e.g. PCBs, PCDDs and
514 other POPs) as well as biological responses to contamination (Sinkkonen and Paasivirta
515 2000; Nardi et al. 2017). The European sea bass is a cold-water fish moving from the open
516 sea to estuaries and it is representative of European Atlantic and Mediterranean areas.

517

518 Another advantage of the sea bass lies in the egg type and embryos characteristics. Indeed,
519 when considering early life stage tests, it is obvious that the characteristics of the eggs and
520 larvae highly influence responses to chemical exposure. In a comparative study on fish

521 eggs, large differences in morphology and structure were found between pelagic and
522 demersal eggs (Lønning et al. 1988). Free-floating pelagic eggs, as in sea bass, are
523 characterized by a thin chorion and a rather fast cleavage (Lønning et al. 1988; Siddique et
524 al. 2017). Demersal eggs instead have a thicker complex chorion, while the yolk is
525 characterized by a high lipid content, which allows eggs to hatch at a more advanced stage
526 than pelagic eggs (Lønning et al. 1988). All model fish species recommended by the OECD
527 guidelines lay adhesive, demersal eggs (Marrable 1965; Benoit and Carlson 1977;
528 Middaugh 1981; Raimondo et al. 2009; Naruse et al. 2011; OECD 2013), which give birth
529 to more developed larvae in comparison to pelagic species. Such differences in
530 eleutheroembryo morphology can impact the responses to chemical exposure, supporting
531 the need to consider different species in routine toxicology tests.

532

533 Finally, it is important to mentioned that, in contrast to model organisms, where individuals
534 generally come from selected strains, in the present study eggs were produced in
535 aquaculture facilities. Laboratory strains are specifically selected, and that has an impact on
536 genetic diversity (Allendorf and Phelps 1980; Aho et al. 2006; Suurväli et al. 2020). Instead,
537 eggs provided by aquaculture facilities, in addition to be of easy access, present the
538 advantage to show higher genetic diversity. Therefore, they might be more representative of
539 wild populations.

540

541 **Conclusion**

542

543 Creating new protocols and procedures for introducing native species into routine
544 toxicology tests is a challenge. This study shows the suitability of the European sea bass in
545 an early-life toxicity test. An easy and affordable exposure protocol was developed and
546 tested using 3,4-DCA as reference molecule. Sea bass appears to be as sensitive to 3,4-DCA
547 (96 h LC50) as other common model organisms such as zebrafish, fathead minnow or
548 guppy. A successful behavioral test was performed while no significant behavioral
549 disruption was detected in larvae exposed to a low concentration of 3,4-DCA. Future studies
550 investigating additional molecules would reinforce the use of the European sea bass larvae
551 in standardized toxicity testing.

552

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846 **Tables**

847

848 Table 1: LC_x values at 96 h and 168 h calculated for each experiment separately and combined. Data
 849 are given as estimated values (mg/L) [95 % credible interval]. All data were acquired using
 850 MOSAIC *GUTS-fit*.

Experiment	LC ₁₀		LC ₅₀	
	96 h	168h	96 h	168h
1	3.09 [2.79 ; 3.48]	1.61 [1.47 ; 1.75]	3.87 [3.68 ; ^a]	2.08 [1.97 ; 2.19]
2	0.65 [0.53 ; 0.79]	0.46 [0.37 ; 0.52]	1.57 [1.4 ; 1.78]	0.73 [0.66 ; 0.79]
3	0.39 [0.31 ; 0.461]	0.15 [^a ; 0.20]	1.35 [1.2 ; 1.54]	0.41 [0.36 ; 0.46]
1:3	0.57 [0.48 ; 0.68]	0.22 [0.18 ; 0.26]	2.04 [1.89 ; 2.22]	0.79 [0.73 ; 0.85]

851 ^a - not detectable

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853

854 **Table 2:** Descriptive statistics of behavioral data. Analyses were run after outliers' detection and
 855 elimination. n: group size; IQR: interquartile range

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Endpoint	Group	n	Median [IQR]	Coefficient of variation
<i>Total distance (cm)</i>	control	28	76.15 [55.43-122.0]	48.39 %
	0.25 mg/L	14	107.2 [53.48 - 124.0]	48.92 %
<i>Periphery distance (cm)</i>	control	28	63.50 [46.51 - 110.8]	53.00 %
	0.25 mg/L	14	75.70 [40.95 – 114.3]	50.40 %
<i>Central-zone distance (cm)</i>	control	28	8.54 [6.96 – 17.40]	58.66 %
	0.25 mg/L	14	6.94 [4.45 – 14.66]	81.57 %
<i>Time spent in the peripheral zone (s)</i>	control	28	264.9 [253.2 - 275.9]	17.74 %
	0.25 mg/L	14	274.1 [243 - 286.4]	11.04 %

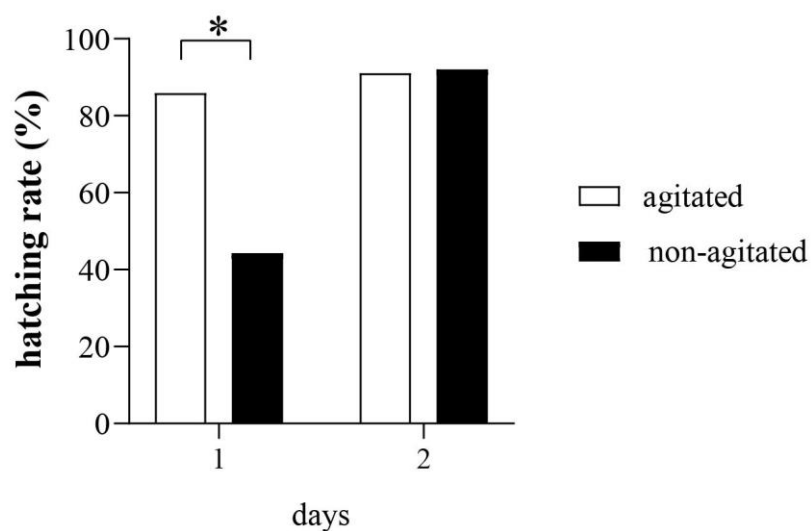
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859 **Figure legends**

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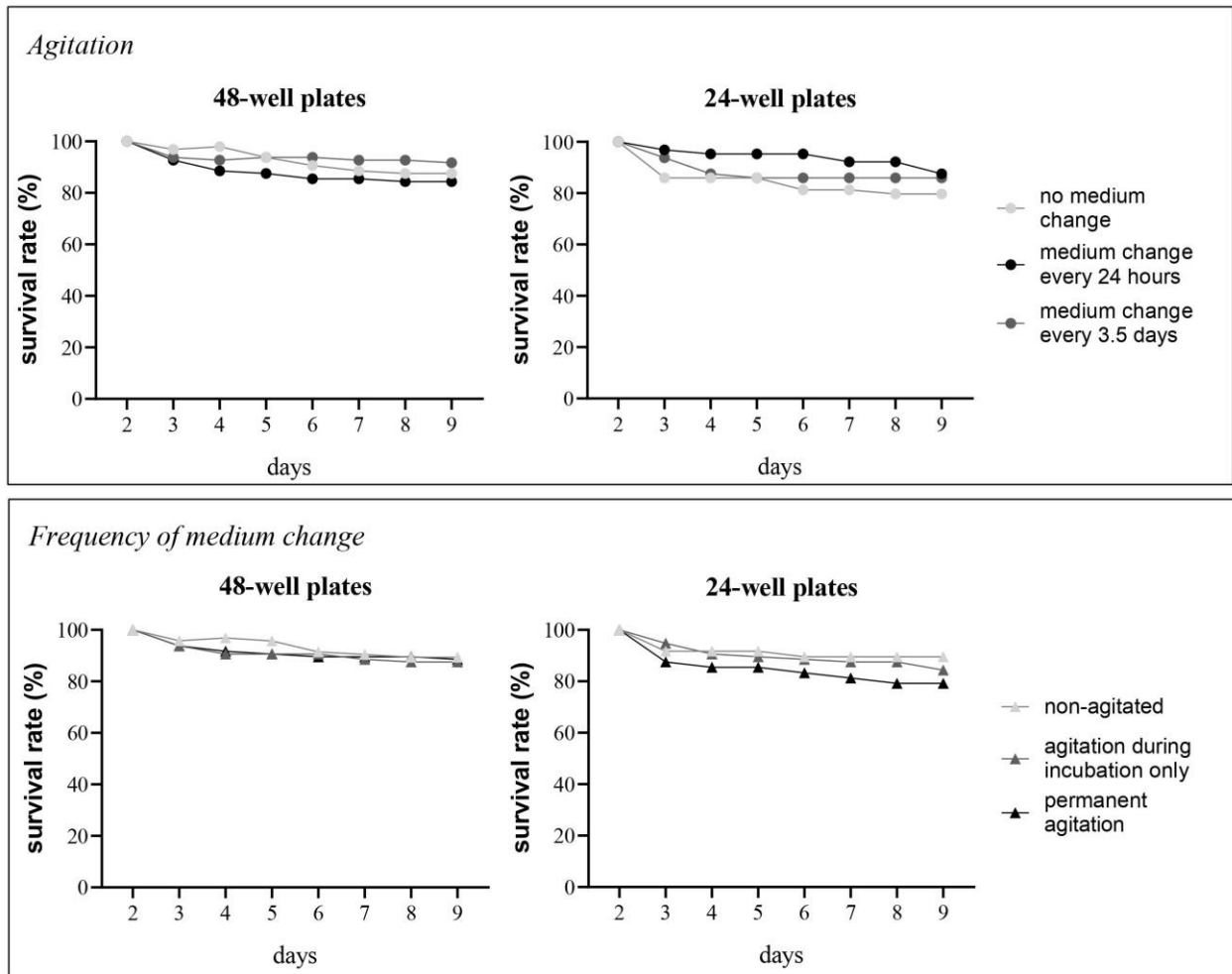
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864 **Fig. 1** Hatching rate under agitated and non-agitated conditions of sea bass eggs (preliminary

865 experiment 1). Values represent the daily percentage of hatched eggs per condition. * = $p <$

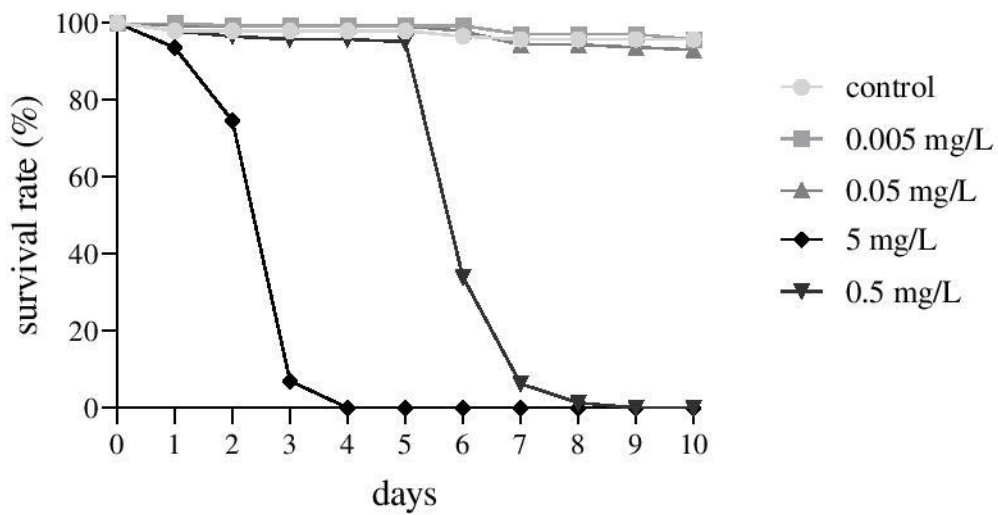
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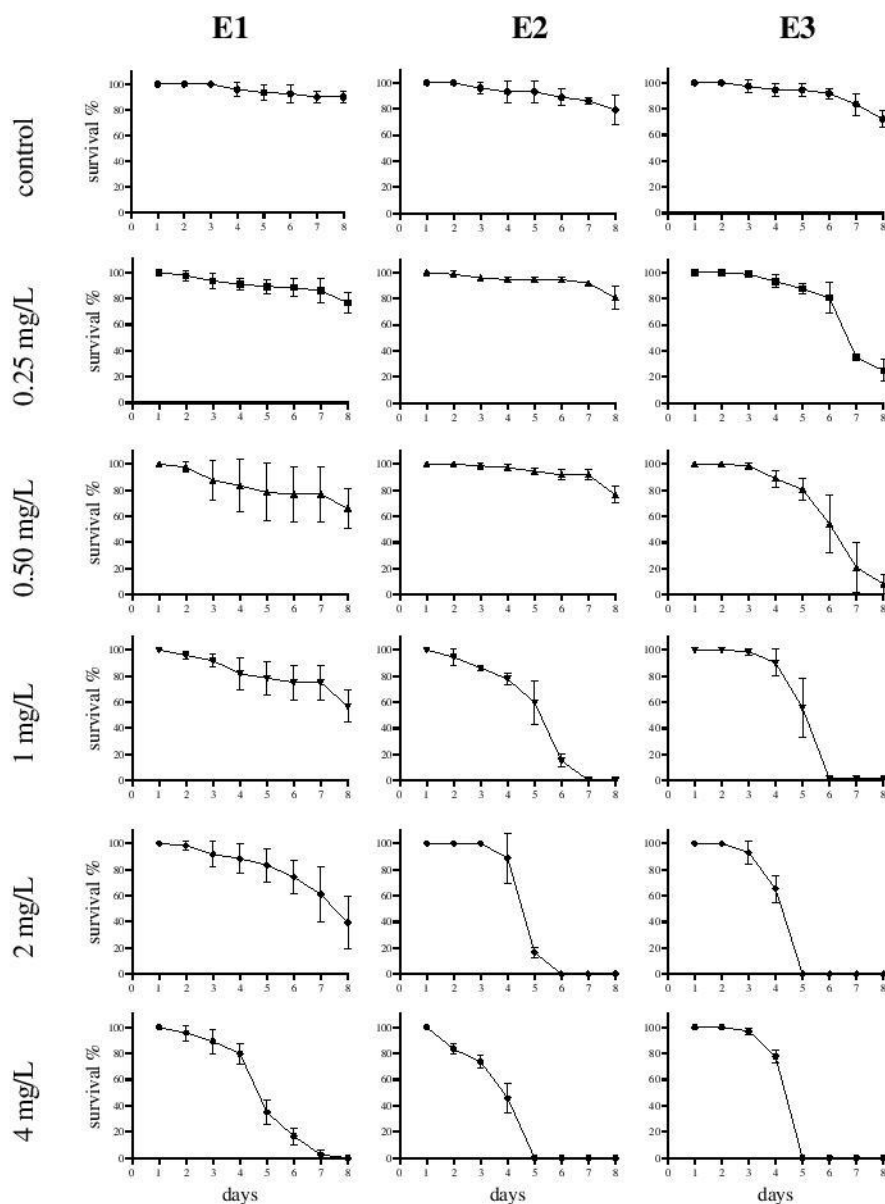
868 **Fig. 2** Survival rate (%) of sea bass larvae placed in 48- and 24-well plates under different
 869 conditions of agitation and medium change frequencies in preliminary experiment 1. Values
 870 represent the daily % of alive larvae per condition from day 2 to day 9.

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873 **Fig. 3** Survival rate (%) of sea bass larvae exposed to different concentrations of 3,4-DCA
 874 concentrations (0.005 – 5 mg/L; preliminary experiment 2). Values are presented as daily
 875 percentage of alive larvae per condition.



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877 **Fig. 4** Survival rate (%) of sea bass larvae exposed to increasing concentrations of 3,4-DCA (0.25 -
 878 4 mg/L). Values are presented as percentage of alive larvae per condition (mean \pm SD). E1=
 879 Experiment 1; E2= Experiment 2; E3= Experiment 3.

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881 Annexes

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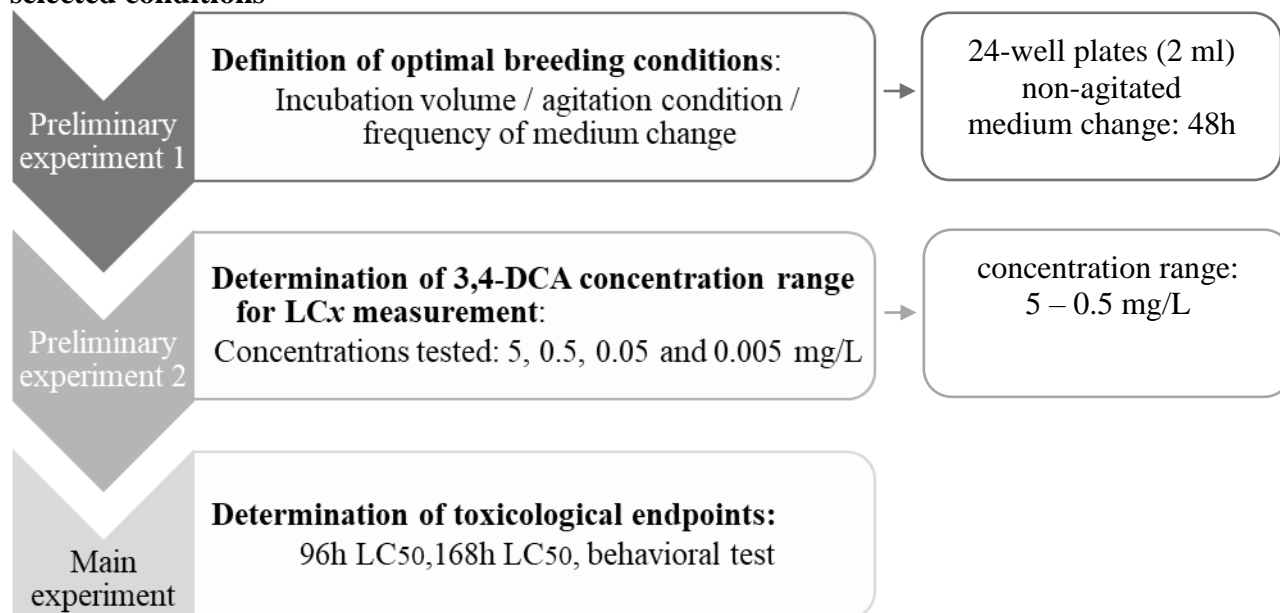
884 **Annex I:** Flow-chart showing all steps implemented in this study to establish an exposure protocol
885 for sea bass eleutheroembryos. At first (preliminary experiment 1) optimal conditions of exposure
886 were identified comparing several factors as different incubation volumes, agitation conditions and
887 frequencies of medium change. Once those factors defined, larvae were exposed to 3,4-DCA as
888 reference molecules. A second experimental step (preliminary experiment 2) was then carried out
889 exposing larvae to a wide range of 3,4-DCA concentrations to detect in which concentration range
890 the 96h and 168h LC₅₀ would fall. Once the concentration range defined, three independents
891 replicated of the established protocol (main experiment) were carried out in two different
892 laboratories to compare 96h and 168h LC₅₀. In the last experiment, behavioral analyses were added
893 as additional toxicological endpoint.

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selected conditions



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900 **Annex II:** Graphical representation of the experimental design for “Preliminary design 1”. To
901 define the best test conditions, sea bass larvae were exposed in either 24- or 48-well plates, in 2 and
902 4 mL of sea water respectively. Each plate was subjected to three different agitation conditions
903 (non-agitated, permanent agitation or agitation during incubation only) and within each plate 3
904 different frequencies of medium change were tested (no change, change every 24h or every 3.5
905 days).

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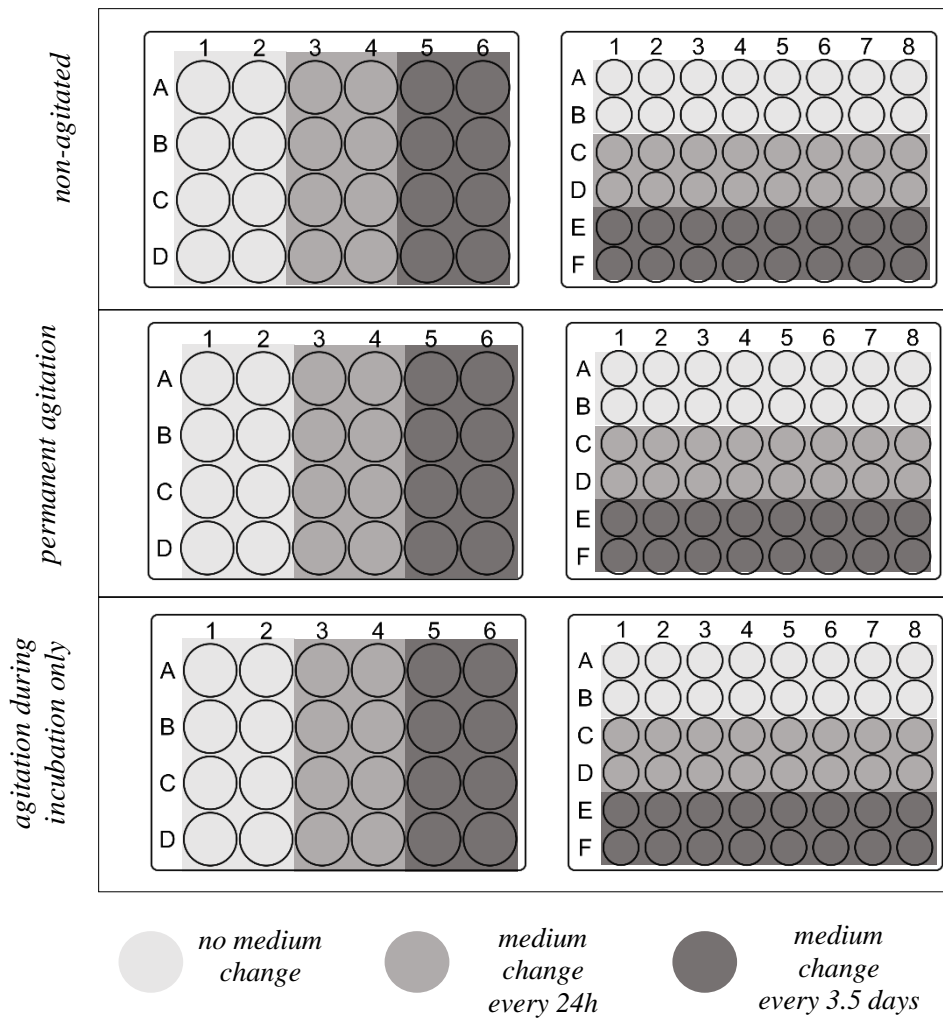
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934 **Annex III:** Description of natural sea water characteristics at the sampling location: Platform
935 6200310, Luc sur mer, France (49.3438;-0.3074). Values are presented as min-max.

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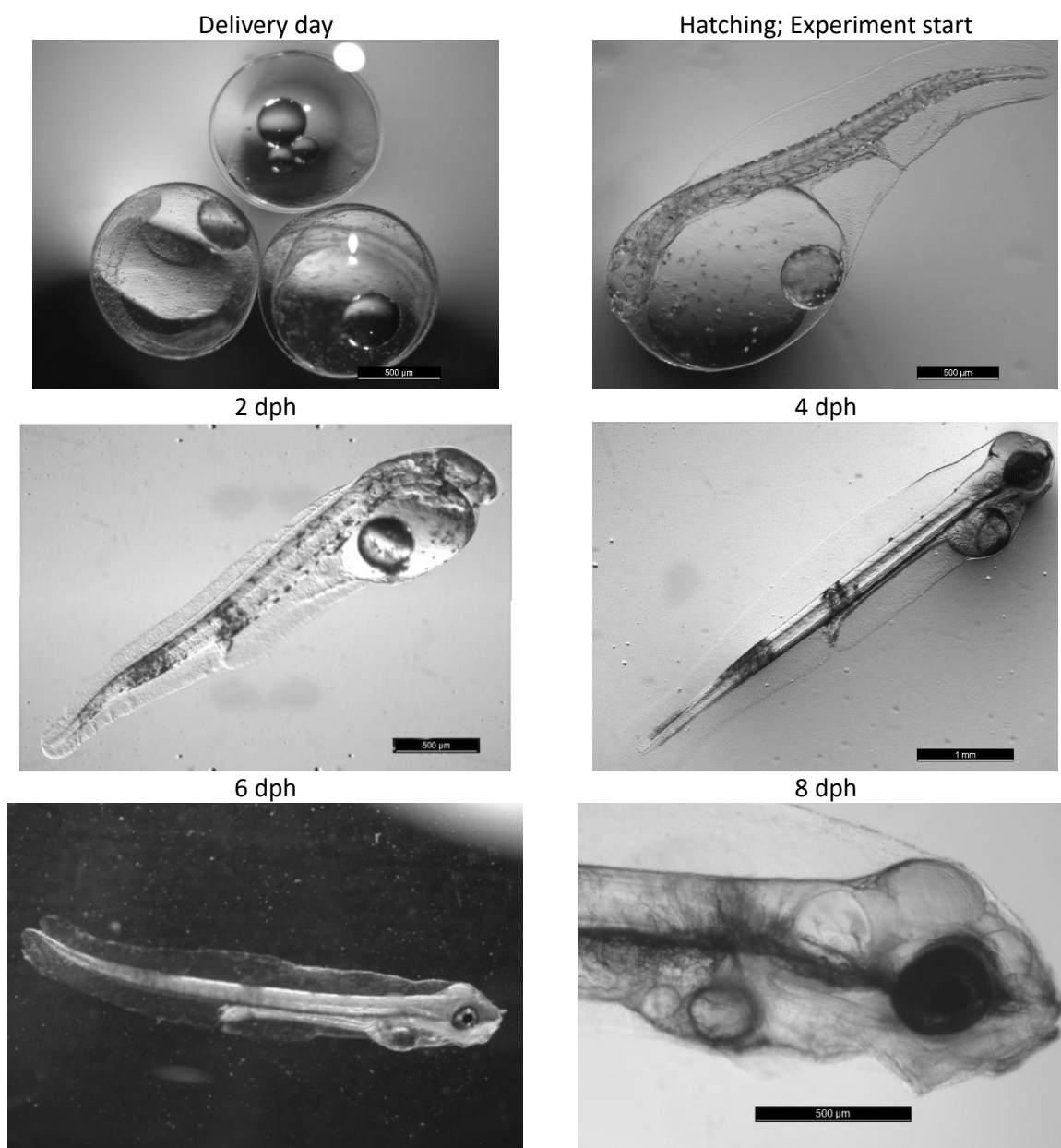
Date	Units	05.2018	07.2018	11.2018
Salinity	<i>psu</i>	28.99 - 32.68	29.97 – 33.35	32.53 – 34.79
Sea temperature	°C	9.68 - 16.82	16.8 – 22.21	10.02 – 13.74
Electrical conductivity	<i>S/m</i>	0.01 - 4.02	4.13 – 4.70	3.65 – 4.05
Dissolved oxygen	<i>ppm</i>	4.68 - 8.99	3.92 – 9.07	5.18 – 5.9
Oxygen saturation	<i>ppm</i>	78.44 - 149.85	74.76 – 150.13	85.61 – 95.58
Potential of hydrogen	<i>pH</i>	7.39 - 7.69	7.19 – 7.92	7.61 – 7.81
Turbidity	<i>NTU</i>	0.01 - 102.95	2.59 – 121.45	0.74 – 24.28

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939 **Annex IV:** Follow-up of larval development from egg's delivery day to 8 dph. Pictures were taken
940 with Leica MZ 10F microscope coupled with a Leica DFC425 C camera. dph: days post hatching

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