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

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Abstract :

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

Keywords : Early-life stage toxicity test, Temperate conditions, Sea bass larvae, 3,4-Dichloroaniline, Behavioral test, 96 h LC50

Introduction

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

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

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

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

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

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

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

Material and Methods

1. *Test species*

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

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

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

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

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

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

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

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

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

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

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

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

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

4. Main Experiments

4.1 LCx determination

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

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

4.2 Behavioral test

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

Recordings were analyzed using a video tracking software (Ethovision XT 10, Noldus). Each well was virtually divided into a central (½ well diameter) and a peripheral zone. An increased time spent in the inner or outer zone (*i.e.* thigmotaxis) would indicate an increased exploratory behavior or anxiety-like behavior, respectively. Behavioral endpoints included distance traveled and time spent in each well-zone.

5. Statistics

Data were computed using R software (version 3.5.1).

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

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

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

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

Results

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

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

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

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

Dissolved oxygen and nitrite levels were measured every 48 h. Average concentrations corresponded to 7.38 mg/L for O_2 and 0.03 mg/L for NO_2^- and no evidence of any detrimental condition was observed.

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

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

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

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

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

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

3. Main Experiments

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

3.1 Experiment 1

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

In this experiment, at 96 h, the LC_{50} and the LC_{10} values obtained for 3,4-DCA were 3.87 mg/L and 3.09 mg/L, respectively (Table 1). At 168 h, the values obtained for LC were: LC_{50}=2.08 mg/L and LC_{10} =1.61 mg/L.

3.2 Experiment 2

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

In this experiment, LC*x* values obtained for 3,4-DCA were: LC_{50} = 1.57 mg/L and LC_{10} = 0.65 mg/L at 96 h; LC_{50} =0.73 mg/L and LC_{10} = 0.46mg/L at 168 h (Table 1).

3.3 Experiment 3

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

LC*x* values obtained for 3,4-DCA were LC_{50} = 1.35 mg/L and LC_{10} = 0.39mg/L at 96 h; LC_{50} =0.41mg/L and LC_{10} = 0.15mg/L at 168 h (Table 1).

3.4 Behavioral test

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

Discussion

1. *Development of an exposure protocol*

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

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

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

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

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

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

1.1 3,4-DCA exposure and LC_{50}

Once the conditions designed, experiments with the reference molecule 3,4-DCA were performed to define LC*x* values. When pooling all three experiments together, the average 96 h LC_{50} obtained corresponds to 2.04 mg/L (95 % interval of confidence: 1.89 - 2.22).

LC_{50} experiments were carried out respectively in May, July and November. Even if the management of the reproductive cycle by hormonal induction or manipulation of environmental factors is well established in sea bass (Mylonas and Zohar 2000), offspring fitness may vary between spontaneous and induced spawns. For example, Mañanós et al. (1997) showed a significant reduction in hatching rate and larval survival between spawns from fish maintained under natural conditions and fish maintained under manipulated temperature and photoperiods. In sea bass, the natural spawning season starts in January and ends up in June for the coldest climates (Haffray et al. 2006). In our FEET assays, experiment 1 (i.e. performed in May) showed a higher survival rate if compared to experiments 2 and 3 (performed outside the natural breeding season). Differences in egg's fitness might also arise from genitors' choice in hatcheries. Nevertheless, the confidence interval of the overall 96h LC_{50} (2.04 mg/L) remains narrow (1.89-2.22), showing good repeatability of our assay despite possible seasonal or genetic influence on larvae fitness.

96h LC_{50} in sea bass larvae for 3,4-DCA, appears similar to values obtained in other fish species such as rainbow trout larvae (96 h LC_{50}: 1.94 mg/L; Hodson 1985), perch larvae (96 h LC_{50}: 3.1 mg/L; Schäfers and Nagel 1991) and zebrafish larvae (96 h LC_{50}: 2.7 mg/L; Busquet et al. 2014). In a developmental study on rare minnow, 72 h LC_{50} corresponded to 4.1 mg/L for embryos and 1.1 mg/L for larvae (Zhu et al. 2013). These values suggest that larvae are more sensitive to 3,4-DCA exposure than older fishes. Indeed, in juvenile and adult organisms, the reported 96 h LC_{50} ranged from 6.99 mg/L in juvenile fathead minnow (Call et al. 1987) or 8.5 mg/L in adult zebrafish (Becker et al. 1990) and 2.7 mg/L in adult rainbow trout (Crossland 1988). Adema and Vink (1981) reported 96 h LC_{50} values for several species including to freshwater young guppy (8.5 to 9 mg/L), seawater young guppy (5 mg/L), adult seawater guppy (3.5 mg/L), adult gobi (2.4 mg/L) and adult European plaice (4.6 mg/L).

At 168 h (7 days), the average LC_{50} measured in sea bass larvae dropped down to 0.79 mg/L. This is in accordance with the description of 3,4-DCA made by Schäfers and Nagel (1993), who defined it as a molecule of great interest due to its toxicity at low concentration in extended exposures. In perch larvae (96 h LC_{50}: 3.1 mg/L, 6 days LC_{50}: 1.5 mg/L; Schäfers and Nagel 1993), European plaice (96 h LC_{50}: 4.6 mg/L, 7 days LC_{50}: 1.7 mg/L; Adema and Vink 1981), as well as in juvenile guppy (96h LC_{50}: 8.5 mg/L, 14 days LC_{50}: 6.8 mg/L; Adema and Vink 1981), a decrease in LC_{50} was measured after extended exposure. On the other hand, guppy (adult: 96 h LC_{50}: 4.6 mg/L, 7 days LC_{50}: 1.7 mg/L; freshwater young: 96 h LC_{50}: 8.7-9.0 mg/L, 7 days LC_{50}: 8.5-8.2 mg/L; seawater young: 96 h LC_{50}: 5.0 mg/L, 7 days LC_{50}: 4.6 mg/L; Adema and Vink 1981), and gobi (96 h LC_{50}: 2.4 mg/L, 7 days LC_{50}: 2.2 mg/L; Adema and Vink 1981) showed quite similar LC_{50} after 96h or 7 days of exposure. Considering the LC_{50} at day 7, sea bass appears more sensitive to 3,4-DCA exposure than the organisms reported above.

LC_x values are precious toxicological parameters. Values reported here (96h LC_{50}: 2.04 mg/L; 168h LC_{50}: 0.79 mg/L) can be used to assess the success and repeatability of our test protocol.

1.2 Behavior

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

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

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

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

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

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

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

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

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

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

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

Conclusion

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

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Tables

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

	LC_{10}		LC_{50}	
Experiment	**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]

[a] - not detectable

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

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

Figure legends

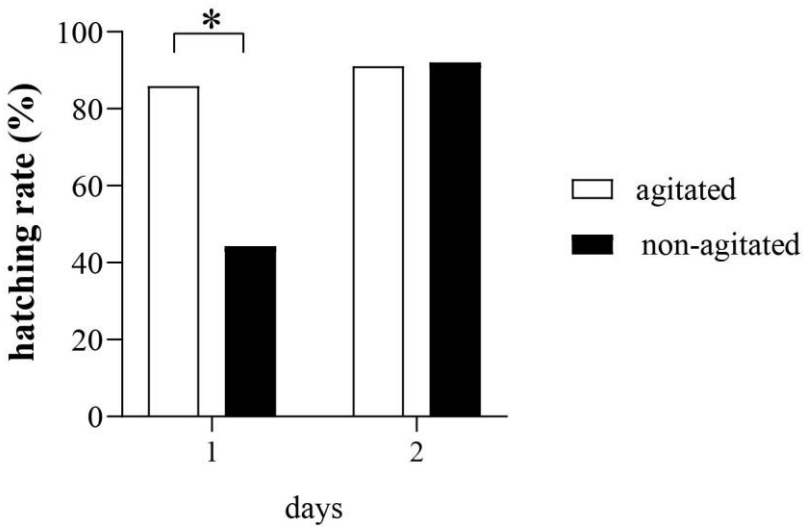
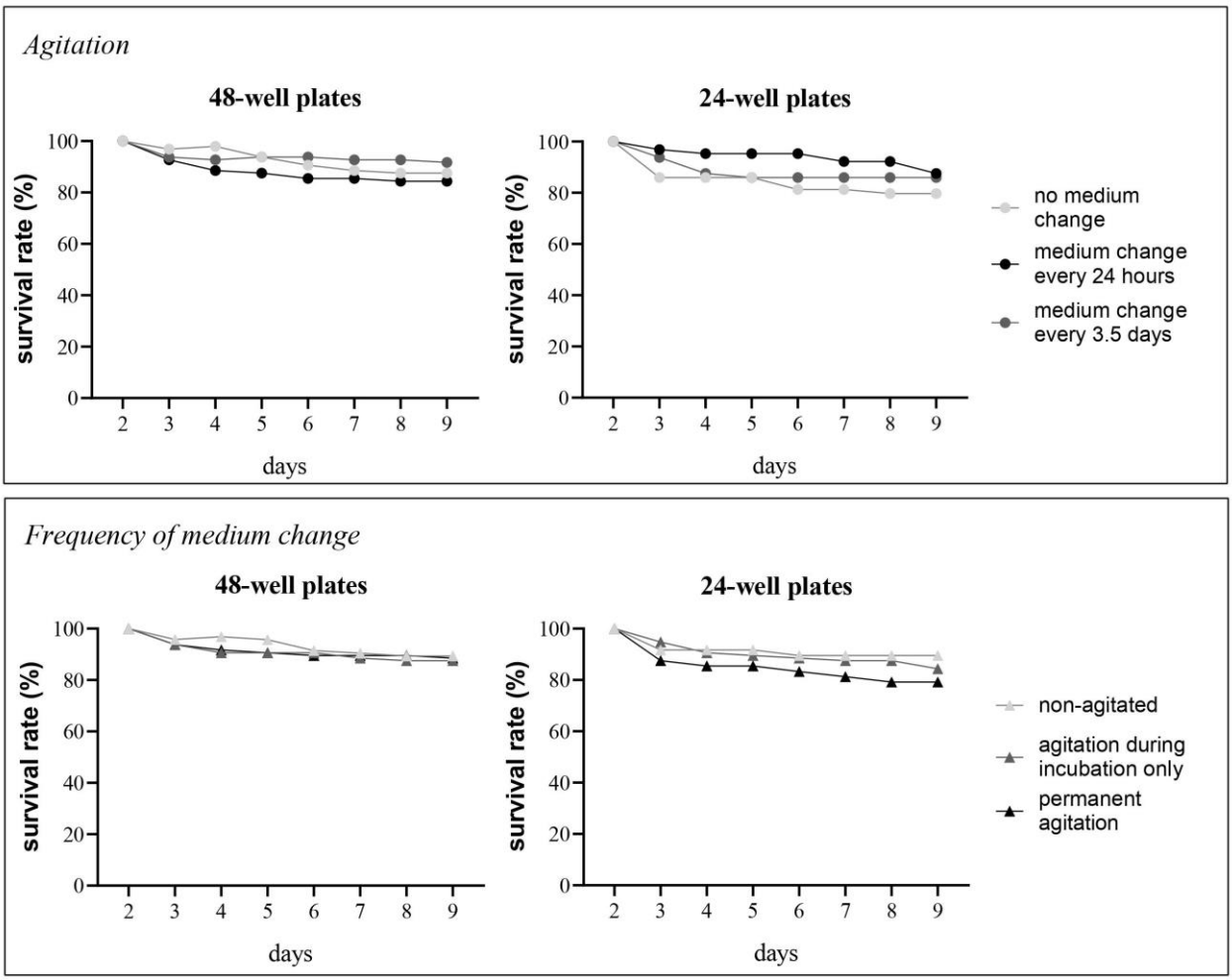


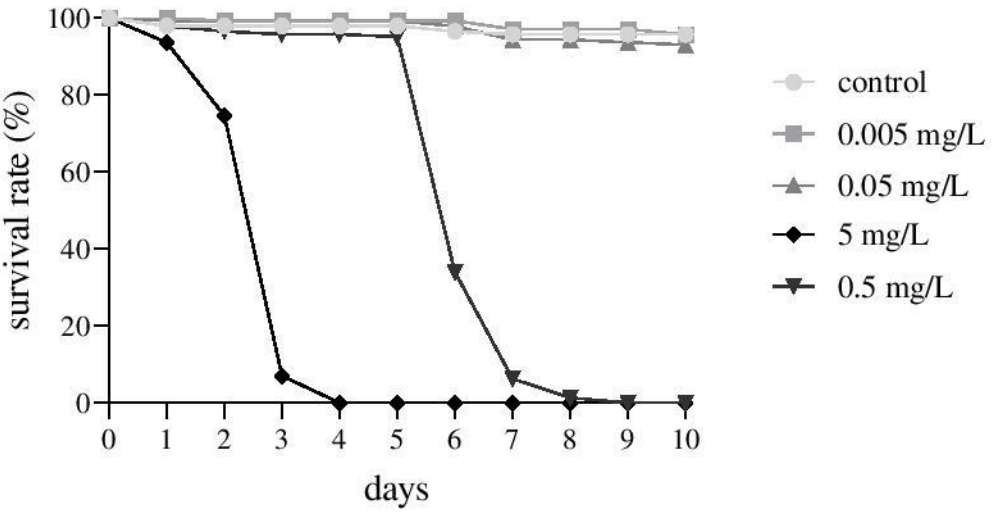
Fig. 1 Hatching rate under agitated and non-agitated conditions of sea bass eggs (preliminary experiment 1). Values represent the daily percentage of hatched eggs per condition. * = $p < 0.001$



867

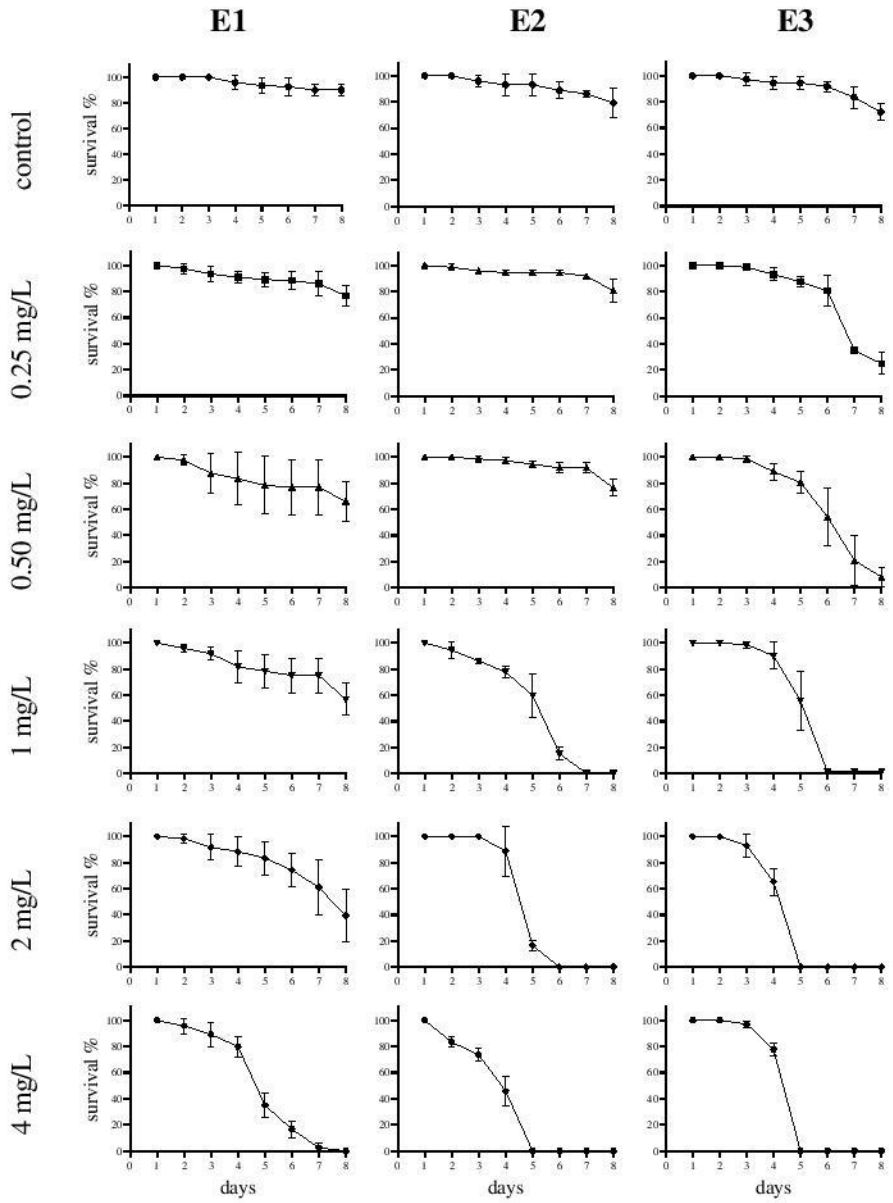
868 **Fig. 2** Survival rate (%) of sea bass larvae placed in 48- and 24-well plates under different
869 conditions of agitation and medium change frequencies in preliminary experiment 1. Values
870 represent the daily % of alive larvae per condition from day 2 to day 9.

871



872

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.



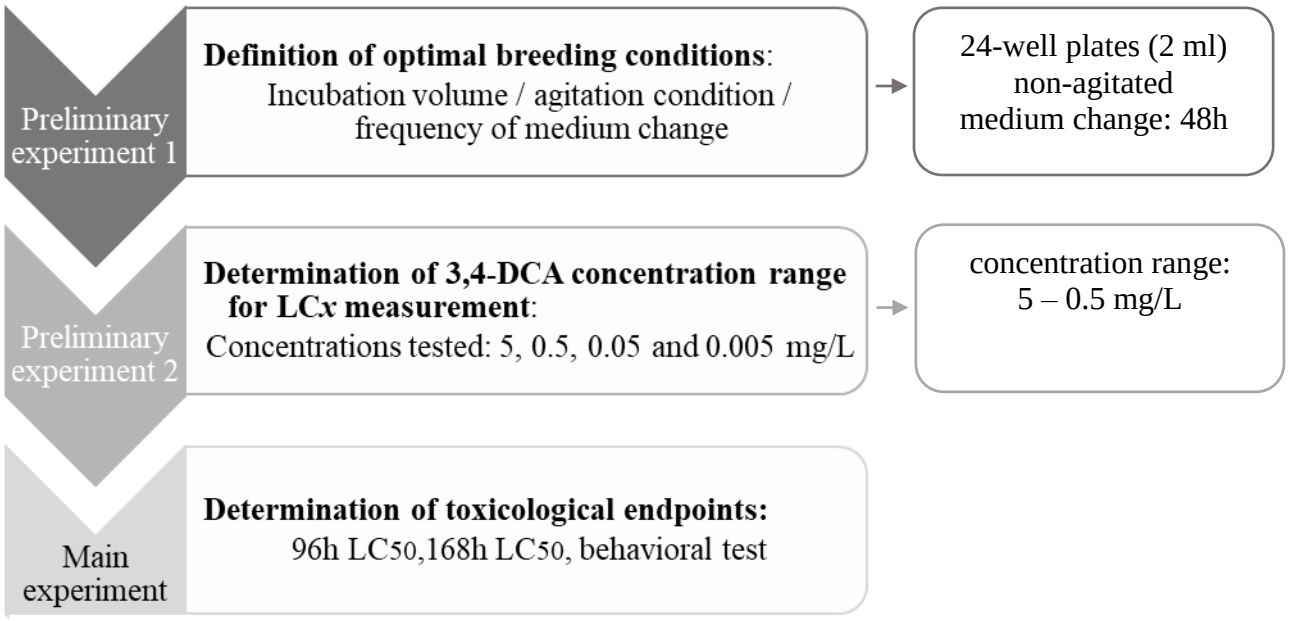
876

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 ± SD). E1=
879 Experiment 1; E2= Experiment 2; E3= Experiment 3.

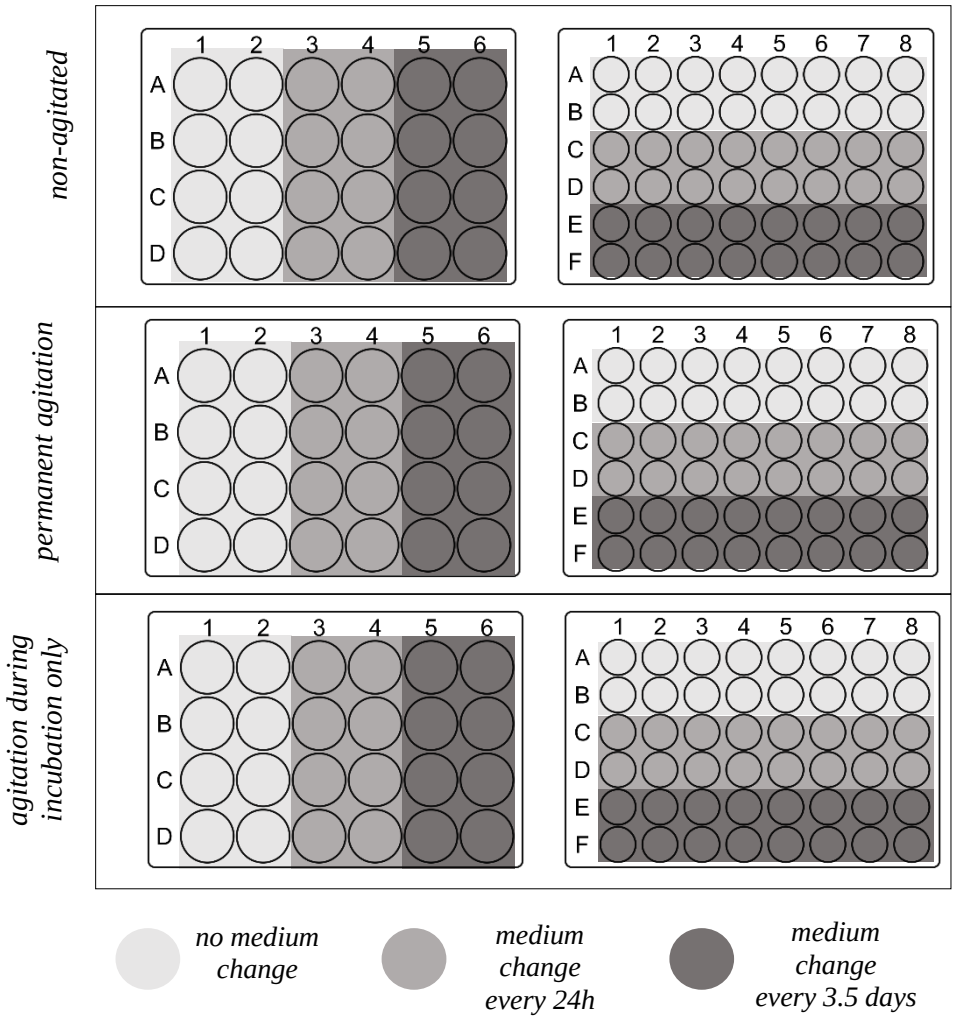
880

Annexes

Annex I: Flow-chart showing all steps implemented in this study to establish an exposure protocol for sea bass eleutheroembryos. At first (preliminary experiment 1) optimal conditions of exposure were identified comparing several factors as different incubation volumes, agitation conditions and frequencies of medium change. Once those factors defined, larvae were exposed to 3,4-DCA as reference molecules. A second experimental step (preliminary experiment 2) was then carried out exposing larvae to a wide range of 3,4-DCA concentrations to detect in which concentration rage the 96h and 168h LC_{50} would fall. Once the concentration range defined, three independents replicated of the established protocol (main experiment) were carried out in two different laboratories to compare 96h and 168h LC_{50}. In the last experiment, behavioral analyses were added as additional toxicological endpoint.



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



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

Date	**Units**	**05.2018**	**07.2018**	**11.2018**
Salinity	*psu*	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	*S/m*	0.01 - 4.02	4.13 – 4.70	3.65 – 4.05
Dissolved oxygen	*ppm*	4.68 - 8.99	3.92 – 9.07	5.18 – 5.9
Oxygen saturation	*ppm*	78.44 - 149.85	74.76 – 150.13	85.61 – 95.58
Potential of hydrogen	*pH*	7.39 - 7.69	7.19 – 7.92	7.61 – 7.81
Turbidity	*NTU*	0.01 - 102.95	2.59 – 121.45	0.74 – 24.28

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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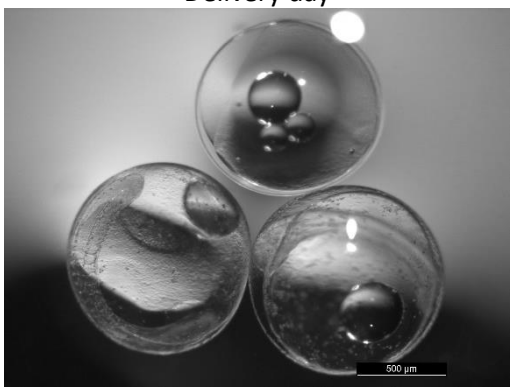
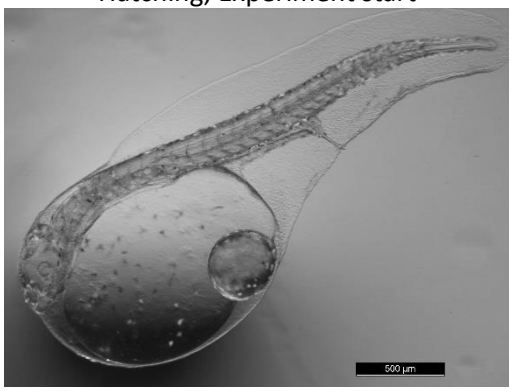
942

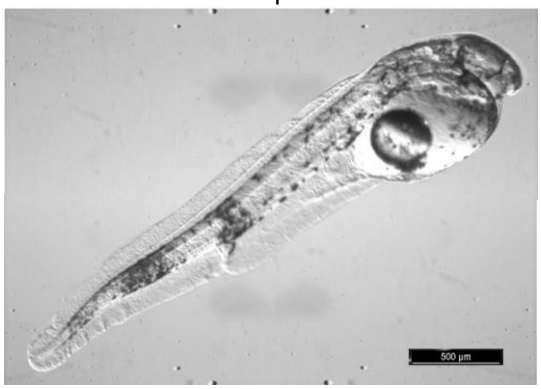
Delivery day



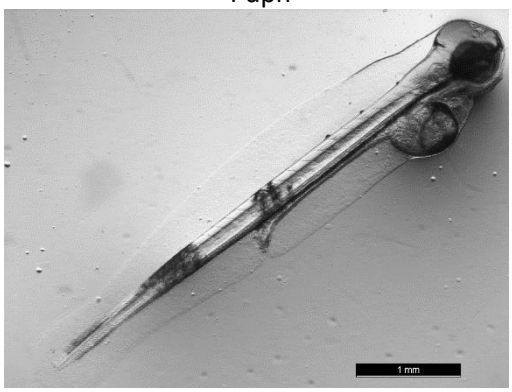
Hatching; Experiment start



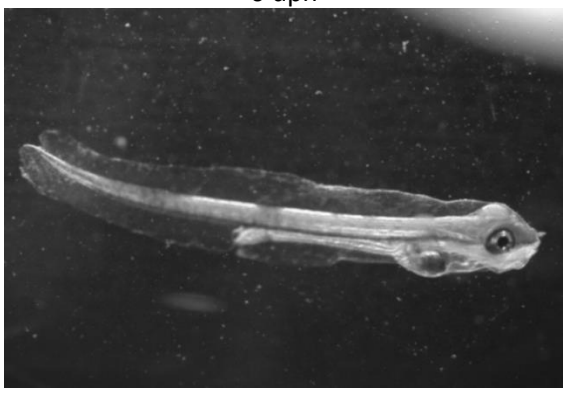
2 dph



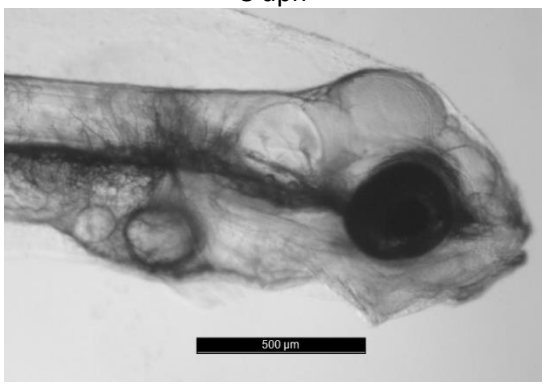
4 dph



6 dph



8 dph



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