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

Running Title: Bacterial Infectivity Models in striped catfish.

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Conflict of interest statement

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

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Data Availability Statement

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

Abstract

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

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

1. INTRODUCTION

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

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

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

Performing *in vivo* bacterial challenge studies for fish species under experimental conditions is difficult to standardise between studies (Nordmo & Ramstad, 1997; Nordmo, Sevatdal & Ramstad, 1997). This is often due to variation in strain pathogenicity, concentration, exposure route of the pathogen and consideration must be given to the variation in the age, size and species of the fish host. All of these factors heavily influence the expected outcome of clinical signs of disease and morbidity similar to those experience in natural infections (Crumlish et al., 2010; Thinh et al., 2009). Pathogen exposure methods in fish include injection, oral, hyperosmotic immersion, direct immersion, and cohabitation (Bell et al. 1984; Elliott et al. 1991), with injection being the most widely adopted method used in aquaculture. Pathogen exposure through injection remains the most favoured transmission route as it allows exact dose per fish to be known and reduced variation between individual fish. Immersion

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

2. MATERIALS AND METHODS

2.1 Fish

The fish used for the experimental studies were obtained from a stock population held in the Aquaculture Research Facility (ARF), University of Stirling. These fish were purchased from a farm in central Thailand and had been health certified as free from BNP from the Department of Fisheries (DOF) Thailand prior to shipment to the UK. The fish were maintained in 200L fibreglass tanks at 28^{o}C ± 2^{o}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^{o}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^{o}C) in a shaking incubator

(Kuhner shaker, ISF-1-W, Switzerland). After 24h, the bacterial broth suspension was centrifuged at 3,500 rpm (Sanyo NSE Mistral 2000R, Japan) and the cell pellet re-suspended and adjusted to give an optical density (OD_{600nm}) value of 1 using 0.85% sterile saline. The viable colony counts were performed using the Miles and Misra method (Miles et al. 1938) and then 10-fold serial dilutions performed to give approximately 1 x 10^7 cfu mL^{-1} for the challenge studies.

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

2.4 Challenge experimental design

From the immersion pilot study results, a second immersion challenge was performed with more refined bacterial pathogen exposure time (Table 1). In the second study, 4 treatment groups with 3 replicate tanks per treatment group each containing 10 fish per tank (Table 2). Fish in treatment groups 1-3 were exposed to a single concentration of *E. ictaluri* at approximately 1 x 10^7 cfu ml^{-1} for 30 seconds, 1 minute or 2 minute duration (Table 2).

2.5 Cohabitation experimental design

Co-habitation studies are considered the most natural route of bacterial exposure. Under experimental condition, this requires the introduction of an infected “seed” fish 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 niave 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 ± 2^{o}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^{o}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

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

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

3.2 Cumulative percentage mortality in cohabitation experiment

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

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

3.3 Clinical signs and gross pathology

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

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

mortality. Internally, the affected fish presented grossly with white lesions (1-2 mm diameter) distributed throughout the spleen and the kidney (Fig 3). Later, white lesions also occurred in the liver of infected fish. The abdomen was swollen and abdominal dropsy was present with fluid in the peritoneal cavity. Spleen and kidney were enlarged.

Large areas of cellular necrosis and haemorrhage were present in the spleen and kidney from the moribund fish sampled. Necrotic kidney tubules were observed in all fish exposed to *E. ictaluri* (Fig 4). Multiple extensive areas of necrosis were observed in the head kidney of affected fish presenting with clinical signs of BNP. The spleen also showed extensive confluent areas of necrosis within the parenchyma.

The chromatin in the nucleus of liver cells was distributed irregularly through the cytoplasm indicative of nuclear fragmentation of a cell undergoing apoptosis (Fig 5). Cellular inflammation and necrosis were observed in the liver of infected fish in all bacterial treatment groups. Some areas of liver showed the process of karyolysis which resulted in the complete dissolution of the chromatin of a dying cell because of enzymatic degradation resulting in necrosis. This was preceded by karyorrhexis (Fig 5).

No pathological changes were observed in fish in all control groups.

Pure cultures of bacteria identified as *E. ictaluri* were recovered from moribund and fresh dead fish. Rate of re-isolation in moribund and dead fish of the bacterial group was 100%. No mortalities/morbidity, clinical signs of disease or bacteria were observed or recovered from the control group or any of the survivors.

3.4 Phenotypic and genomic identification

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

challenge strain. They were all identified as Gram negative, non-motile short or varied length rods, fermentative on O/F and oxidase negative with an API 20E profile of 4004000. These gave β-haemolysis when cultured on sheep blood agar and no H_2S, acid or gas was produced when inoculated onto TSI slopes. Generally, the phenotype of the bacteria recovered from 96 moribund and dead fish that all presented with typical clinical signs of BNP was consistent with the other members of the genus *Edwardsiella* and was identified as *E. ictaluri*.

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

4 DISCUSSION

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

In the immersion challenges performed in this study, mortality rates proved to be a valuable indicator of the challenge concentration received, and in agreement with Murray et al., (1992). In the present study, mortalities were obtained in all treatment groups except the controls and these mortalities appeared to be concentration dependent, which was not unexpected. In this study, the mortalities were 100% even at 5 min immersion, showing that for experimental studies on pathogenesis or evaluating prevention and treatment control strategies, the mortalities were very high using this route of pathogen exposure and concentration of bacteria. Other experimental challenge studies performed in striped catfish using the same immersion route presented mortalities as high as in the present study by using prolonged immersion time for 30 minutes to 1 hour. Immersion of 1.2 x 10^6 cfu ml^{-1} of *E. ictaluri* in 1 hour caused 100% mortality of yellow catfish (Ye, Li, Qiao, & Li, 2009). The LD_{60} of *E. ictaluri* for striped catfish was 1x 10^6 cfu ml^{-1} for 1 hour immersion and 3.5 x 10^6 cfu ml^{-1} in ip-injected fish (Thinh et al., 2009). Another study reported that an immersion challenge dose of 1 x 10^8 cfu ml^{-1} for 1 hour or 1 x 10^6 cfu ml^{-1} in i.p.-injected fish gave more than 80% fish mortality (Crumlish et al. 2010). It may be that the duration of exposure by immersion may be too stressful for the fish, thus exacerbating the final morality rates, hence the need for a more refined and natural pathogen exposure route.

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

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

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Figure legends

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

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

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

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

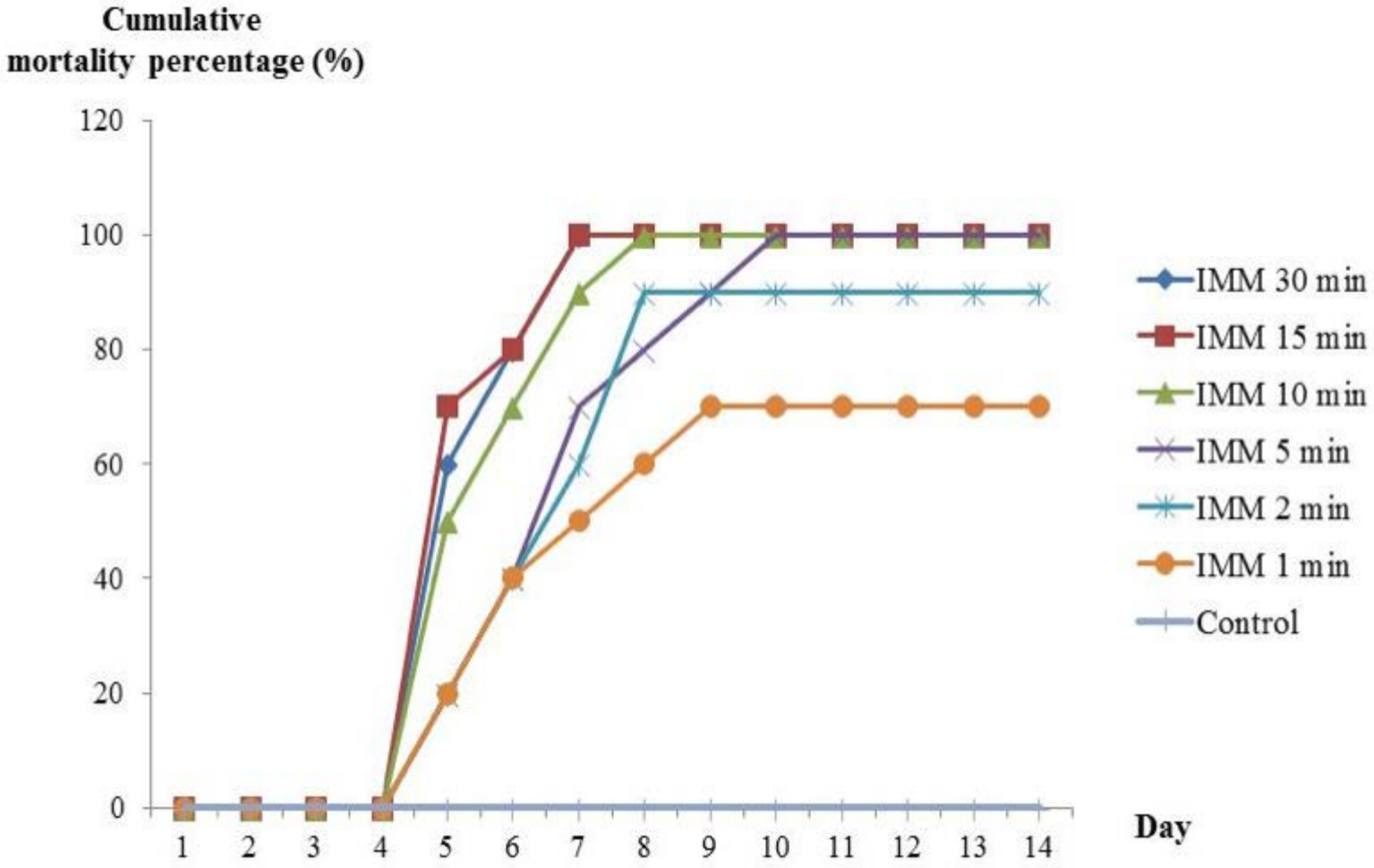
FIGURE 5 Liver and hepatopancreas cytopathology of fish infected with *E. ictaluri* exposed for 30 second (B) showed cellular inflammation with some Pyknotic nuclei (P) cells compared with control un-infected fish (A). The liver of infected fish showed severve necrosis (N).

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

Treatment group	No. Fish per Group	Bacterial concentration (cfu mL^{-1})	Exposure time (s)
1	30	1×10^7	30
2	30	1×10^7	60
3	30	1×10^7	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)	$1x10^6$ cfu $fish^{-1}$
1b	i.p. injection (control)	0.1 ml of 0.85% sterile saline
2a	Immersion (bacteria)	$1x10^7$ cfu mL^{-1} for 15 min
2b	Immersion (control)	0.85% sterile saline


Cumulative mortality percentage (%)
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Day
IMM 30 min
IMM 15 min
IMM 10 min
IMM 5 min
IMM 2 min
IMM 1 min
Control

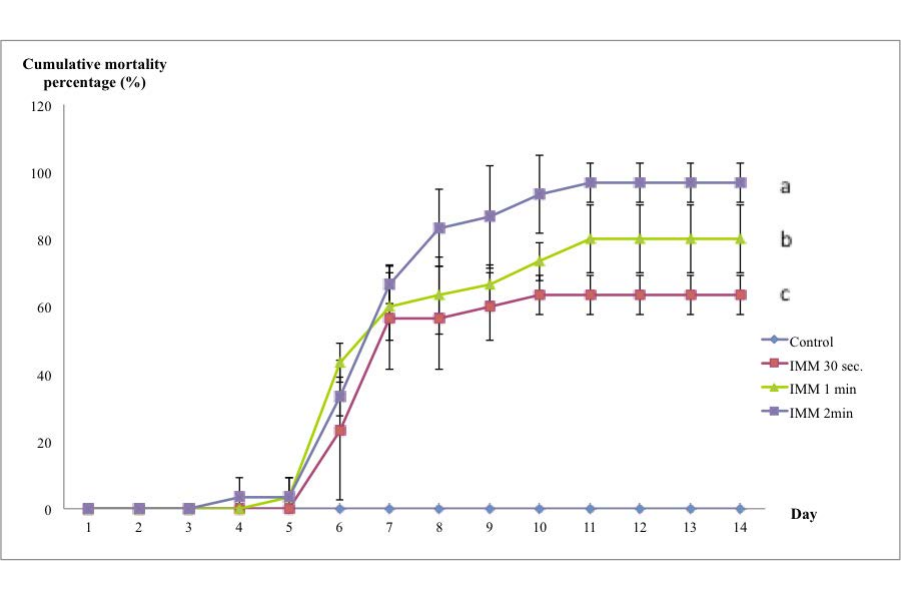
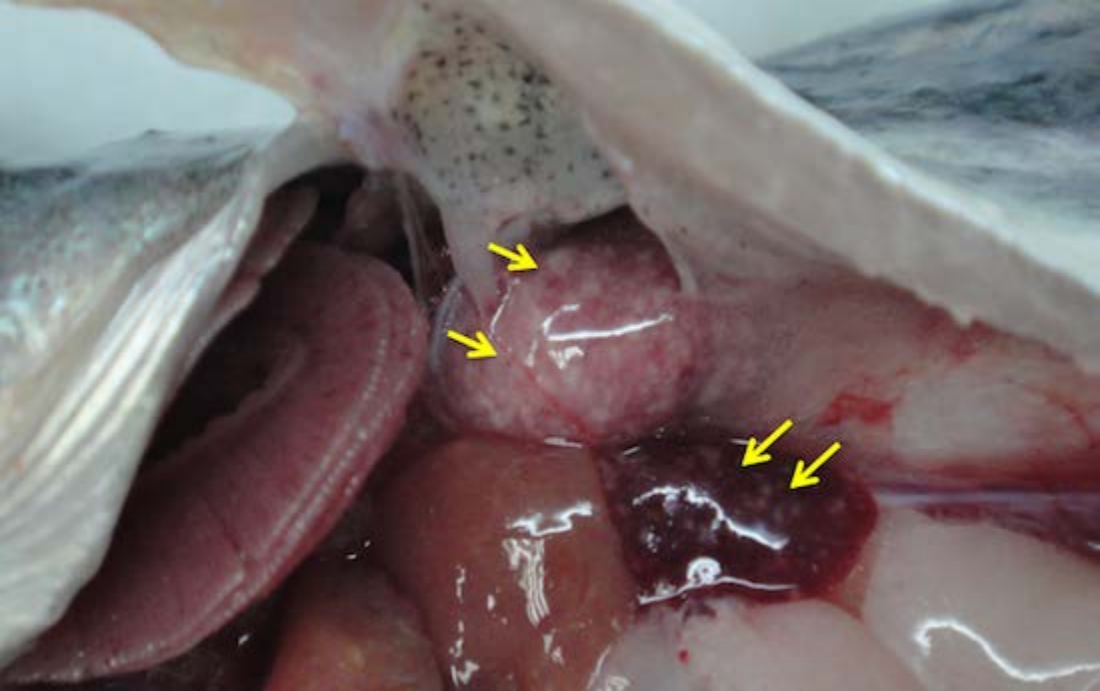
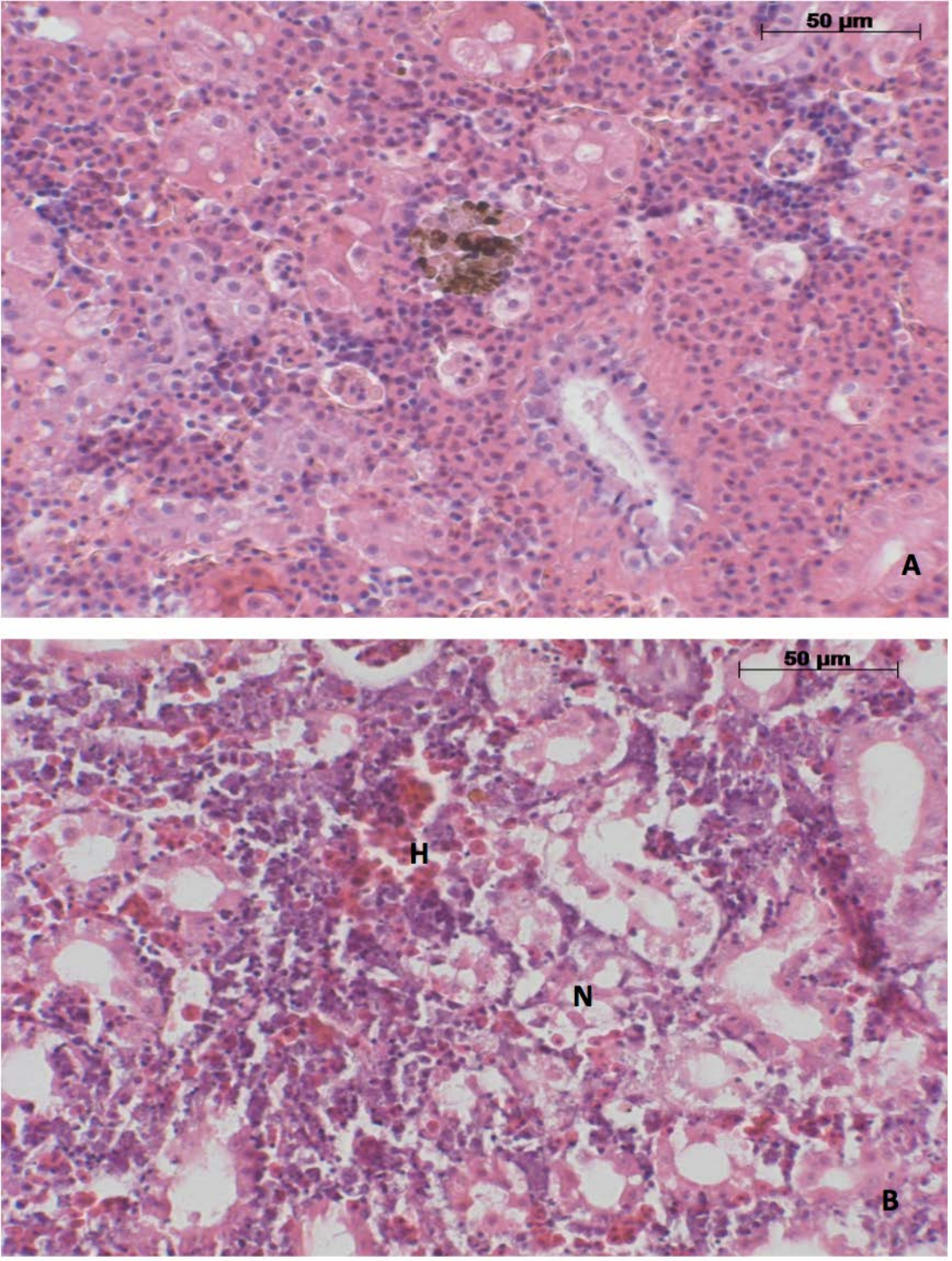
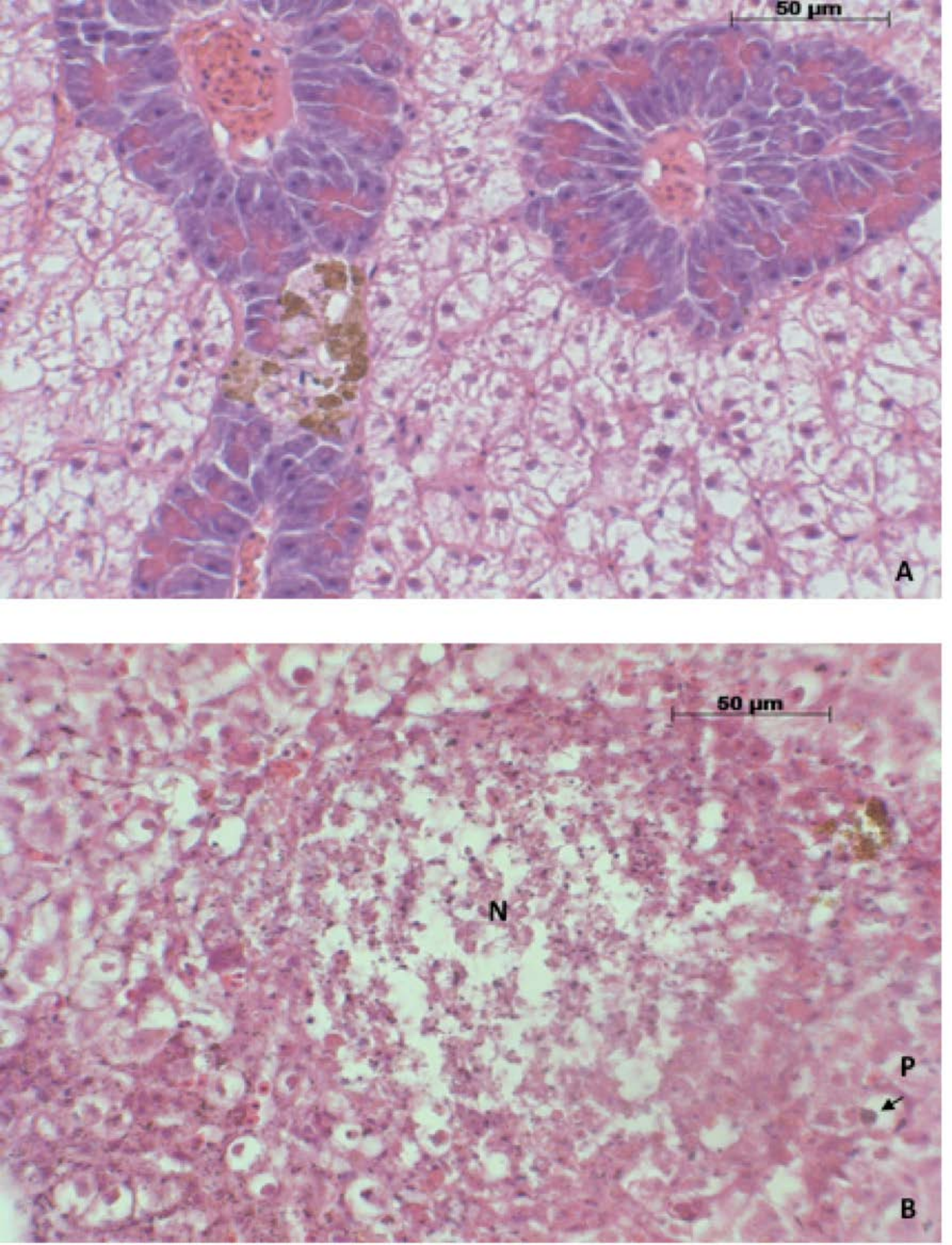

Cumulative mortality percentage (%)
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Day
a
b
c
Control
IMM 30 sec.
IMM 1 min
IMM 2min

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 (IMM for 15 min)	1	7	22
	2	7	22
1b (i.p. injection)	1	5	33
	2	5	44
2a (IMM control)	1		0
2b (i.p. injection control)	1		0




50 µm
A
50 µm
H
N
B


50 µm
A
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