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1 **Establishing Bacterial Infectivity Models in Striped Catfish *Pangasianodon***  
2 ***hypophthalmus* (Sauvage) with *Edwardsiella ictaluri***

3 Running Title: Bacterial Infectivity Models in striped catfish.

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10 **Conflict of interest statement**

11 All authors approved the manuscript, this submission and declared no known conflicts  
12 of interest associated with this publication.

13 **Acknowledgement**

14 The authors would like to thank the Ministry of Education and Training, Vietnam who  
15 provided the funding for the study. The authors would like to thank Prof. Barbara  
16 Nowak, University of Tasmania, Australia and Endeavour Fellowship and Scholarship  
17 (Australia) for assistance with editing.

18 **Data Availability Statement**

19 The data that support the findings of this study are available from the corresponding  
20 author upon reasonable request.

## 21 **Abstract**

22 A bacterial infectivity challenge model of *Edwardsiella ictaluri* in striped catfish was  
23 developed. All experiments were conducted using a bacterial isolate of *Edwardsiella*  
24 *ictaluri* that had been recovered during a natural outbreak of Bacillary Necrosis of  
25 Pangasianodon (BNP) in farmed striped catfish *Pangasianodon hypophthalmus* in  
26 Vietnam. Time of immersion in  $10^7$  CFU.ml<sup>-1</sup> had significant effect on mortality. The  
27 immersion bacterial dose of  $10^7$  CFU ml<sup>-1</sup> for 30 s resulted in a cumulative percentage  
28 mortality of 63%. Three to 4 days post-bacterial challenge, fish showed gross clinical  
29 signs of natural BNP and *E. ictaluri* was recovered and identified from these fish.  
30 Moreover, a cohabitation challenge was evaluated as an alternative challenge method,  
31 although the mortalities among the infected fish were lower at around 15-40%. This  
32 study confirmed the horizontal transmission of *E. ictaluri* in striped catfish and  
33 elucidated that cohabitation challenge could be used in reproducing the disease under  
34 controlled conditions.

35 **Keywords:** *Pangasianodon hypophthalmus*, *Edwardsiella ictaluri*, Bacillary Necrosis of  
36 *Pangasianodon*, immersion challenge, cohabitation challenge

## 37 **1. INTRODUCTION**

38 Bacillary necrosis of Pangasianodon (BNP), one of the most serious diseases of striped  
39 catfish in Vietnam. It was first described in 2001 (Ferguson et al., 2001) and *E. ictaluri*  
40 was identified as the causative agent in 2002 (Crumlish, Dung, Turnbull, Ngoc, &  
41 Ferguson, 2002) and aetiology confirmed through experimental studies in 2010  
42 (Crumlish, Thanh, Koesling, Tung, & Gravningen, 2010). Affected farms in Vietnam

43 reported 50-90% mortality during a natural outbreak (Dung, Crumlish, Ngoc, Tinh, &  
44 Thy, 2004).

45 Over the last 20 years the farmed Vietnamese striped catfish (*Pangasianodon*  
46 *hypophthalmus*) has increased significantly and in 2018, over 1.4 million tonnes of  
47 catfish were farmed and sold globally (VASEP, 2019). Bacterial disease outbreaks due to  
48 *Edwardsiella ictaluri* continue to be one of the biggest threats to the sector (Phu,  
49 Phuong, Scippo, & Dalsgaard, 2015), however the lack of alternatives to fish infectivity  
50 models in aquaculture, there remains a reliance on the use of fish experiments to  
51 understand pathogenesis and evaluate treatment and prevention strategies for bacterial  
52 diseases. Such models have been established and tested for *E. ictaluri* in non-Pangasius  
53 species with varying degrees of success (Iwanowicz, Griffin, Cartwright, & Blazer, 2006;  
54 Pasnik, Evans, & Klesius, 2007; Tinh et al., 2009).

55 Performing *in vivo* bacterial challenge studies for fish species under experimental  
56 conditions is difficult to standardise between studies (Nordmo & Ramstad, 1997;  
57 Nordmo, Sevatdal & Ramstad, 1997). This is often due to variation in strain  
58 pathogenicity, concentration, exposure route of the pathogen and consideration must  
59 be given to the variation in the age, size and species of the fish host. All of these factors  
60 heavily influence the expected outcome of clinical signs of disease and morbidity similar  
61 to those experience in natural infections (Crumlish et al., 2010; Tinh et al., 2009).

62 Pathogen exposure methods in fish include injection, oral, hyperosmotic immersion,  
63 direct immersion, and cohabitation (Bell et al. 1984; Elliott et al. 1991), with injection  
64 being the most widely adopted method used in aquaculture. Pathogen exposure  
65 through injection remains the most favoured transmission route as it allows exact dose  
66 per fish to be known and reduced variation between individual fish. Immersion

67 (McCarthy et al. 1984; Nordmo et al., 1997) and cohabitation studies (Bell, Higgs, &  
68 Traxler, 1984, Nordmo et al, 1997) have shown promise as pathogen exposure routes as  
69 they require less handling and represent a more natural route of pathogen entry than  
70 injection. However, these methods are often more difficult to control and to  
71 standardise (Aoki, Kondo, Kawai, & Oshima, 2005; Nordmo & Ramstad, 1997) because it  
72 is difficult to know the individual uptake per fish and therefore the variation is larger which does  
73 actually mimic better than natural infection. Therefore, it requires longer exposure times to  
74 the pathogen, which can result in poor reproducibility between experimental studies,  
75 even using the same pathogen. Very little work has been done to standardize *in vivo*  
76 challenge tests using non-injection exposure routes generally in aquaculture but  
77 specifically with *E. ictaluri*. A robust and reliable challenge model is required for  
78 infectivity studies of *E. ictaluri* in *P. hypophthalmus* to determine changes in  
79 pathogenicity and host susceptibility as well as refinement of prevention and treatment  
80 strategies against infection. The aim of this study was to refine an immersion and co-  
81 habitation challenge model for *E. ictaluri* infection in striped catfish, performed under  
82 experimental conditions, to provide improved options when studying aquatic  
83 pathogenesis, infectivity and treatments.

## 84 **2. MATERIALS AND METHODS**

### 85 **2.1 Fish**

86 The fish used for the experimental studies were obtained from a stock population held  
87 in the Aquaculture Research Facility (ARF), University of Stirling. These fish were  
88 purchased from a farm in central Thailand and had been health certified as free from  
89 BNP from the Department of Fisheries (DOF) Thailand prior to shipment to the UK. The  
90 fish were maintained in 200L fibreglass tanks at  $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$ , and fed a commercial

91 salmonid diet (Skretting, Norway). In total for the challenge experiments, 10 fish per  
92 treatment group were allocated to 100L tanks with an average weight of  $15 \pm 2$  g. The  
93 fish were starved for 24h prior to pathogen exposure.

## 94 **2.2 Bacterial strain**

95 A bacterial strain of *E. ictaluri* recovered from a natural outbreak of BNP in Vietnamese  
96 *P. hypophthalmus* was used for all challenge studies. This isolate was identified as *E.*  
97 *ictaluri* following the primary identification tests and biochemical profiles described in  
98 Crumlish et al., (2002). A species-specific polymerase chain reaction (PCR) targeting to  
99 the upstream region of the fimbrial gene was performed for rapid identification of *E.*  
100 *ictaluri* following the methods of Sakai, Yuasa, Sano, & Iida, (2009)

## 101 **2.3 Bacterial challenge study**

102 Prior to performing the challenge experiments, the *E. ictaluri* strain was passaged  
103 through naive fish, twice. The bacterial suspension was grown in Tryptone Soya Broth  
104 (TSB, Oxoid, England) at 28°C, centrifuged and re-suspended in sterile 0.85% NaCl water  
105 to give a high bacterial concentration. One hundred microliters of the suspension was  
106 then injected by intraperitoneal injection (i.p.) into each fish and recovered from  
107 moribund/dead fish directly from the spleen and kidney onto Tryptone Soya Agar (TSA,  
108 Oxoid UK). This procedure was repeated twice, and the identification of the isolate  
109 recovered from the fish was confirmed as described above and then used for the  
110 subsequent challenge experiments. This is called bacterial passage with the purpose was  
111 to enhance virulence of the pathogen post-storage.

112 The challenge inoculum was produced by adding 3-5 colonies of pure *E. ictaluri* isolate  
113 (ex-passage 2) grown on TSA into 50 ml of sterile Tryptone Soya Broth (TSB, Oxoid UK).  
114 This was then incubated to mid logarithmic phase (140 rpm, 28°C) in a shaking incubator

115 (Kuhner shaker, ISF-1-W, Switzerland). After 24h, the bacterial broth suspension was  
116 centrifuged at 3,500 rpm (Sanyo NSE Mistral 2000R, Japan) and the cell pellet re-  
117 suspended and adjusted to give an optical density (OD<sub>600nm</sub>) value of 1 using 0.85%  
118 sterile saline. The viable colony counts were performed using the Miles and Misra  
119 method (Miles et al. 1938) and then 10-fold serial dilutions performed to give  
120 approximately  $1 \times 10^7$  cfu mL<sup>-1</sup> for the challenge studies.

121 To determine the immersion exposure time a pilot study was performed using 5  
122 treatment groups with n=10 fish per group and in treatment groups 1-6, all fish were  
123 exposed to a single concentration of  $1 \times 10^7$  cfu mL<sup>-1</sup> for either 1, 2, 5, 10, 15 and 30  
124 minutes. The control fish group was not exposed to the bacteria but instead the same  
125 volume of sterile saline was added to the tank and fish exposed for 30 min before being  
126 transferred to their original tanks.

#### 127 **2.4 Challenge experimental design**

128 From the immersion pilot study results, a second immersion challenge was performed  
129 with more refined bacterial pathogen exposure time (Table 1). In the second study, 4  
130 treatment groups with 3 replicate tanks per treatment group each containing 10 fish per  
131 tank (Table 2). Fish in treatment groups 1-3 were exposed to a single concentration of *E.*  
132 *ictaluri* at approximately  $1 \times 10^7$  cfu mL<sup>-1</sup> for 30 seconds, 1 minute or 2 minute duration  
133 (Table 2).

#### 134 **2.5 Cohabitation experimental design**

135 Co-habitation studies are considered the most natural route of bacterial exposure.  
136 Under experimental condition, this requires the introduction of an infected “seed” fish  
137 which is then co-habited with the naive fish. All seed fish in this study were identifiable

138 from the naïve fish by removing the adipose fin. The experimental studies and designs  
139 are described in Table 2. Briefly, each tank has 1 “seed” fish and 9 naïve fish. There were  
140 2 treatment groups, Treatment group 1a had the “seed” fish exposed to the *E. ictaluri* by  
141 i.p injection and then placed with the naïve fish in the same tank. The control tank  
142 (Treatment group 1b) for this exposure route had the “seed” fish given 0.85% sterile  
143 saline by i.p. injection (control). In Treatment group 2a the “seed” fish was exposed to  
144 the *E. ictaluri* by immersion for 15 min and then added to the naïve fish whereas the  
145 control Treatment group 2b, the “seed” fish was not exposed to *E. ictaluri* but to same  
146 volume of sterile 0.85% sterile saline for 15 min.

147 Water temperature was  $26 \pm 2^\circ\text{C}$  and the duration of the study was 15 days for all of the  
148 challenge studies. Water was aerated using an air stone and the fish were fed *ad*  
149 *libitum*. The water temperature and mortality/morbidity was checked and recorded 4  
150 times per day as per standard practise within the University of Stirling Aquarium  
151 Facilities. Moribund and freshly dead fish were necropsied and examined grossly for any  
152 external and internal clinical signs of disease. Bacterial samples were aseptically taken  
153 from the kidney and spleen of each fish onto TSA plates, incubated at  $28^\circ\text{C}$ . These were  
154 checked daily for a maximum of 7 days for bacterial growth and purity. At the end of the  
155 challenge period, 50% of all surviving fish per treatment group were removed and  
156 examined for gross clinical signs of disease and sampled for bacteria culture as  
157 described above.

#### 158 **Ethics statement**

159 All experiments were conducted with the approval of the University of Stirling Ethics  
160 Committee and performed under Home Office Licence 60/3949.

161 All experimental protocols were adopted in this study in accordance with the UK  
162 legislation under the Animals (Scientific Procedures) Act 1986 Amendment Regulations  
163 (SI 2012/3039).

## 164 **2.6 Statistical analysis**

165 Parametric assumptions were checked using Levene's test for homogeneity of variances  
166 and Shapiro-Wilk's test for normality. The samples with homogenous variances were  
167 analyzed using ANOVA followed by Duncan test, while Dunnett's T3 test was used for  
168 the samples with unequal variances. As data were normal-distributed and  
169 homoscedastic, the cumulative percentage mortalities between treatment groups were  
170 compared by using one-way ANOVA, followed by the Duncan test. All the tests were  
171 performed using the SPSS program release 17.5. Differences were considered  
172 statistically significant if  $p < 0.05$ .

## 173 **3. RESULTS**

### 174 **3.1 Cumulative Percentage Mortality in Fish Exposed by Immersion Route (Pilot Study)**

175 Mortalities were observed in all fish groups receiving the bacteria by immersion for all  
176 exposure times (Fig. 1). The mortality curves were similar for each of the treatment  
177 group exposed to the *E. ictaluri*, with the highest total cumulative mortalities (100%)  
178 found in the treatment groups that had been exposed to the bacteria for 5 min or  
179 longer.

180 In the second immersion challenge study the mortality curves were again similar for all  
181 treatment groups (Fig. 2). The longer the exposure time the higher the level of mortality  
182 in the treatment group. The reduction in the exposure time in study 2 shows that  
183 shorter exposure time provide better refinement of the infection process under  
184 experimental conditions. The first mortality occurred at day 3 within the group exposed



185 for 2 min (Fig. 2) and the second mortality was observed in the treatment group  
186 exposed for 1 min at day 4 post bacterial challenge. From day 5 post-bacterial exposure  
187 the mortalities occurred in all treatment groups except the control (Fig. 2). The highest  
188 percentage cumulative mortality (100%) was found in the treatment group exposed to  
189 the bacteria for the longest duration (2 min, Fig. 2).

190 By the end of this experiment, the cumulative mortality was highest in the group  
191 exposed to bacteria for 2 min and was significantly higher than the 1 min immersion  
192 group ( $p = 0.024$ ) and treatment group exposed for 30s ( $p= 0.001$ ). The end-point  
193 mortality (63%) was found in groups that had been exposed to the bacteria for 30s (Fig.  
194 2).

### 195 **3.2 Cumulative percentage mortality in cohabitation experiment**

196 A significantly higher cumulative percentage mortality was observed in the treatment  
197 group (1b) where the seed fish was injected with the bacteria prior to cohabitation  
198 (Table 3,  $p=0.013$ ). Furthermore, the onset of the mortalities occurred faster in the  
199 Treatment group (1b) compared with the Treatment group (1a) where the seed fish  
200 were exposed to the bacterium by immersion (Table 3).

201 No mortalities or morbidity were observed in the seed saline/control fish or any other  
202 fish in the same treatment group (Table 3).

### 203 **3.3 Clinical signs and gross pathology**

204 Within 3 to 4 days post exposure, clinical signs commonly associated with *E. ictaluri*  
205 infection were observed in the fish in both immersion challenges and at day 7 in the  
206 cohabitation experiments (Fig 3).

207 Affected fish in both immersion and cohabitation experiments showed behavioural  
208 changes including erratic swimming in a spiral motion and stopped feeding prior to

209 mortality. Internally, the affected fish presented grossly with white lesions (1-2 mm  
210 diameter) distributed throughout the spleen and the kidney (Fig 3). Later, white lesions  
211 also occurred in the liver of infected fish. The abdomen was swollen and abdominal  
212 dropsy was present with fluid in the peritoneal cavity. Spleen and kidney were enlarged.  
213 Large areas of cellular necrosis and haemorrhage were present in the spleen and kidney  
214 from the moribund fish sampled. Necrotic kidney tubules were observed in all fish  
215 exposed to *E. ictaluri* (Fig 4). Multiple extensive areas of necrosis were observed in the  
216 head kidney of affected fish presenting with clinical signs of BNP. The spleen also  
217 showed extensive confluent areas of necrosis within the parenchyma.  
218 The chromatin in the nucleus of liver cells was distributed irregularly through the  
219 cytoplasm indicative of nuclear fragmentation of a cell undergoing apoptosis (Fig 5).  
220 Cellular inflammation and necrosis were observed in the liver of infected fish in all  
221 bacterial treatment groups. Some areas of liver showed the process of karyolysis which  
222 resulted in the complete dissolution of the chromatin of a dying cell because of  
223 enzymatic degradation resulting in necrosis. This was preceded by karyorrhexis (Fig 5).  
224 No pathological changes were observed in fish in all control groups.  
225 Pure cultures of bacteria identified as *E. ictaluri* were recovered from moribund and  
226 fresh dead fish. Rate of re-isolation in moribund and dead fish of the bacterial group was  
227 100%. No mortalities/morbidity, clinical signs of disease or bacteria were observed or  
228 recovered from the control group or any of the survivors.

### 229 **3.4 Phenotypic and genomic identification**

230 The isolated strains from 96 moribund and fresh dead fish recovered during these  
231 challenge studies showed almost identical phenotypic characteristics with the original

232 challenge strain. They were all identified as Gram negative, non-motile short or varied  
233 length rods, fermentative on O/F and oxidase negative with an API 20E profile of  
234 4004000. These gave  $\beta$ -haemolysis when cultured on sheep blood agar and no H<sub>2</sub>S, acid  
235 or gas was produced when inoculated onto TSI slopes. Generally, the phenotype of the  
236 bacteria recovered from 96 moribund and dead fish that all presented with typical  
237 clinical signs of BNP was consistent with the other members of the genus *Edwardsiella*  
238 and was identified as *E. ictaluri*.

239 All of the *E. ictaluri* strains recovered from the experimentally exposed fish expressing  
240 clinical signs of BNP were confirmed positive by PCR as they provided a single molecular  
241 band at 470 bp.

#### 242 **4 DISCUSSION**

243 The results of this study produced a successful immersion and co-habitation challenge  
244 model for the bacterial infection, BNP. The bacterium recovered and identified from the  
245 affected fish during the challenge study was identified as *E. ictaluri*, and was considered  
246 homogeneous in identification and moribund fish showed similar signs to those  
247 described for both natural and previous experimental BNP infections (Crumlish et al.,  
248 2010; Ferguson et al., 2001; Ho, Areechon, Srisapoome, & Mahasawasde, 2008). These  
249 fish challenge studies confirmed Koch's postulates for new exposure routes, that are  
250 considered more natural compared with the traditional i.p. injection route. In the  
251 second immersion study, to comply with the 3R's when working with experimental  
252 animals the lowest number of fish were used in the control group which did not affect  
253 the statistical validity of the study

254 In the immersion challenges performed in this study, mortality rates proved to be a  
255 valuable indicator of the challenge concentration received, and in agreement with  
256 Murray et al., (1992). In the present study, mortalities were obtained in all treatment  
257 groups except the controls and these mortalities appeared to be concentration  
258 dependent, which was not unexpected. In this study, the mortalities were 100% even at  
259 5 min immersion, showing that for experimental studies on pathogenesis or evaluating  
260 prevention and treatment control strategies, the mortalities were very high using this  
261 route of pathogen exposure and concentration of bacteria. Other experimental  
262 challenge studies performed in striped catfish using the same immersion route  
263 presented mortalities as high as in the present study by using prolonged immersion time  
264 for 30 minutes to 1 hour. Immersion of  $1.2 \times 10^6$  cfu ml<sup>-1</sup> of *E. ictaluri* in 1 hour caused  
265 100% mortality of yellow catfish (Ye, Li, Qiao, & Li, 2009). The LD<sub>60</sub> of *E. ictaluri* for  
266 striped catfish was  $1 \times 10^6$  cfu ml<sup>-1</sup> for 1 hour immersion and  $3.5 \times 10^6$  cfu ml<sup>-1</sup> in ip-  
267 injected fish (Thin et al., 2009). Another study reported that an immersion challenge  
268 dose of  $1 \times 10^8$  cfu ml<sup>-1</sup> for 1 hour or  $1 \times 10^6$  cfu ml<sup>-1</sup> in i.p.-injected fish gave more than  
269 80% fish mortality (Crumlish et al. 2010). It may be that the duration of exposure by  
270 immersion may be too stressful for the fish, thus exacerbating the final mortality rates,  
271 hence the need for a more refined and natural pathogen exposure route.

272 In all *in vivo* pathogen challenge studies, fish are subjected to the additional stress of  
273 handling or prolonged exposure to the pathogen (Alcorn, Murray, Pascho, & Varney,  
274 2005). In this study, the short exposure time of 30 seconds was sufficient to establish an  
275 infection as shown from the presence of clinical signs, mortalities, bacterial recovery  
276 and histology results.

277 Comparison of the data provided in this study showed that the range of organs affected  
278 and the nature of the host response was similar when an infection is created through a  
279 high level single pulse exposure (injected) or a high level stable aquatic bath exposure.  
280 In addition, the fish exposed to the bacterium had similar behavioral, clinical signs and  
281 histology changes of liver and kidney to those described for both natural and  
282 experimental BNP infections (Ferguson et al. 2001; Crumlish et al. 2002; Ho et al. 2008;  
283 Crumlish et al. 2010).

284 Whilst pathogen uptake was not explored in the study presented, it may be that the skin  
285 is the first route of entry, simply a matter of opportunity rather than actual tissue  
286 specificity. Menanteau-Ledouble, Karsi, & Lawrence, (2011) revealed that *E. ictaluri*  
287 entered channel catfish through the skin instead of penetrating the fish through  
288 intestine, nares, or gills.

289 The most natural exposure route for fish infectivity studies is co-habitation, however  
290 few, if any fish models exist for bacterial co-habitation studies. In the data presented,  
291 lower final mortality figures were achieved by co-habitation, irrespective of the  
292 exposure route of the “seed” fish and a difference was observed in the time to mortality  
293 between the i.p. and immersion exposure of the “seed” fish. However, the mortality of  
294 striped catfish exposed to *E. ictaluri* in both immersion challenge and cohabitation  
295 challenge experiments stopped at day 12, and the study terminated by day 15. These  
296 factors complied with Amend, (1981) which defined the end point as two days beyond  
297 the day that the last fish specifically died from the infection. Our data would therefore  
298 support a refinement in the experimental designs for future *in vivo E. ictaluri* challenge  
299 studies performed in *P. hypothalamus* catfish.

300 Cohabitation challenge permits the determination of crossover infections within a group  
301 of infected and un-infected fish (Murray et al., 1992). However, it takes significantly  
302 longer time between the introduction of the infected seed fish and the onset of  
303 mortality among the challenged fish than by immersion (Alcorn et al., 2005). Physical  
304 contact is considered a risk factor for transmission of any pathogen in the water body  
305 (Cvitanich, Garate, & Smith, 1991; Gaunt et al., 2006; P. Klesius, 1994; Shotts, Blazer, &  
306 Waltman, 1986)). Whilst the results of the co-habitation method developed in this study  
307 clearly showed mortalities with clinical signs of disease and recovery of the infectious  
308 agent, it was difficult to determine the challenge dose received by the naïve fish.  
309 Nevertheless, our data showed that it is possible to achieve infection through  
310 cohabitation where the seed fish were challenged by i.p injection or through immersion.  
311 In the present study, the mortality of striped the contact cohabitant by i.p. injection  
312 (38.89% ) or immersion (22.22%) confirmed the importance of physical contact as a  
313 vector in horizontal transmission of *E. ictaluri* among striped catfish thus early removal  
314 of infected fish might be important in reducing the infection of *E. ictaluri* in naïve striped  
315 catfish at the farm level. The high density of striped catfish applied in grow out farming  
316 (Phan et al. 2009; 2011) can cause an increased severity of infection with this bacterium  
317 where the infection spreads rapidly to healthy fish in the same pond and contiguous  
318 ponds once the BNP occurs.

319 In conclusion, the present study fulfilled the study aims and produced two non-injection  
320 challenge models: immersion and cohabitation. An adequate level of challenge was  
321 achieved in the immersion challenge, which provided a minimum of 60% mortality of  
322 the infected fish suggesting that this method was reproducible and reliable alternative.  
323 Although the end-point mortality of co-habitation experiments was lower than expected

324 these models would be extremely useful in investigating alternatives to antibiotics or  
325 oral deliver of products at early stages of infection. Both of these methods would be  
326 suitable for investigating pathogenesis of *E. ictaluri* infections in *P. hypophthalmus*.

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### 432 **Figure legends**

433 **FIGURE 1** Cumulative percentage mortalities in the immersion exposure group (pilot  
434 study) (IMM = immersion).

435 **FIGURE 2** Cumulative percentage mortalities in the immersion challenged groups with *E.*  
436 *ictaluri* for 30 s (IMM 30 s), 1 min (IMM 1 min), and 2 min (IMM 2 min) compared with  
437 the control group. Means with the same letters are not significantly different ( $p=0.07$ ;  
438 0.12).

439 **FIGURE 3** White lesions (arrows) were presented in the anterior kidney and spleen of  
440 infected fish.

441 **FIGURE 4** Kidney from fish infected with *E. ictaluri* exposed for 30 second showed  
442 necrosis (N) and haemorrhagic areas (H) compared with un-infected fish in control  
443 groups (A).

444 **FIGURE 5** Liver and hepatopancreas cytopathology of fish infected with *E. ictaluri* exposed for 30  
445 second (B) showed cellular inflammation with some Pyknotic nuclei (P) cells compared with  
446 control un-infected fish (A). The liver of infected fish showed severe necrosis (N).

447

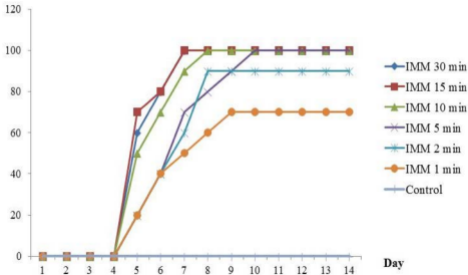
**TABLE 1** Challenge experimental design demonstrating the concentration of *E. ictaluri*, exposure time, number of fish and replicate tanks per treatment group.

Treatment group	No. Fish per Group	Bacterial concentration (cfu mL <sup>-1</sup> )	Exposure time (s)
1	30	1 x 10 <sup>7</sup>	30
2	30	1 x 10 <sup>7</sup>	60
3	30	1 x 10 <sup>7</sup>	120
Control	10	0.85% sterile saline	120

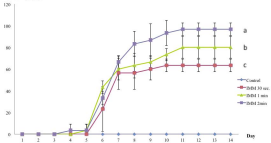
**TABLE 2** Experimental design for the direct contact cohabitation challenge according to the concentration of *E. ictaluri*, the method of experimental infection of seed fish, number of naive fish per treatment group.

Treatment group	Infection route (seed fish)	Bacterial concentration
1a	i.p. injection (bacteria)	$1 \times 10^6$ cfu fish <sup>-1</sup>
1b	i.p. injection (control)	0.1 ml of 0.85% sterile saline
2a	Immersion (bacteria)	$1 \times 10^7$ cfu mL <sup>-1</sup> for 15 min
2b	Immersion (control)	0.85% sterile saline

**Cumulative mortality percentage (%)**



Cumulative mortality percentage (%)



**TABLE 3** Mortality among groups of challenged striped catfish with various controls or *E. ictaluri* infection followed by a cohabitation challenge. The first mortality was recorded as day post-challenge.

Treatment	Replicate No.	Day first mortality	Final cumulative mortality (%)
1a	1	7	22
(IMM for 15 min )	2	7	22
1b	1	5	33
(i.p. injection)	2	5	44
2a (IMM control)	1		0
2b (i.p. injection control)	1		0

