Environmental conditions influence susceptibility of striped catfish *Pangasianodon hypophthalmus* (Sauvage) to *Edwardsiella ictaluri*

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Abstract

Over the last 20 years the production farmed Vietnamese striped catfish (*Pangasianodon hypophthalmus*) has increased significantly and in 2016, over 1.2 million tonnes of catfish were farmed and sold globally. Bacterial disease outbreaks due to *Edwardsiella ictaluri* continue to be one of the biggest threats to the sector, however, little is known on how the environmental conditions affect the survival of the fish during disease outbreaks. Growth of 14 *Edwardsiella ictaluri* strains recovered from natural disease outbreaks occurring in 4 provinces in Vietnam between 2002-2011 was investigated *in vitro* under different pH and salt concentrations. The results showed that a pH value of 6.5, NaCl concentration of 0.5% was optimal for the growth of the bacteria *in vitro*. The effect of varied pH and salt concentrations on the susceptibility of striped catfish to *E. ictaluri* infection was also studied *in vivo* following an immersion bacterial challenge (1.1 x 10^7 cfu ml^{-1} *E. ictaluri* for 30 s). The cumulative mortality of striped catfish in water at pH 5.5 and pH 6.5 was significantly higher than that of fish maintained in more alkaline water ($p<0.05$). The cumulative mortality of the striped catfish maintained in 0.5% NaCl was significantly lower than those kept in

0%, 1 % and 1.5 % NaCl ($p<0.05$). This study identified the effect of pH and salinity changes on the susceptibility of striped catfish to *E. ictaluri* infections.

Keywords: *Edwardsiella ictaluri*, *Pangasianodon hypophthalmus*, environmental conditions, pH, salinity.

1. Introduction

Aquaculture is currently the fastest growing food production sector globally with the most rapid growth being visible in the Asian sector (Jennings et al., 2016). The freshwater catfish *Pangasianodon hypopthalamus* remains one of Vietnam's top seafood products, with most farms located in the Mekong Delta. The farmed catfish products are exported to almost 140 countries, including the USA and countries of the EU (Halls and Johns, 2013). Since 2006, *P. hypopthalmus* production has increased in Vietnam but the sustainable development of the sector is constantly threatened by infectious disease outbreaks (De Silva and Phuong, 2011). Bacterial diseases have been reported as the major infections affecting Vietnamese striped catfish farming (De Silva and Phuong, 2011), where outbreaks can account for up to 50% of total losses in these farms compared to non-infectious and infectious causes (Phuong et al., 2007). Bacillary necrosis of Pangasius (BNP) is a bacterial infection caused by the gram-negative bacterium *Edwardsiella ictaluri* (Crumlish et al., 2002) and is considered as the most serious disease occurring in striped catfish (Crumlish and Dung, 2006; De Silva and Phuong, 2011; Phan et al., 2011). The influence of environmental conditions on infectivity of *E. ictaluri* remains unclear. Experimental studies have shown that thermal fluctuation was the most significant precursor to establishment of *E. ictaluri* infection in channel catfish (Baxa-Antonio et al., 1992; Francis-Floyd et al., 1987;

Plumb and Shoemaker, 1995) and high salinity altered the host response increasing susceptibility to infection (Uribe et al. 2011).

The Mekong Delta is forecast to be severely impacted by climate change, where a rise in sea levels will increase the salinity and change the pH of the large downstream region of striped catfish farming area (Nguyen et al., 2014; Nguyen et al., 2017). In Vietnamese farming systems, outbreaks of BNP are reported throughout the production cycle, but mortalities peak during seasons when the water quality changes rapidly, which correlated with the onset of the wet season, and increased rainfall in Vietnam (Luu, 2013; Phan et al., 2011; Phuong et al., 2007).

Given the importance of environmental conditions on the host-pathogen interaction, the aim of this study was to determine the survival and growth of *E. ictaluri in vitro* and evaluate how these environmental conditions may influence pathogenicity during an *in vivo* experimental challenge in *P. hypophthalmus*.

2. Materials and methods

2.1. Source and identification of bacterial strains

A total of 14 *E. ictaluri* isolates were included in the *in vitro* screening study, all recovered from 14 different clinical disease outbreaks of pangasius catfish (*P. hypothalamus*) distributed in four provinces in Vietnam (Vinh Long, Can Tho, An Giang and Dong Thap province). These bacteria were all collected from natural disease outbreaks occurring between 2002 to 2011 (Table 1) and given the degree of homogeneity in genotypic profiles between the different strains, isolates used were representative of the six groups identified from the Pulsed Field Gel Electrophoresis (PFGE) study and for 4 provinces (data not shown) and arbitrarily selected for use in

the *in vitro* studies. The isolates applied in this study were representative of the different temporal and geographical presence of the infectious disease (Table 1). All strains had been previous identified by routine bacteriology methods following Frerichs and Millar (1993) and 16 S rRNA gene sequencing and stored as purified strains deposited on cryo-preservative in commercially prepared Protect bead vials (Technical Service Consultant Ltd, UK) at -70°C until required. To confirm viability and purity from storage, a single bead per strain was grown in 10 mL of Tryptone Soya Broth (TSB, Oxoid UK) under vigorous shaking at 28°C for 24h and pure cultures confirmed by plating onto Tryptone Soya Agar (TSA, Oxoid UK) and primary identification tests performed with motility, oxidase, methyl Red, Voges-Proskauer, Triple Sugar Iron Agar (TSI), Lysine decarboxylase, Arginine decarboxylase, Ornithine decarboxylase and DNAse activity following methods described in Frerichs & Millar (1993) and Crumlish (2002). Motility test was performed with the wet-mount technique, haemolysis was assessed on 5% sheep blood agar. Fermentation of carbohydrates was assessed using purple broth base (Difco, UK) with 5% glucose, fructose, galactose, glycerol, maltose, manose, or ribose added.

The biochemical profiles of the isolates were determined using the commercially available kit API 20E (Biomerieux, UK) where the kit was used following the manufacturer's instruction except inoculated strips were incubated at 28°C and results read after 48h. The *E. ictaluri* type strain (NCIMB 12733) was purchased from the National Collection of Industrial and Marine Bacteria and used as an internal control for the *in vitro* screening.

Table 1. List of *Edwardsiella ictaluri* isolates according to the geographical region and year of isolation.

Province	Isolate ID	Year Recovered*
An Giang	042	2006
	049	2008
	070	2011
Can Tho	008	2002
	021	2004
	062	2010
Dong Thap	026	2005
	045	2007
	055	2009
	076	2011
Vinh Long	036	2005
	037	2005
	074	2011
	079	2011

* = each strain represents a different farm even in the same year and province.

2.2. Bacterial preparation for in vitro assays

From a pure bacterial growth plate on TSA, a single colony was removed and placed directly into 5 mL of sterile TSB and incubated overnight at 28°C in the shaking incubator (Kuhner shaker, ISF-1-W, Switzerland; 140 rpm). After 24h the bacterial suspension was centrifuged at 3,500 rpm (Sanyo NSE Mistral 2000R, Japan) and washed twice in sterile phosphate-buffered saline (PBS), containing 0.02 M phosphate and 0.15 M NaCl, and the cell pellet re-suspended in sterile saline (0.85% NaCl) to achieve an OD_{600nm} value of 1, which was expected to give 1×10^9 cfu mL^{-1} based on

standard bacterial growth curves (data not presented). The Miles & Misra method provided viable colony counts (Miles et al. 1938). Briefly, the bacterial suspension of OD_{600nm} value of 1 was serially 10x diluted by adding 1x of suspension to 9x of diluent. The dilutions were made to 10^{-8}. Three TSA plates were prepared for each dilution series. Plates were divided into 6 equal sectors which were labelled with the dilutions from 10^{-3} to 10^{-8}. In each section, 1 x 20 µL of the appropriate dilution was dropped onto the surface of the agar. The plates were left upright on the bench to dry before inversion and incubation at 28°C for 24 hours. Colonies were counted in the sector where the highest number of full-size discrete colonies can be seen. The number of colony forming units (CFU) per mL were calculated by average number of colonies for a dilution x 50 x dilution factor. Then 10-fold serial dilutions were performed to give approximately 1 x 10^7 cfu mL^{-1} concentration per strain tested. This bacterial concentration was used for all of the tolerance assays performed in this study. The actual bacterial concentration used in the in vitro studies was evaluated by viable colony counts method above.

2.3. Tolerance of bacterial growth to varied NaCl and pH conditions, in vitro

2.3.1. NaCl tolerance assay

One hundred microliters of pure *E. ictaluri* suspension at (10^7cfu mL^{-1}) was aseptically inoculated into 30 mL of sterile TSB with 6 NaCl concentrations (0, 0.5, 1.0, 1.5, 2.5 and 4.0% NaCl) and grown in a shaking incubator (Kuhner shaker, ISF-1-W, Switzerland) at 28°C, 140 rpm for 24 hours. A pH of 6.5 was used as the pH standard for all NaCl treatments investigated. The un-inoculated TSB broth (containing 0.5% NaCl) was used as the negative control (Plumb & Vinitnantharat 1989; Benson 2002). Each salt tolerance assay was performed in triplicate per isolate tested. After 24 hour

incubation at 28°C, the optical density (OD_{600nm}) was measured and viable colony counts were performed as previously described in 2.2.

2.3.2. pH tolerance assay

Farm data on the pH ranges in the striped catfish ponds both outwith and during disease outbreaks were used as a guide for the assay performed (un published data of survey Phuoc 2011). The pH range of 4.5, 5.5, 6.5, 8.5 and 9.5 was used in this assay. The bacterial broth suspensions were prepared as previously described and 100uL of the bacterial suspension inoculated into 30 ml of TSB at each of the pH levels being tested. All samples were incubated as described above while the pH of TSB (7.5) and un-inoculated tubes of TSB were used as an internal and a negative control, respectively. Prior to adding in the bacteria, the pH values were adjusted using 1N HCl or 1N KOH (Oxoid, UK) and measured by pH meter (Metter Toledo, Fisher Scientific) both prior and after autoclaving. Each pH tolerance assay was performed in triplicate. The densities of all strains under different pH values were defined after incubating for 24 hours at 28°C by spectrophotometry (OD_{600nm}) (Jenway™ 630 501, Thermo Scientific) and viable colony counts performed as previously described in 2.2. The NaCl concentration in TSB broth (0.5% NaCl) was used as reference concentration for all treatments.

2.4. In vivo challenge.

2.4.1. Source of the fish

Apparently healthy fish (*P. hypophthalmus*) were transported from The National Breeding Centre for Southern Freshwater Aquaculture at An Thai Trung Commune, Cai Be district, Tien Giang province, Vietnam to the Applied Hydrobiology Laboratory of International University, Ho Chi Minh National University, Ho Chi Minh city, Vietnam.

The fish had been starved for 1 day prior to being transported by air-conditioned car. The transportation time was 3 hours and fish were maintained in 4000 L fibreglass tanks using continuous flow-through water at 0.38 L min^{-1} at 28°C ± 2°C, and fed commercial catfish diet (Catfish 2 T502, Uni-President Co., Vietnam) for 14 days in the aquaria, prior to use. Fish used in this study were between 15-20g and health checks of fish prior to experimental challenge were performed by sampling the kidney of 5 fish directly onto TSA and checking for bacterial growth.

2.4.2. Bacterial challenge strain

A single bacterial strain of *E. ictaluri* (isolate 360) was used for all *in vivo* experiments. This isolate was identified as *E. ictaluri* (Crumlish et al. 2002) and had been used in previous infectivity trials (Crumlish et al. 2010). To enhance pathogenicity after long-term storage, the *E. ictaluri* strain was passaged through naive fish by intra peritoneal (i.p.) injection. Moribund fish were sampled for bacterial recovery from the kidney. This process was repeated twice. The isolate (called ex-passage 2) recovered from the fish was identified as described previously and used for the *in vivo* fish experimental challenge studies.

The *E. ictaluri* challenge inoculum was grown in 50 mL of sterile TSB (pH 6.5) with 4 different % added NaCl of 0%, 0.5%, 1%, and 1.5% by adding NaCl (Oxoid, UK) or in TSB (0.5% NaCl) at pH 5.5, 6.5, 7.5 or 8.5. The pH was adjusted using 1N HCl or 1N KOH (Oxoid, UK) and bacteria were grown in an orbital shaking incubator at 28°C, 140 rpm for 24 hours. After 24h the bacterial suspension was prepared to achieve an OD_{600nm} value of 1 and then 10-fold serial dilutions performed to give approximately 1 x 10^7cfu mL^{-1} for the *in vivo* studies (Ngoc Phuoc N., et al., 2020). The actual bacterial concentration was determined by viable colony counts as previously described in 2.2.

2.4.3. In vivo Experimental design

All fish (*P. hypophthalmus*) were held in 50 L tanks and exposed to the bacteria by immersion for 30 seconds. Fish were immersed in the 10L tanks containing bacteria at 1.1 x 10^7 cfu mL^{-1}, removed after 30 seconds and placed into the flow-through experimental tanks (50 L) and observed for 14 days. The bacterial concentration was determined from previous pilot studies where fish had been held at 0% added NaCl and pH = 7.5 and was designed to give 60% total mortalities (data not shown). All fish and treatment groups were randomly allocated. Each treatment group had 3 replicate tanks containing 10 fish per tank (n=30 fish per treatment group). The control group had duplicate tanks with 10 fish per tank (n=20 fish) and a total of 260 fish were used for all experiments.

For the NaCl treatment groups, fish were held in tanks containing 0, 0.5, 1 or 1.5% added NaCl for 2 weeks before and after receiving the challenge as described above. The challenge bacteria were grown in the same NaCl concentrations as the fish. The control fish group received the same treatment were maintained at 0% added NaCl but were not exposed to bacteria.

For the pH treatment groups, a range of 4.5, 5.5, 6.5, 7.5 and 8.5 was used in the *in vivo* challenge. This range reflected the pH values reported from catfish farms (unpublished data) and following recommendations from Wurts & Durborow (1992). Fish were maintained in water at either pH at 5.5, 6.5, 7.5 or 8.5 for 2 weeks before and after exposure to the *E. ictaluri* bacteria, as described above. Again the *E. ictaluri* was grown at pH 5.5, 6.5, 7.5 or 8.5 (*in vitro*). The control fish group received the same treatment but were maintained at pH 7.5 and received no bacteria.

After exposure to the bacteria, fish were kept in 50 L plastic tanks using continuous flow-through water at 0.38 L min^{-1}, a 12 h light: 12 h dark cycle and water temperature at 26 ± 2°C for 15 days. Aeration was supplied through an air stone to each tank and the fish were fed with a commercial diet (Catfish 2 T502, Uni-President Co., Vietnam) to apparent satiation twice daily. The desired pH and NaCl concentration of the tank water were adjusted using 1N HCl or 1N KOH or NaCl (Oxoid, UK). The water temperature, salinity and pH was checked daily using a portable pH meter (pH/temperature Hanna Model-HI98190, Rumani) and a refractometer (Atago Model 2491-master's, Japan). Moribund/dead fish were removed daily and samples for histopathology and bacteriology taken following methods described in Crumlish et al., (2010). At the end of the challenge period, 50% of all surviving fish per treatment group were examined for gross clinical signs of disease and sampled for bacterial recovery.

2.5. Statistical analysis

Parametric assumptions (the bacterial growth as measured from OD values of different isolates over the NaCl or pH ranges tested) were evaluated using Levene's test for homogeneity of variances and Shapiro–Wilk's test for normality.

As data were normally distributed and homoscedastic, the growth rates (OD) of different isolates at pH 7.5 and 8.5 or different concentration of NaCl (0.5 and 1%) were compared using one-way ANOVA, followed by Tukey test. For non-normal distributed data, the growth rates (OD) of different isolates at pH values (4.5, 5.5, 6.5 and 9.5) and NaCl concentrations (0%, 1.5%, 2.5% and 4%) were compared by Kruskal-Wallis. The multiple comparisons and correlation analyses between growth rate (OD) of isolate at different pH and NaCl concentration were conducted using 2-way Anova

with (isolate and pH) or (isolate and NaCl) concentration as fixed factors, the OD value of each pH or each concentration of NaCl was treated as dependent variable. The survival rates between treatment groups exposed to the bacteria in vivo were compared by one-way ANOVA, and estimation of survival times was analysed using Kaplan-Meier curves.

All analysis was performed using the SPSS program 20.0, and significance identified as $P \leq 0.05$.

2.6. Ethical Considerations

All studies were approved following the ethical review processes at University of Stirling. The *in vivo* fish trials were performed in Vietnam, but all studies were conducted according to the ethical approval processes of the Home Office Licence 60/3949.

3. Results

3.1. Tolerance of E. ictaluri to NaCl and pH, as judged by OD and viable recovery

All bacterial isolates examined in this study grew in TSB at 0 to 1.5% NaCl and no growth or viable bacterial recovery was observed at 2.5 % and 4% NaCl (Fig. 1).

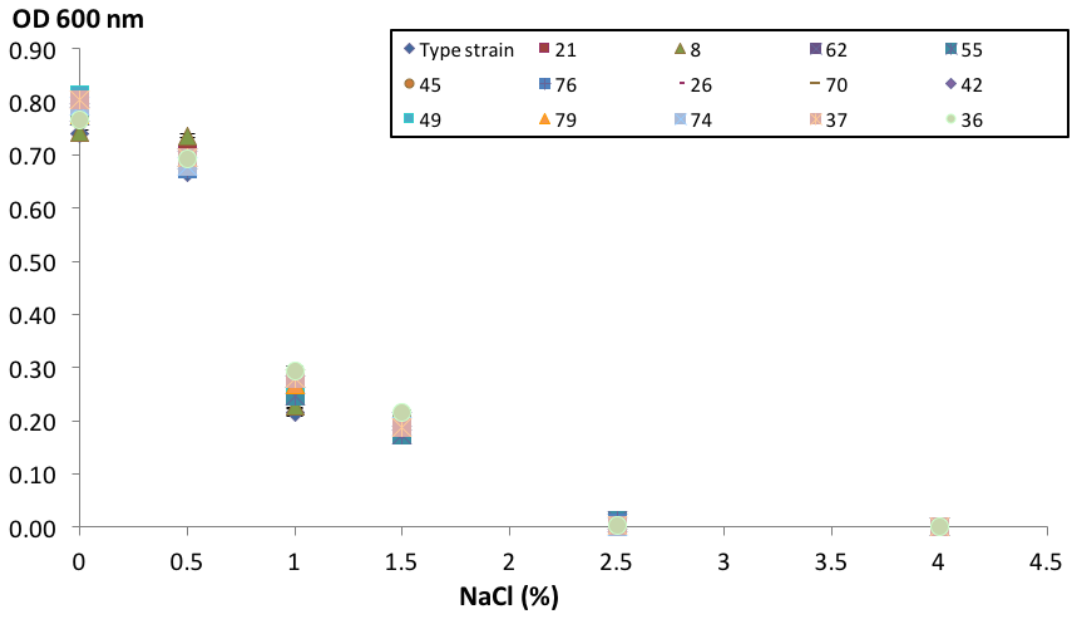

Fig. 1. The OD value of *Edwardsiella ictaluri* grown in 0-4% NaCl, *in vitro*. The type strain is American *E. ictaluri* 12733 from the National Collection of Industrial and Marine Bacteria (NCIMB).

Although all of the *E. ictaluri* isolates grew in 0 to 1.5% added NaCl, the better growth was observed in the treatments of 0% or 0.5% added NaCl as determined by absorbance values (Fig. 1). Lower bacterial growth was observed for all isolates cultured in NaCl concentrations of 1% and above (Fig. 1). With the exception of one isolate (isolate 8), all of the Vietnamese *E. ictaluri* had statistically higher absorbance (growth) at 1% and 1.5% added NaCl compared to the strains grown at 2.5% (p=0.017; p=0.05, respectively) and at 4% added NaCl (p=0.06; p=0.00, respectively). No significant difference in bacterial growth was observed in the strains grown at 2.5% (p= 0.733) and 4% added NaCl (p=1).

All isolates grew in TSB with pH from 4.5 to 8.5 but higher OD value (growth) was observed at pH 5.5 and 6.5 compared to those at pH 4.5, 7.5, 8.5 and 9.5 (Fig. 2). Growth of the isolates examined was statistically greater when isolates were

inoculated at pH 6.5 than those in any other pH treatments (Fig. 2). The Vietnamese *E. ictaluri* isolates had a better growth than the USA NCIMB type strain at pH 4.5 (p=0.03), pH 5.5 (p= 0.04), pH 6.5 (p= 0.04), pH 7.5 (p=0.017), and pH 8.5 (p-0.00) as judged by higher OD value. No significant difference was found between growth of isolates at pH 9.5 (p=0.206). At the highest pH value, all isolates remained viable but non-culturable, and became culturable once transferred to normal TSA (0.5% NaCl, pH 6.5) and incubated 3 days at 28°C.

For all isolates tested, no differences were observed in the colony or micromorphology of the bacteria grown in different levels of NaCl or pH.

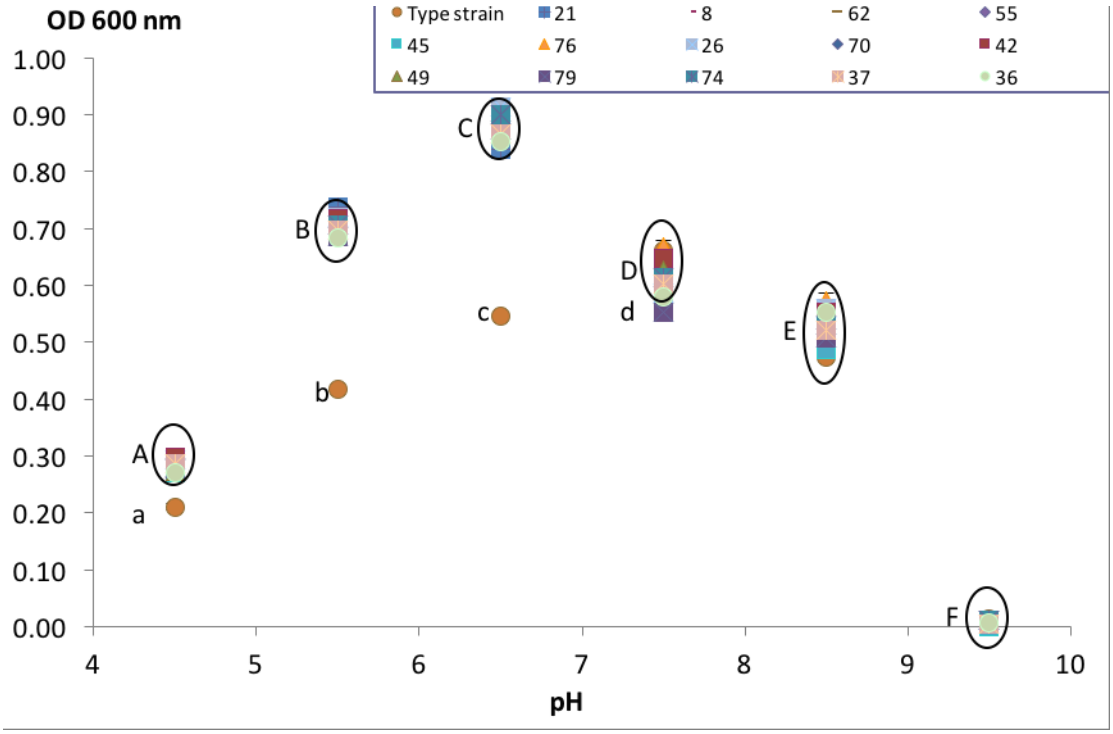

Fig. 2. The OD value of *Edwardsiella ictaluri* in different pH conditions, *in vitro*. Means with the same letter are not significantly different (p>0.05). Mean with same letter but different style (upper case and low case) are significantly different ($p \leq 0.05$). The type strain is American *E. ictaluri* 12733 from the National Collection of Industrial and Marine Bacteria (NCIMB).

3.2. Survival in the challenge test of striped catfish maintained at varied water salinities or pH

The highest fish survival (60%) was recorded in the treatment group held in 0.5% added NaCl, which was twice the survival rate of the fish held in 0% NaCl (30%) (Fig. 3), however, this was not statistically significant (p= 0.064). All fish died in the 1.5% added NaCl treatment group (Fig. 3). Survival rate of the fish in the treatment receiving 1% NaCl was only 10%. The survival of fish in the treatment group receiving 1.5 % added NaCl and in the treatment of 1% added NaCl was lower than those in treatment of 0.5% added NaCl (p= 0.000; p=0.000, respectively) or treatment of 0% NaCl (p=0.000; p =0.026, respectively) (Fig. 3).

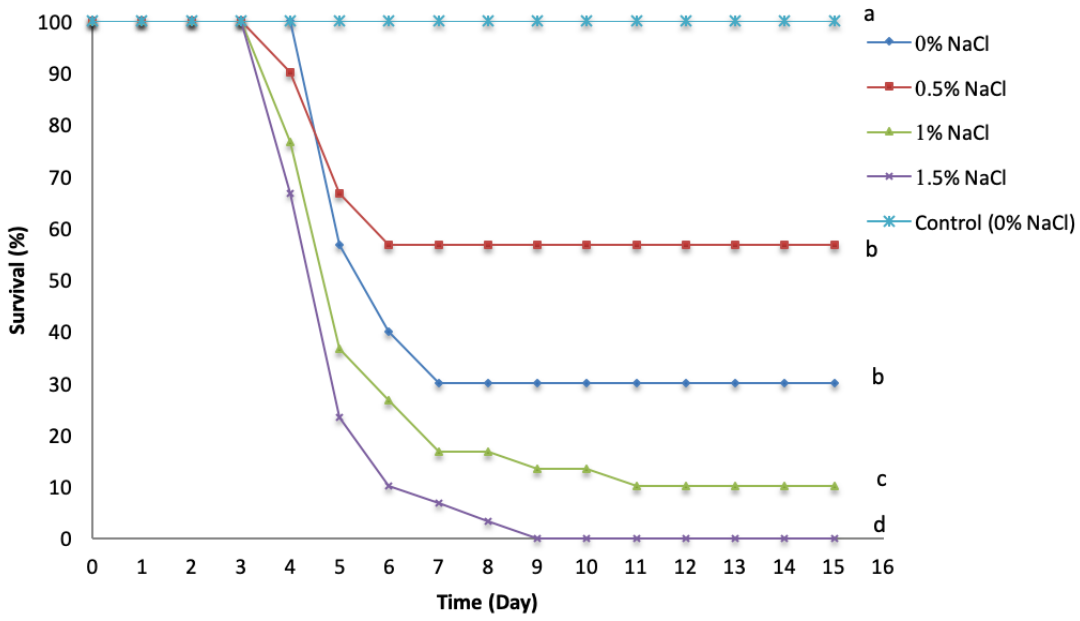

Fig. 3. Survival of striped catfish in different salinities after immersion exposure to *E. ictaluri* for 30 seconds. Different letters indicate significantly different treatments (p<0.05).

Fish survival was pH dependent, as the lowest survival was observed in the fish exposed to the lowest pH value (pH 5.5) and was statistically significantly lower than those in the fish held at pH 6.5 (p=0.01), or pH 7.5 (p=0.00), or pH 8.5 (p=0.00) (Figure 4). The survival of fish exposed to bacteria at pH 8.5 was highest (53.3%) but was not significant different with treatment pH 7.5 (p=0.08).

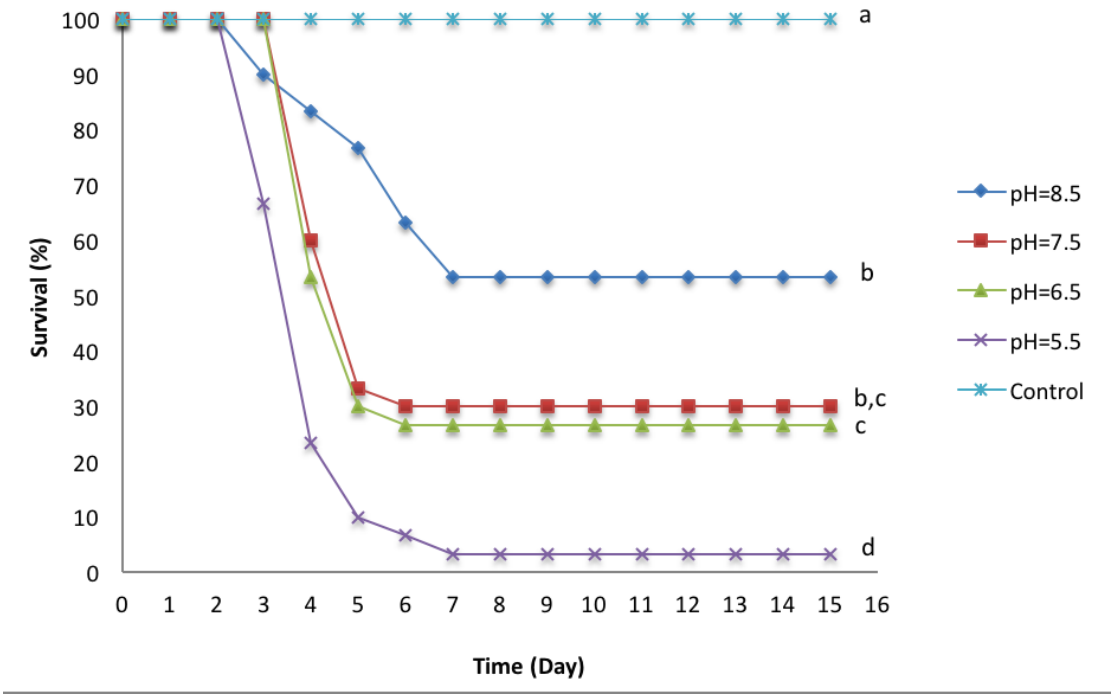

Fig. 4. Survival of fish exposed to *E. ictaluri* for 30 seconds under different pHs and the control group. Different letters indicate significantly different treatments (p<0.05).

3.3. Clinical signs and gross pathology

Moribund/dead fish experimentally exposed to *E. ictaluri* under different pH and NaCl concentration showed clinical signs of BNP disease similar to naturally infected fish with typical clinical signs of white lesions observed grossly in the kidney and liver within 4 days post exposure (Fig. 5).

Typical pathological changes of cellular inflammation and large areas of necrosis were observed in the spleen and kidney of infected fish exposed to different salinities and pH (results not shown). No mortalities/morbidity, clinical signs of disease or histopathological changes were seen in the control fish group or the survivors.

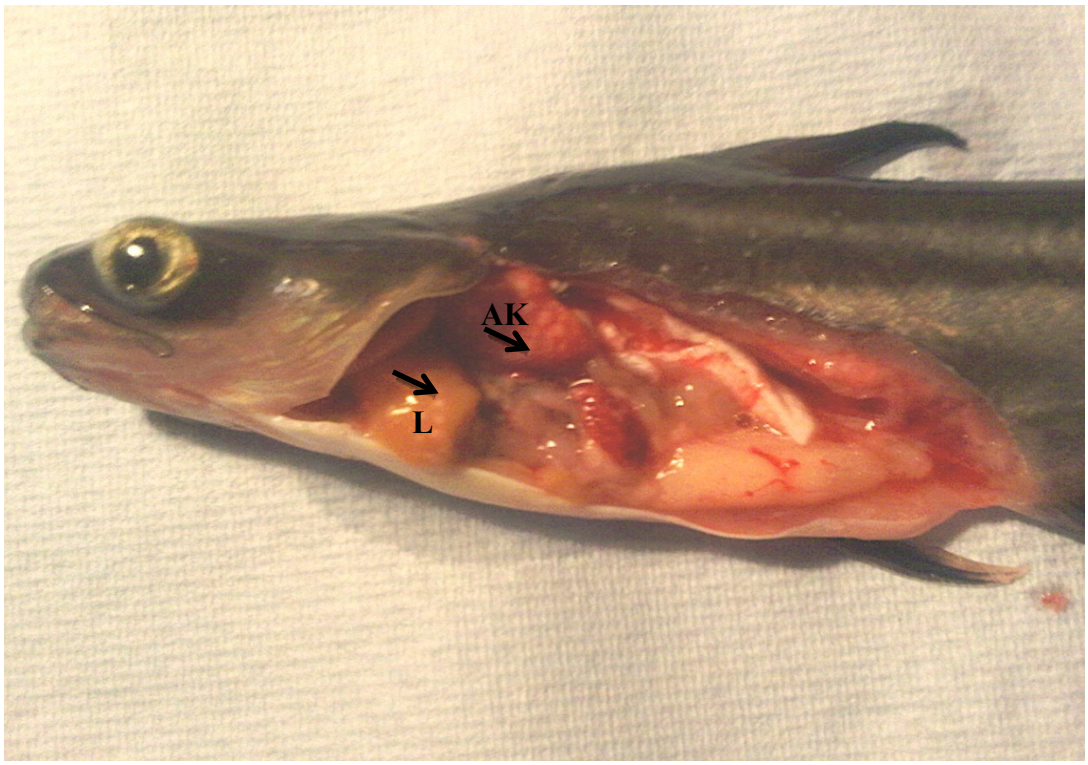

Fig. 5. Typical gross presentation of moribund fish exposed to bacteria, white lesions (arrows) observed in the anterior kidney (AK) and liver (L).

3.4. Bacteria identification in the experimental fish groups

All bacterial isolates recovered from affected fish with clinical signs of BNP from all experiments performed showed almost identical phenotypic characteristics to the original challenge strain. All isolates were described as small gram-negative rods, formed semi-transparent, round colonies on TSA and were cytochrome-oxidase negative. They were positive for lysine decarboxylase and only fermentation was observed using glucose as the substrate. The API 20E biochemical profile was 400400

for all isolates recovered during the experimental challenge studies performed, which confirmed *E. ictaluri*. Pure cultures were recovered from the kidney of moribund fish in all experiments where fish had clinical signs of BNP disease. No *E. ictaluri* was recovered from the surviving or control fish sampled at the end of the study period for any experimental groups.

4. Discussion

Although little has been published on NaCl or pH tolerances and *E. ictaluri* infections in striped catfish, the results from this study would support increased susceptibility to infection when fish are kept in water at low pH and high salinity conditions. This agreed with previous studies where environmental conditions (pH, salinity) in the water were considered to favour the expression of virulence factors in USA *E. ictaluri* strains recovered from infected channel catfish (Rogge and Thune, 2011).

Sodium chloride tolerance of Vietnamese *E. ictaluri* isolates recovered from striped catfish (*P. hypophthalmus) in vitro* was similar to that reported from the USA *E. ictaluri* isolates recovered from channel catfish (*Ictalurus punctatus*) (Hawke et al., 1981; Plumb and Vinitnantharat, 1989; Waltman and Shotts, 1986). The NaCl tolerance of Vietnamese *E. ictaluri* isolates investigated in this study was in agreement with the previous findings that *E. ictaluri* can grow in vitro at 1.5% but not in 2% NaCl, thus supporting *E. ictaluri* as a freshwater pathogen able to tolerate brackish water conditions (Plumb and Vinitnantharat, 1989; Waltman et al., 1986).

In this study, the fish groups held at the lower NaCl concentrations (0 or 0.5% NaCl), had significantly reduced mortality/morbidity when experimentally infected with *E. ictaluri*. This was in agreement with Plumb and Shoemaker (1995) who demonstrated

significantly lower mortalities in Channel catfish naturally infected with *E. ictaluri* when held in lower concentrations of NaCl. An incremental increase in NaCl concentration from 0 to 0.5% significantly decreased the mortality in the striped catfish experimentally infected with *E. ictaluri*. Furthermore, the best growth rates of the *E. ictaluri in vitro* were observed when the bacteria were cultured at the lower concentration of NaCl (0 or 0.5% NaCl), however in the *in vivo* bacterial challenge study, the survival of fish was highest at 0.5% NaCl, suggesting that the striped catfish benefit physiologically from 0.5% NaCl thus increasing resistance. No measurements of the host-pathogen interaction were made during the study, but it may be that the lower salinity is better for the catfish host and does not preferentially enhance virulence factors for the bacteria. This is supported by the greater increase in mortality observed in the striped catfish held at 1% and 1.5% NaCl, perhaps suggesting that the higher NaCl concentration affects both the growth of *E. ictaluri* but is more damaging to osmoregulatory functions of the catfish leading to increased disease susceptibility. Until now, the effect of NaCl concentration on striped catfish in Vietnam has not been investigated in relation to diseases susceptibility or even host physiology. However, Allen (1969) showed that a 1% NaCl concentration or less, permitted normal growth and survival of channel catfish (Allen, 1969).

NaCl has been commonly used in striped catfish farming as a therapeutant (Crumlish and Dung, 2006; Phan et al., 2011; Phan et al., 2009). The amount of NaCl reportedly used by Vietnamese fish farmers varied from 300kg to 500kg per 20 000m^3 per 1 to 2 weeks (unpublished data). When NaCl is added to the freshwater ponds during the production cycle as a proxy treatment or putative preventive measure, this may lead to an increase in the NaCl tolerance of striped catfish. In the study presented, there

were no mortalities or morbidity experienced in the catfish groups when held at higher salinity levels (1 and 1.5% added NaCl) during the acclimation prior to bacterial exposure. The addition of 0.5% added NaCl within the experimental facilities did not significantly affect the behaviour or apparent health of the *P. hypophthalmus* and increased the survival of fish when experimentally challenged with *E. ictaluri*. Low NaCl can be applied in ponds where natural salinity water is available for giving the better survival rate of freshwater striped catfish.

The pH was considered as one of an important factors influencing the susceptibility of Chanel catfish to stress-induced Edwardsiellosis (Baxa-Antonio et al., 1992; Mqolomba and Plumb, 1992). Data generated in this study from the *in vitro* work showed the optimum pH for growth of *E. ictaluri in vitro* was between 5.5 and 6.5 in contrast to the previous finding of Plumb and Vinitnantharat, (1989) who found that a pH of 7-7.5 was the optimum growth condition for USA *E. ictaluri*. In this study, Vietnamese isolates grew better at the lower pH 5.5 compared with pH 7.5. Furthermore, the Vietnamese *E. ictaluri* strains appeared more acid tolerant when tested *in vitro*. When investigating the effect of pH on the virulence of American isolates, Booth et al., (2009) found that *E. ictaluri* produced an acid-inducible urease enzyme to increase its virulence at pH levels equal or less than 4. Moreover, the type III secretion system (T3SS) apparatus gene and the T6SS gene in the *E. ictaluri* , which are virulence factors promoting infectivity in channel catfish were more activated at lower pH 5.5 (Booth et al., 2009; Rogge and Thune, 2011; Rogge et al., 2013; Thune et al., 2007). In this study, we did not investigate the expression of urease from bacteria maintained at different pH nor did we determine the effect of pH on expression of virulence genes. It is important to investigate this characteristic further particularly as peak *E. ictaluri*

infections resulting in heavy mortality in farmed striped catfish in Vietnam have been reported and observed during the rainy season when pH of water was lower than 6.5 (Anh et al., 2010; Giang H.T., 2008).

Furthermore, the availability of a urea source in the fishponds from uneaten feed and fish waste could easily be stimulating the activity of *E. ictaluri* urease resulting in enhanced survival, growth and virulence. Therefore, the "BNP window" as described by the Vietnamese fish farmers may be dependent on the pH of the aqueous environment. The results provided from this study would support this hypothesis on the pH dependent window of BNP infections, thus higher mortalities during seasonal variations.

At the highest pH values tested in this study the *E. ictaluri* strains remained viable but non-culturable, however, when incubated in more "favourable" conditions i.e. at lower pH they became culturable again. This may also support the mechanisms to enhance prolongued survival of the *E. ictaluri* bacterial loads in the Vietnamese catfish farms. Although pH 9.5 inhibited the growth of this bacterium under *in vitro* conditions it was unrealistic to use this value as it would be dangerous to fish because of the rise in blood NH_3 levels which would result in a marked increase in body stores of total ammonia and toxicity to fish (Randall and Wright, 1989). Wurts & Durborow (1992) also recommended that the pH range for aquaculture should be between 6.5-9.0 and fish may become stressed and die if the pH drops below 5 or rises above 10.

The wastewater and sludge discharge from striped catfish ponds contribute to the acidification of water in the river and surrounding water areas (Anh et al., 2010) and is considered to contribute to an increase in *E. ictaluri* outbreaks in striped catfish in Vietnam. The findings for the study presented, certainly supported that a lower

survival rate was observed in the fish exposed to the bacteria and held at more acid pH water.

5. Conclusion

This study showed that the infectivity of the bacterium *E. ictaluri* is altered depending on the environmental conditions of the fish. However, further work is required to evaluate the impact of varied salinity and pH conditions to the health and welfare of the striped catfish as this study looked at fish and bacterial survival but did not evaluate the change in host-pathogen interaction or even subsequent alteration of virulence expression of the bacterium and host immune response within these conditions. These data help to establish a relationship between 2 important environmental factors (NaCl and pH) and the susceptibility of striped catfish to *E. ictaluri* infection and lead the way for future studies to evaluate infectivity and host response.

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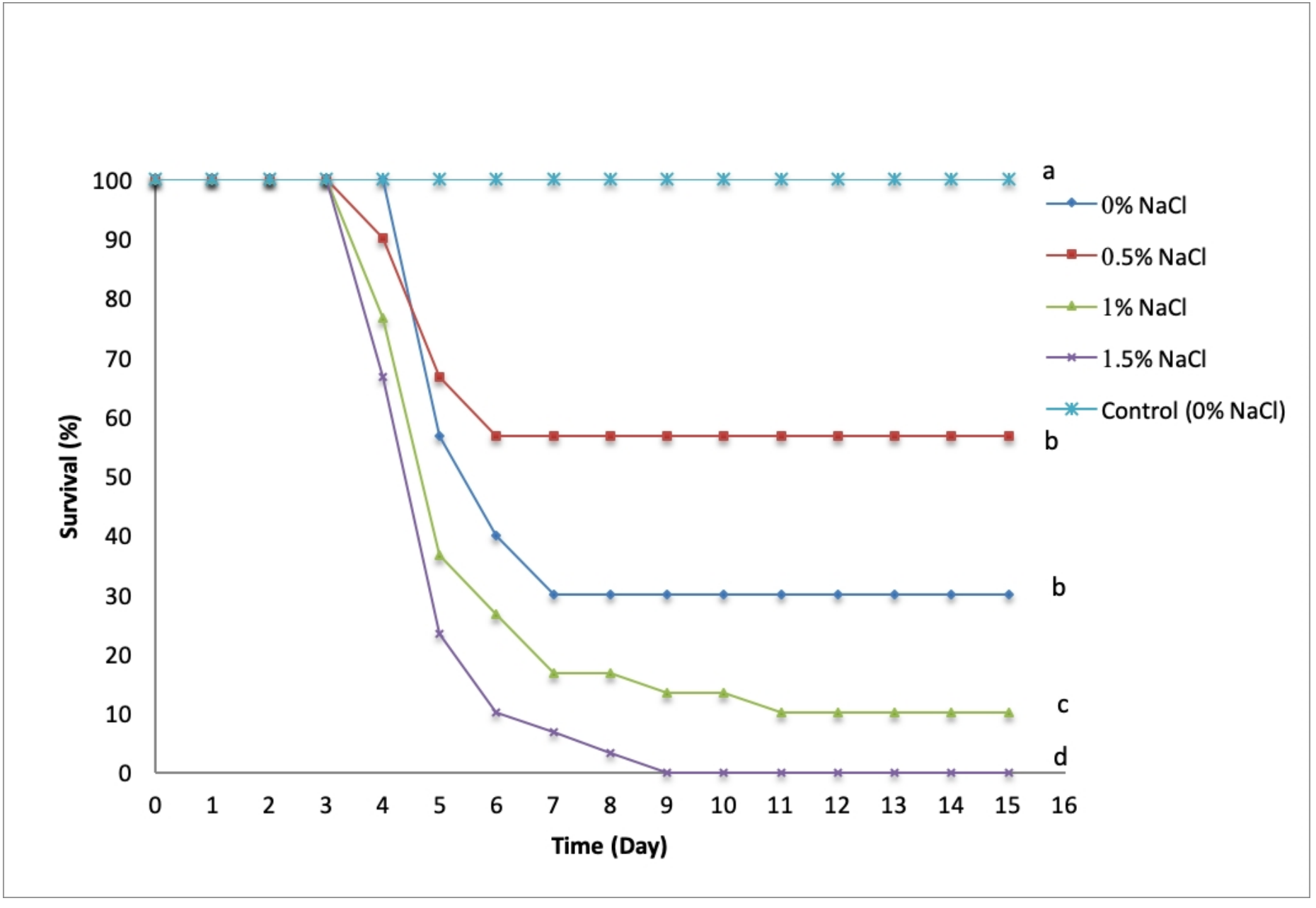
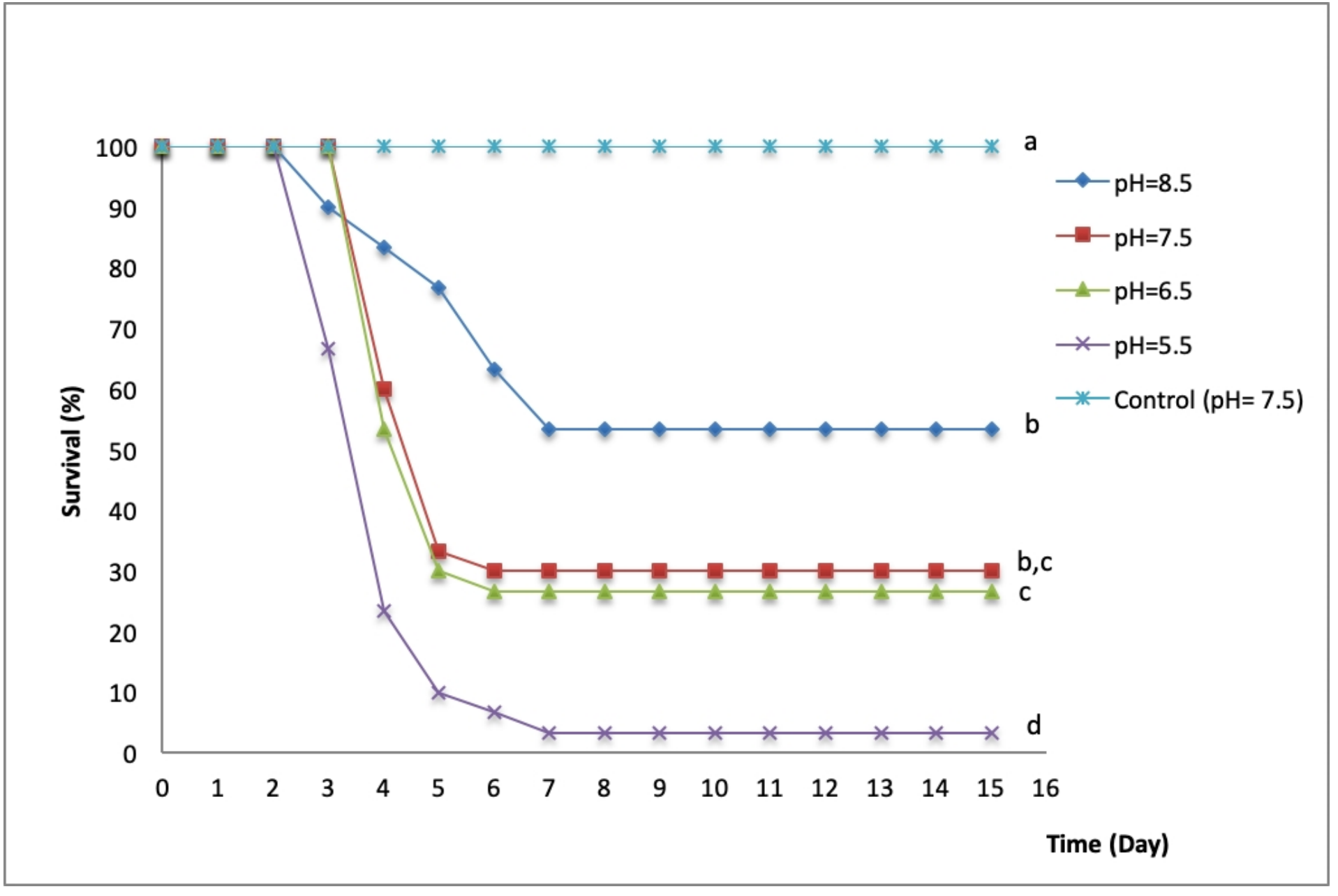
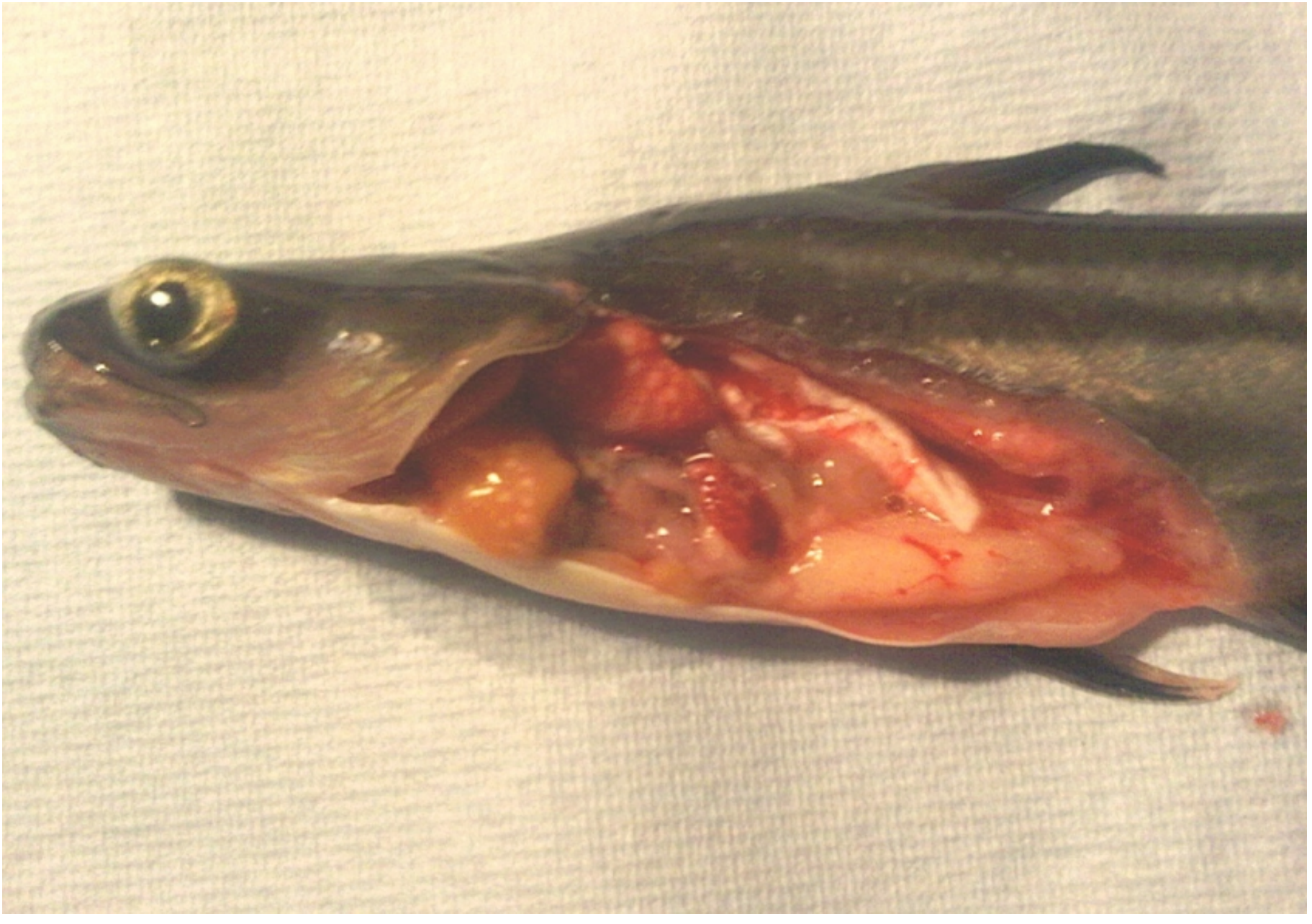

Survival (%)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Time (Day)
a
0% NaCl
0.5% NaCl
1% NaCl
1.5% NaCl
Control (0% NaCl)
b
b
c
d


Survival (%)
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Time (Day)
a
b
b,c
c
d
pH=8.5
pH=7.5
pH=6.5
pH=5.5
Control (pH= 7.5)



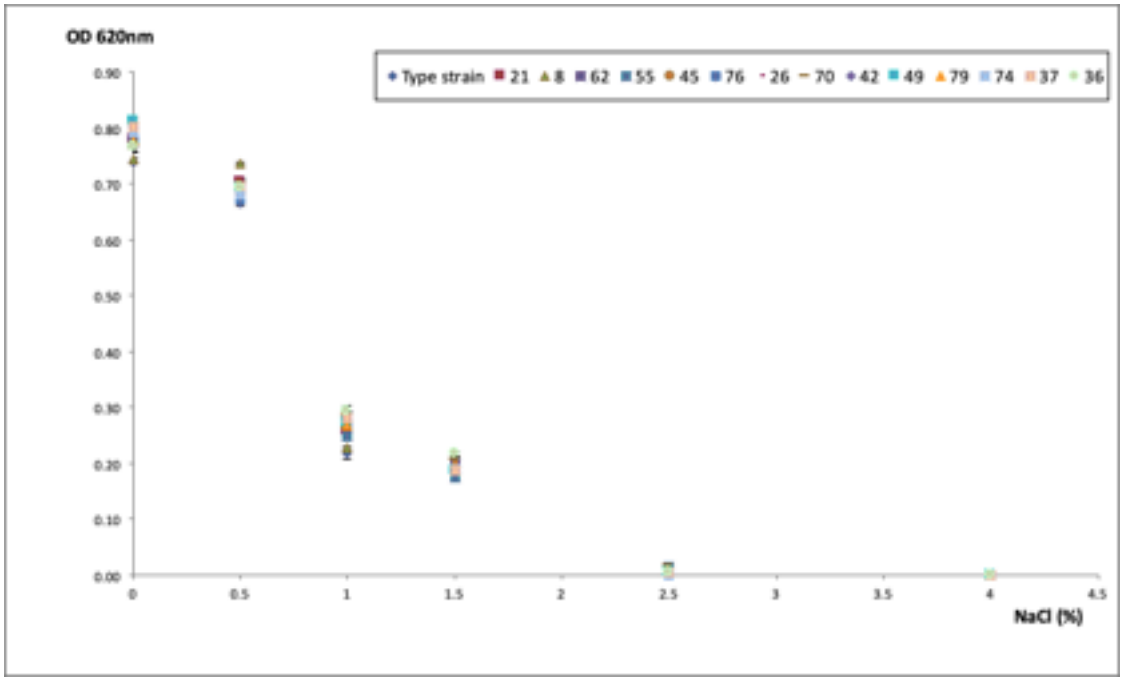


Fig. 1. The OD value of *Edwardsiella ictaluri* grown in 0-4% NaCl, *in vitro*. The type strain is American *E. ictaluri* 12733 from the National Collection of Industrial and Marine Bacteria (NCIMB).

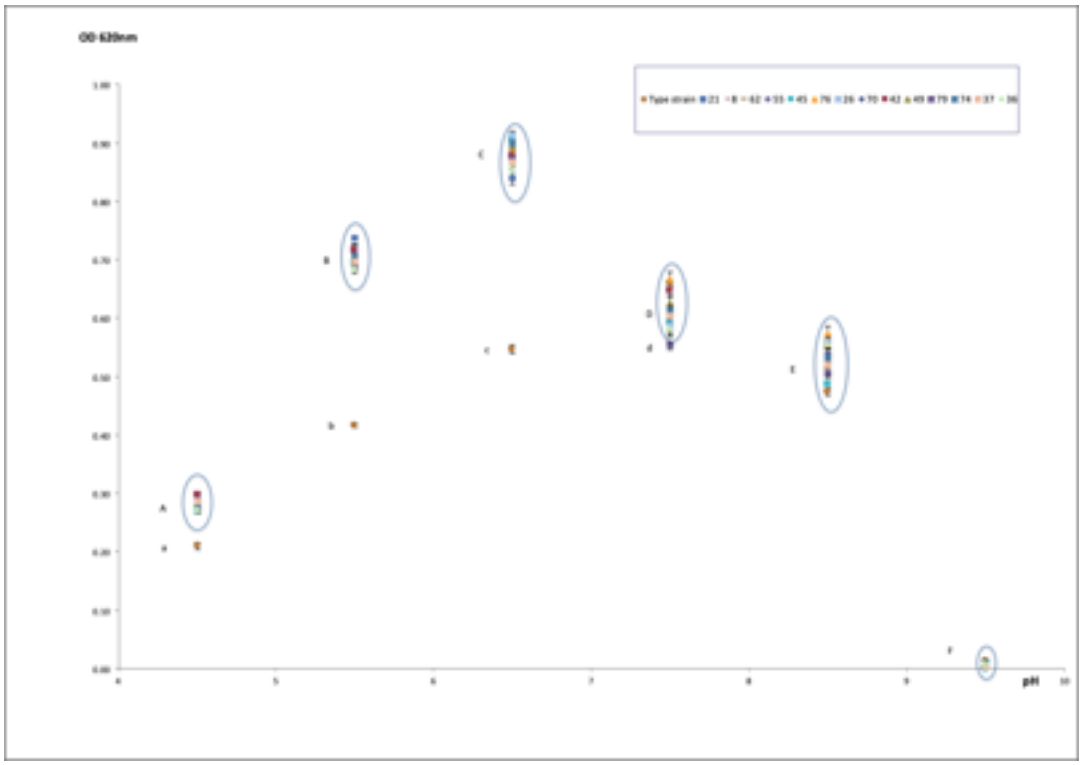


Fig. 2. The OD value of *Edwardsiella ictaluri* in different pH conditions, *in vitro.* Means with the same letter are not significantly different ($p>0.05$). Mean with same letter but different style (up case and low case) are significantly different ($p<0.05$). The type strain is American *E. ictaluri* 12733 from the National Collection of Industrial and Marine Bacteria (NCIMB).

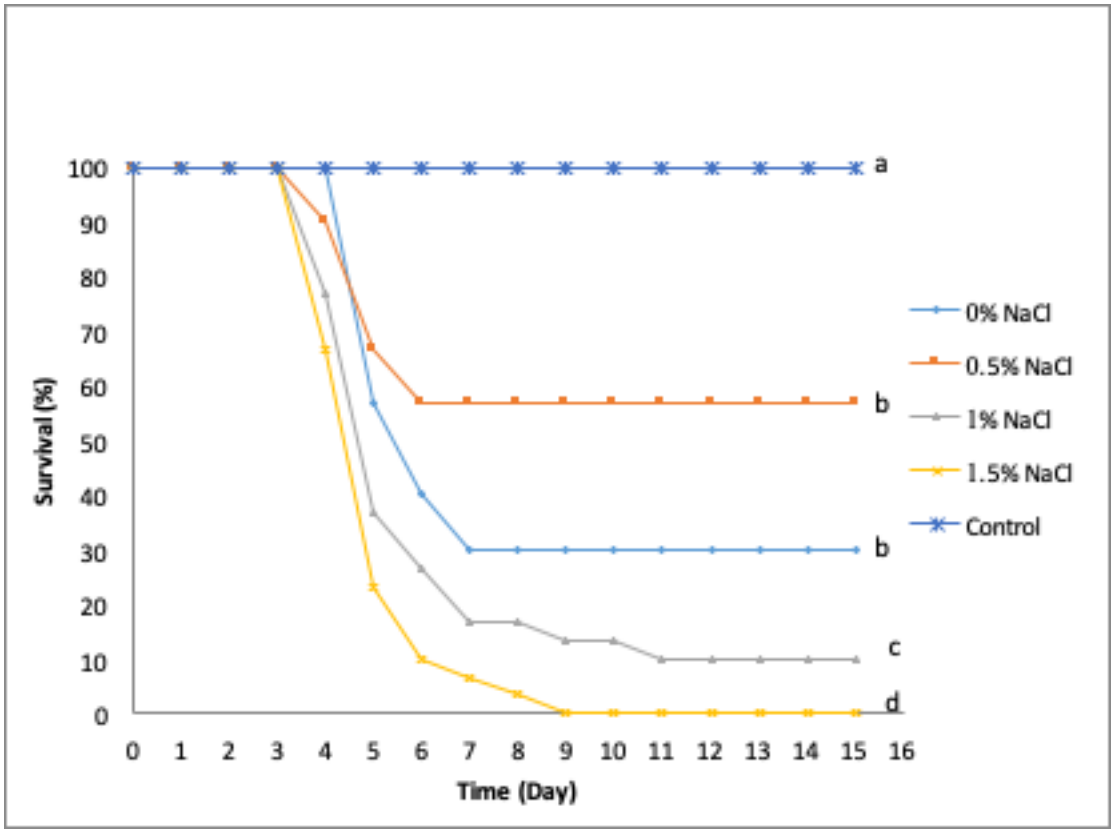


Fig. 3. Survival of striped catfish in different salinities after immersion exposure to *E. ictaluri* for 30 seconds. Different letters indicate significantly different treatments ($p<0.05$).

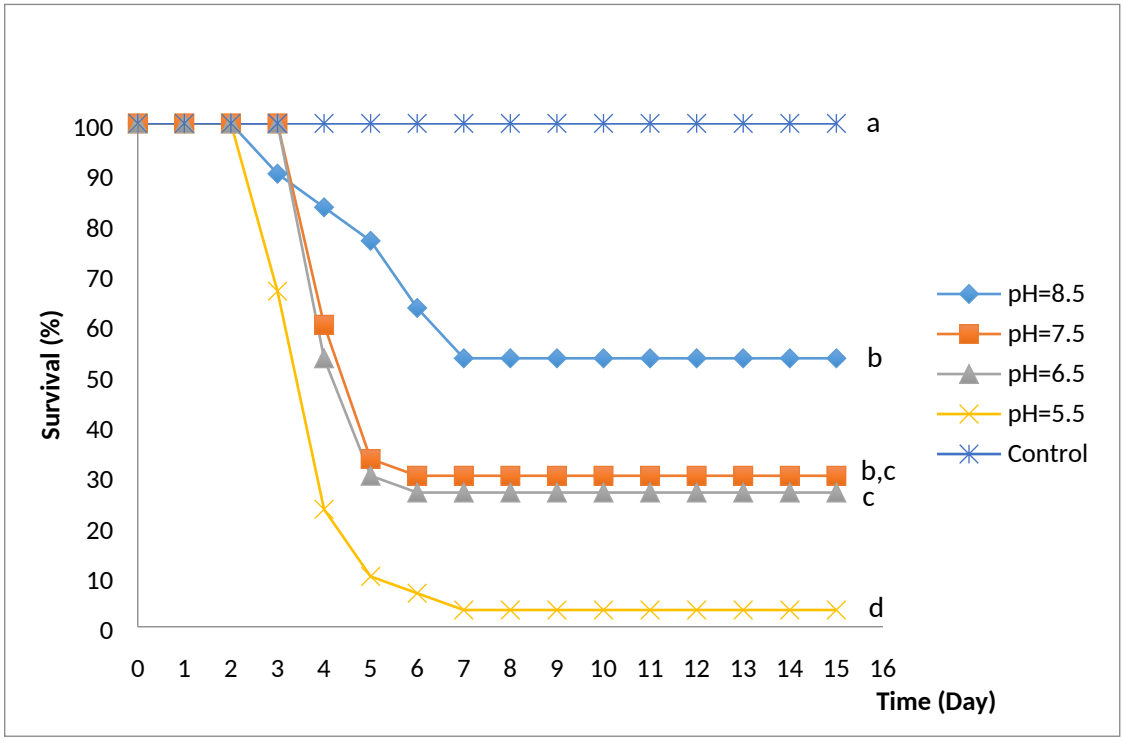


Fig. 4. Survival of fish exposed to *E .ictaluri* for 30 seconds under different pHs and the control group. Different letters indicate significantly different treatments ($p<0.05$).

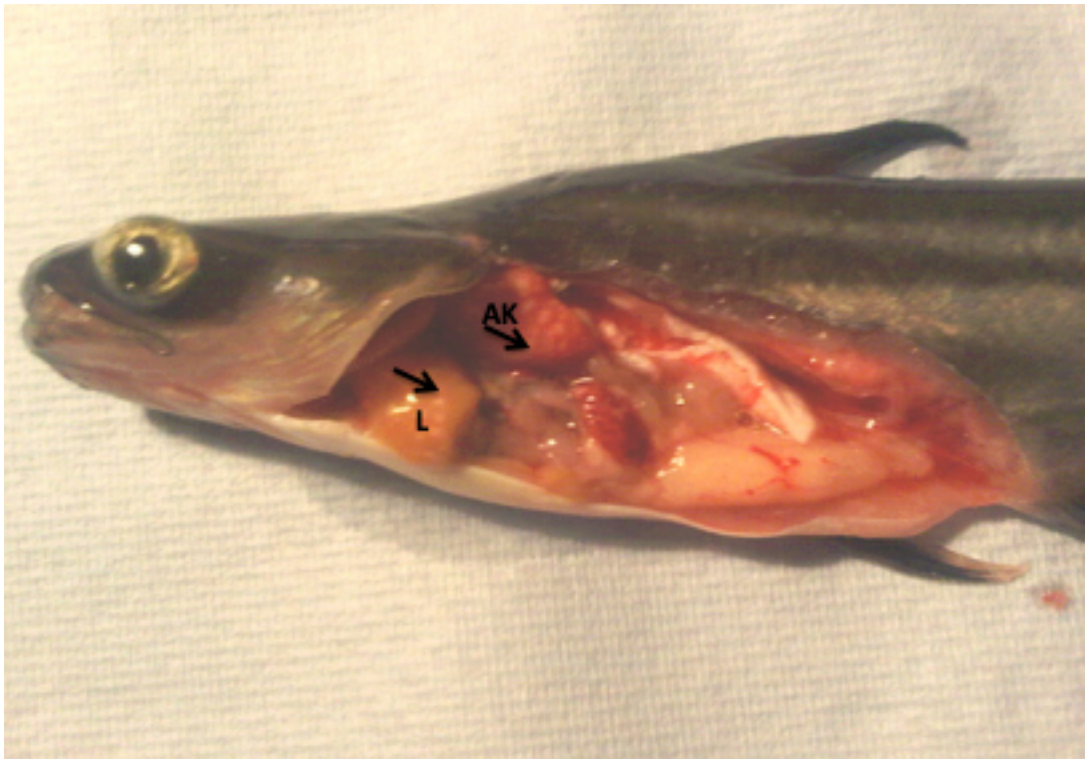
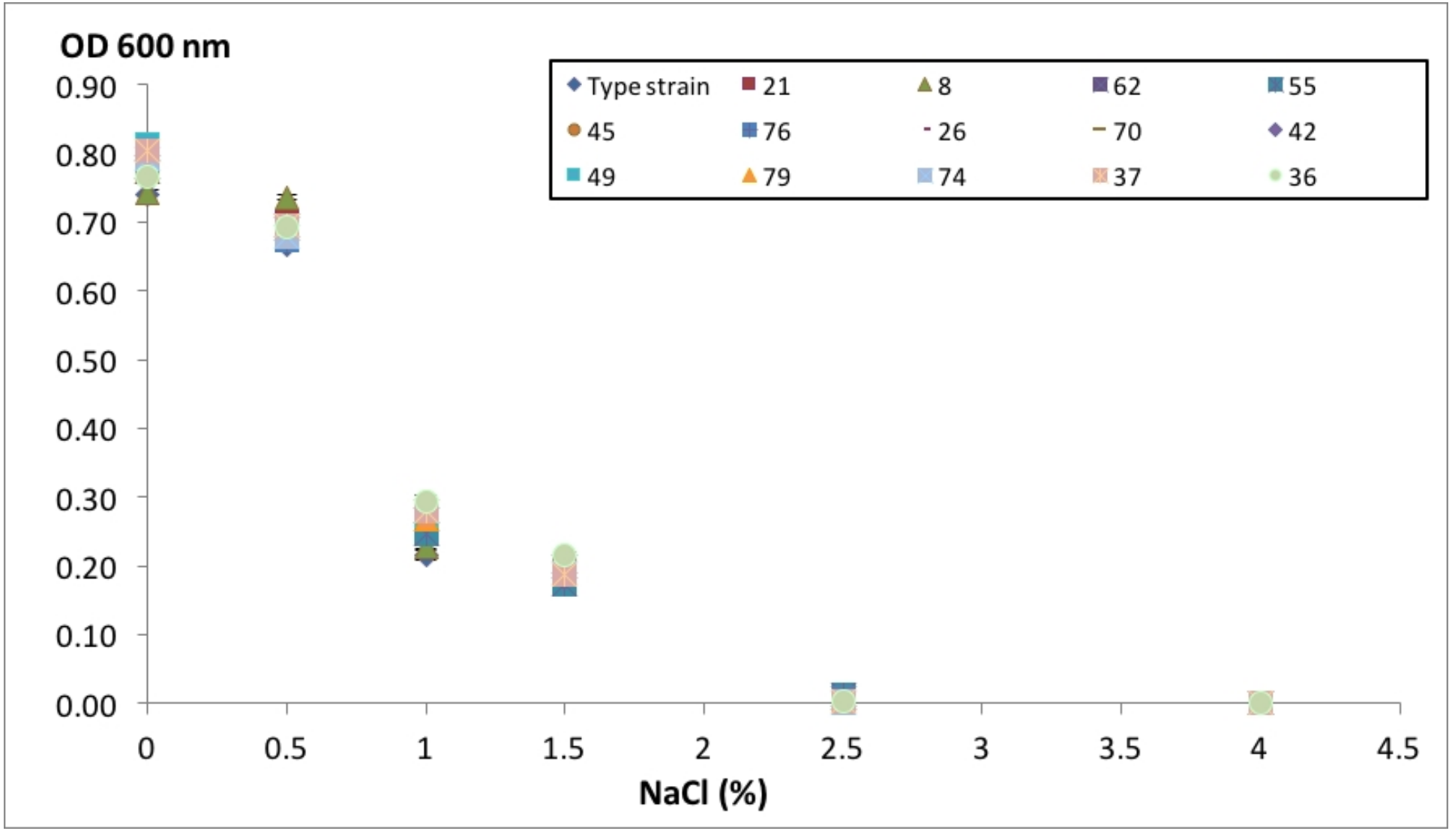


Fig. 5. Typical gross presentation of moribund fish exposed to bacteria, white lesions (arrows) observed in the anterior kidney (AK) and liver (L).


OD 600 nm
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Type strain
21
8
62
55
45
76
26
70
42
49
79
74
37
36
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
NaCl (%)

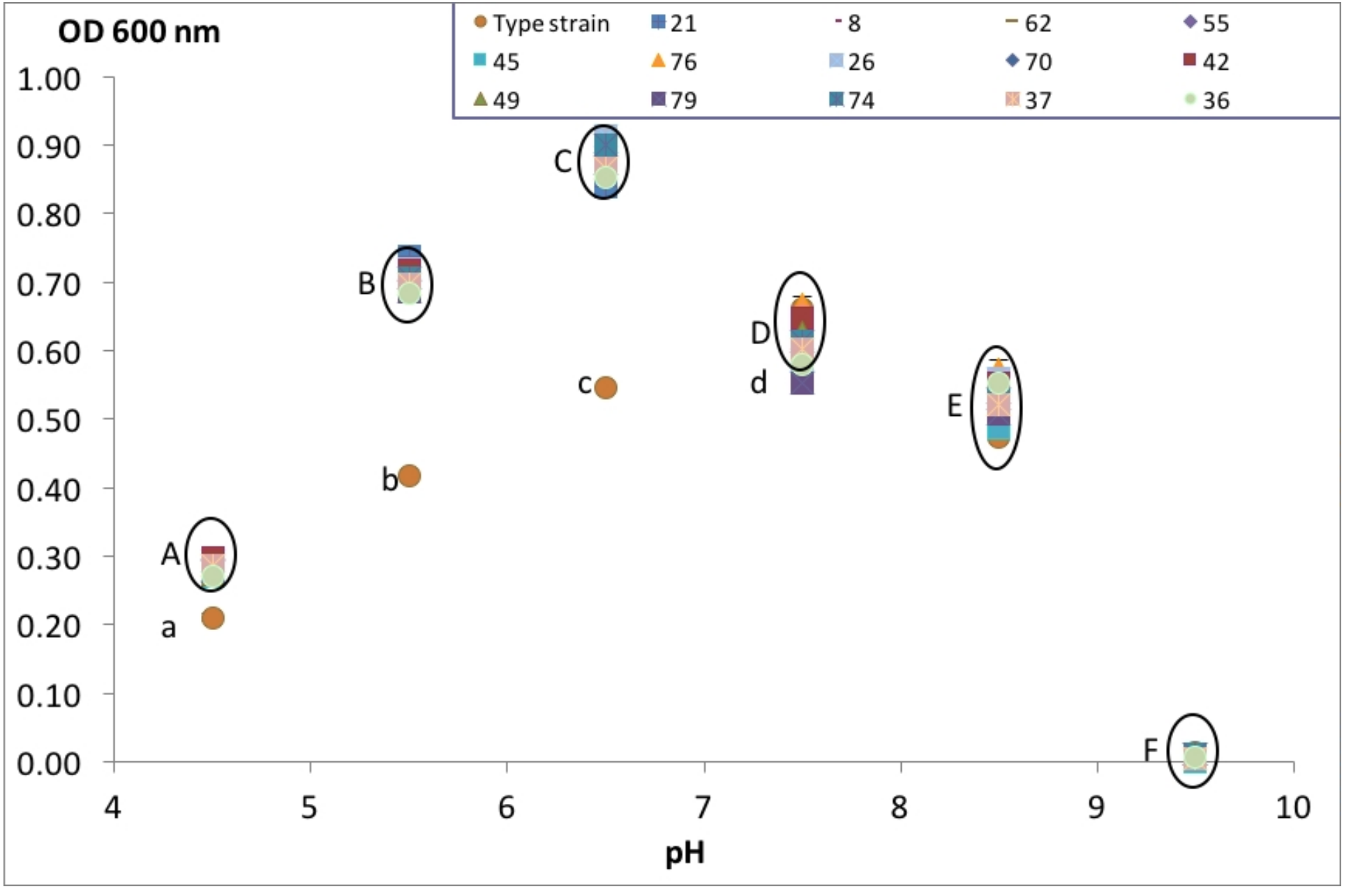

OD 600 nm
Type strain
21
8
62
55
45
76
26
70
42
49
79
74
37
36
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
4
5
6
7
8
9
10
pH
A
a
B
b
C
c
D
d
E
F

Table 1. List of *E. ictaluri* isolates according to the geographical region and year of isolation.

Province	Isolate ID	Year Recovered*
Can Tho	021	2004
	008	2002
	062	2010
Dong Thap	055	2009
	045	2007
	076	2011
	026	2005
An Giang	070	2011
	042	2006
	049	2008
Vinh Long	079	2011
	074	2011
	037	2005
	036	2005

* = each strain represents a different farm even in the same year and province.

Conflict of interest statement

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

Environmental conditions influence susceptibility of striped catfish *Pangasianodon hypophthalmus* (Sauvage) to *Edwardsiella ictaluri*

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The role(s) of all authors to the manuscript:

- Conceptualization: Nguyen Ngoc Phuoc, Randolph Richards, Magrgaret Crumlish
- Methodology: Nguyen Ngoc Phuoc, Magrgaret Crumlish
- Validation: Nguyen Ngoc Phuoc, Magrgaret Crumlish
- Formal Analysis and/or interpretation of data: Nguyen Ngoc Phuoc
- Drafting the manuscript: Nguyen Ngoc Phuoc
- Revising the manuscript critically for important intellectual content: Nguyen Ngoc Phuoc, Randolph Richards and Margaret Crumlish
- Approval of the version of the manuscript to be published: Nguyen Ngoc Phuoc, Randolph Richards and Margaret Crumlish