Disinfection effect of povidone-iodine in aquaculture water of swamp eel (*Monopterus albus*)

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The swamp eel (*Monopterus albus*) is an important commercial farmed fish species in China. However, it is susceptible to *Aeromonas hydrophila* infections, resulting in high mortality and considerable economic loss. Povidone-iodine (PVP-I) is a widely used chemical disinfectant in aquaculture, which can decrease the occurrence of diseases and improve the survival. However, environmental organic matter could affect the bactericidal effectiveness of PVP-I, and the efficacy of PVP-I in aquaculture water is still unknown. In this paper, disinfection assays were conducted to evaluate the effectiveness of PVP-I against the *A. hydrophila* in different types of water. We found that the effective germicidal concentration of PVP-I in outdoor aquaculture water was 25 ppm for 12 hours. In indoor aquaculture water with 10^5 CFU/mL bacteria, 10 ppm and 20 ppm of PVP-I could kill 99 % and 100 % of the bacteria, respectively. The minimal germicidal concentration of PVP-I in Luria-Bertani broth was 4000 ppm, respectively. Available iodine content assay in LB solutions confirmed that the organic substance had negative impact on the effectiveness of PVP-I, which was consistent with the different efficacy of PVP-I in different water samples. Acute toxicity tests showed that the 24h-LC_{50} of PVP-I to swamp eel was 173.82 ppm, which was much lower than the germicidal concentrations in outdoor and indoor aquaculture water, indicating its safety and effectivity to control the *A. hydrophila*. The results indicated PVP-I can be helpful for preventing the transmission of *A. hydrophila* in swamp eel aquaculture.

1 **Disinfection effect of povidone-iodine in aquaculture water of swamp eel**

2 **(*Monopterus albus*)**

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30   **Abstract**

31   The swamp eel (*Monopterus albus*) is an important commercial farmed fish species in China.

32   However, it is susceptible to *Aeromonas hydrophila* infections, resulting in high mortality and

33   considerable economic loss. Povidone-iodine (PVP-I) is a widely used chemical disinfectant in

34   aquaculture, which can decrease the occurrence of diseases and improve the survival. However,

35   environmental organic matter could affect the bactericidal effectiveness of PVP-I, and the

36   efficacy of PVP-I in aquaculture water is still unknown. In this paper, disinfection assays were

37   conducted to evaluate the effectiveness of PVP-I against the *A. hydrophila* in different types of

38   water. We found that the effective germicidal concentration of PVP-I in outdoor aquaculture

39   water (outdoor net cage water) was 25 ppm for 12 hours. In indoor aquaculture water

40   (dechlorinated tap water) with 10^5 CFU/mL bacteria, 10 ppm and 20 ppm of PVP-I could kill 99 %

41   and 100 % of the bacteria, respectively. The minimal germicidal concentration of PVP-I in

42   Luria-Bertani broth was 4000 ppm. Available iodine content assay in LB solutions confirmed

43   that the organic substance had negative impact on the effectiveness of PVP-I, which was

44   consistent with the different efficacy of PVP-I in different water samples. Acute toxicity tests

45   showed that the 24h-LC$_{50}$ of PVP-I to swamp eel was 173.82 ppm, which was much lower than

46   the germicidal concentrations in outdoor and indoor aquaculture water, indicating its safety and

47   effectivity to control the *A. hydrophila*. The results indicated PVP-I can be helpful for preventing

48   the transmission of *A. hydrophila* in swamp eel aquaculture.

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57   **Introduction**

58   The swamp eel (*Monopterus albus*), belongs to Order Synbranchiformes, Family

59   Synbranchidae, and is widely distributed in southern China, Japan, India and other Southeast

60   Asian countries (FishBase, http://fishbase.org/). Due to its great growth performance and rich

61   nutrient content, swamp eel has become a commercially important farmed species in China.

62   However, the intensive and stressful rearing conditions make farmed swamp eels highly

63   susceptible to bacterial pathogens, such as *Aeromonas hydrophila*, which is the main pathogen

64   causing hemorrhagic septicemia and result in high mortality and considerable economic losses

65   (Hossain et al., 2014; Nielsen et al., 2001). The clinical signs of infection include red and

66   swollen anus, visceral congestion, skin erythema and gill hemorrhage (Jagoda et al., 2014).

67   The hemorrhagic septicemia severely restricts the development prospects of the swamp eel

68   farming industry. To prevent the spread of *A. hydrophila* and outbreak of the diseases in

69   aquaculture, the direct and effective method is to reduce the amount of pathogenic bacteria in the

70   aquaculture water. Povidone-iodine (PVP-I) is an important chemical disinfection widely used to

71   disinfect pathogenic organisms and equipments in aquaculture (Scarfe, Lee & O'Bryen, 2006). It

72   has been reported as a broad-spectrum microbicide with potency to inactivate bacteria, fungi,

73   protozoans, several viruses and some spores (Wutzler et al., 2000). Moreover, PVP-I was

74   determined to be an animal drug of low regulatory priority by the Food and Drug Administration,

75   and suggested to act as an egg surface disinfectant due to the lower irritation and toxicity to

76   tissues (USFDA, 2010). However, many environmental factors can affect the efficacy of PVP-I,

77   such as temperature, pH, organic matter, etc. (Amend, 1974). The presence of organic matter can

78   result in a significant decrease in the bactericidal effectiveness of PVP-I (Rodriguez Ferri et al.,

79   2010). By far, most disinfection research has been carried out in sterile water (Chang et al.,

80   2015), and only very few reports mention use of aquaculture water for testing (Hershberger,

81   Pacheco & Gregg, 2008).

82   Swamp eel aquaculture includes outdoor cage culture and indoor tank culture. The former is

83   mainly used for large-scale commercial swamp eel production, and the latter is commonly used

84   in laboratory research. Outdoor aquaculture ponds are complex environments containing large

85   amounts of organic matter and suspended solids (Lin, 2002). However, indoor tank culture

86   mainly uses dechlorinated tap water with water changes every day, so the content of organic

87   matter of indoor culture water is very low. Different contents of organic matter may cause

88   differences in disinfection efficiency of PVP-I (Yoneyama et al., 2006). Accordingly, it is

89   necessary to test whether the organic matter in the aquaculture water is able to cause significant

90   negative effect on disinfection.

91   The aim of this study was to investigate the germicidal effect of PVP-I on *A. hydrophila* in

92   three different solutions and attempt to determine the effective disinfection concentrations to

93   control the transmission of *A. hydrophila*.

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Metrials and methods

Bacterial strain

97   Six isolates of *A. hydrophila* used in this study (Ah 1-6) were isolated from sick *M. albus* by
98   our laboratory and stored at -80 °C until used. Before disinfection assays, all isolates were
99   subcultured at 25 °C overnight on solid-phase Luria-Bertani (LB) agar (10 g/L tryptone, 5 g/L
100 yeast extract, 10 g/L sodium chloride and 15 g/L agar powder).

Water sample preparation

102 Three different water samples were prepared in this study: outdoor net cage water,
103 dechlorinated tap water and LB broth. These three types of water samples represented outdoor
104 aquaculture waters, indoor aquaculture waters and eutrophic waters, respectively. Outdoor
105 aquaculture waters were sampled from four different net cages of a swamp eel farm, and some of
106 the swamp eel in one cage had typical clinical signs of hemorrhagic septicemia. Tap water
107 samples were filtered through 0.22 μm membrane to remove impurities and microbes and
108 autoclaved at 121 °C for 15 min. LB broth was prepared with deionized distilled water.

Germicidal test

110 PVP-I solutions were prepared with different water samples immediately prior to use. For
111 outdoor aquaculture water, 1 mL of each net cage samples were treated with 5 to 100 ppm PVP-I
112 (final concentration) at 25 °C in the dark for different durations (0, 2, 4, 8 and 12 hours).

113 For indoor aquaculture water (tap water) and LB solution, *A. hydrophila* strains were used to
114 evaluate the disinfection effect of PVP-I. Briefly, for indoor aquaculture water, six *A. hydrophila*
115 isolates (Ah 1-6) were incubated in LB liquid media overnight at 25 °C, respectively. Then 5 mL
116 of each culture broth was centrifuged at 12000 rpm for 5 minute at 25 °C. The bacterial pellet
117 was washed with tap water three times and then resuspended and diluted to different

118   concentration (10^3-10^5 CFU/mL). Disinfection treatments were performed by mixing 900 μL of

119   diluted bacteria solutions and 100 μL PVP-I solution to a 1 mL volume containing 10^3-10^5

120   CFU/mL bacteria and 1.25-20 ppm of PVP-I (final concentration). For LB liquid media, only *A.*

121   *hydrophila* Ah1 isolate (isolated from skin) was used, and the disinfection treatments were

122   performed like those for the indoor aquaculture water except that the tap water was replaced by

123   LB liquid media.

124   For all above disinfection trials, treatment tubes were incubated at 25 °C in the dark with

125   gentle mixing. At each nominal exposure time (0, 2, 4, 8 and 12 hours), a 160 μL aliquot was

126   transferred to a sterile 1.5 mL microcentrifuge tube, and the equal volume of sodium thiosulfate

127   (0.004 mol/L) was added to neutralize the PVP-I immediately. After neutralization, the mixed

128   solution was tenfold serially diluted, and then 100 μL of each diluted solution was plated onto

129   LB agar plates in triplicate using sterile beads (Sanders, 2012). Plates were incubated at 25 °C

130   for 24 hours and colonies were counted. Every experiment was repeated three times.

131   **Effect of organic matter on available iodine**

132   LB liquid media was 10-fold serially diluted with ddH$_2$O. Then the 2000 ppm of PVP-I

133   solutions were prepared with serially diluted LB solutions. 1 mL of PVP-I solution was mixed

134   with 2 mL of 0.1% soluble starch solution, and the absorption was measured at 585 nm

135   immediately. The undiluted LB broth and ddH$_2$O were used as controls. The experiment was

136   repeated three times.

137   **Median lethal concentration (LC$_{50}$)**

138   Healthy swamp eel (11-15 g) were obtained from a commercial farm and acclimated in

139   dechlorinated tap water at 25±1 °C in 10 L aquarium tanks for 2 weeks until use. Daily, eels were

140   fed and the tank water replaced with fresh water. The fishes were starved for 24 hours prior to

141   and during the test to reduce the contaminations by fecal and excess food. Five concentrations of

142   PVP-I were chosen for testing purposes (100, 150, 175, 200 and 250 ppm) and a group without

143 PVP-I was used as the control. The duration of exposure was 24 hours. For each concentration

144 and control, three replications were conducted and each replication contained ten swamp eel.

145 Fish were observed every four hours and the dead fish was immediately removed from the test

146 tanks. The LC_{50} value was calculated using the Probit analysis (Lin, 2002; Lu et al., 2017). After

147 the LC_{50} test, the surviving individuals were replaced in dechlorinated tap water without PVP-I,

148 fed and the tank water replaced with fresh water daily. The status and survival of the eels were

149 monitored for at least one month. All animal procedures were conducted according to the

150 guidelines for the care and use of experimental animals established by the Ministry of

151 Agriculture of China (No. SCXK YU2005-0001). Animal Care and Use Committee (ACUC) in

152 Jiangxi Agricultural University specially approved this study.

153 **Statistical analysis**

154 SPSS17.0 was used for data analysis. Significance was evaluated by one-way analysis of

155 variance (ANOVA) using LSD test. A value of $P<0.05$ was considered to indicate a significant

156 difference. Probit analysis was used to calculate the Median lethal dose.

157

158 **Results**

159 **The effect of PVP-I in outdoor aquaculture water**

160 Four outdoor net cage water samples were marked as WA, WB, WC and WD. pH value of

161 these cage water samples were 7.12, 7.25, 7.08 and 7.32, respectively. The water sample WD

162 was taken from a cage in which hemorrhagic septicemia was discovered. According to plate

163 count, the initial cultivable bacterial concentration of these samples were $(0.70\pm0.07)\times10^3$

164 CFU/mL, $(1.59\pm0.23)\times10^3$ CFU/mL, $(0.38\pm0.04)\times10^3$ CFU/mL and $(3.96\pm0.22)\times10^3$ CFU/mL,

165 respectively. The germicidal test showed that low concentrations of PVP-I, such as 5 ppm and 10

166 ppm, could not provide effective disinfection (Fig. 1). When treated with these two

167 concentrations of PVP-I, the average survival decreased in early period, then increased and

168 finally up to about 1.5-2.5 fold and 0.8-1.5 fold of initial bacteria amount, respectively.

169 Increasing the PVP-I concentration to 25 ppm significantly improved the bactericidal effects

170 resulting in 98.08±0.17%, 98.74±0.13%, 96.49±0.03% and 99.66±0.03% mortality in four

171 outdoor water samples for 12 hours, respectively (Fig. 1). However, higher PVP-I concentrations

172 (50-100 ppm) did not significantly increase the sterilization rate (P>0.05), although 80 ppm and

173 100ppm could kill all bacteria in WA and WD samples.

The effect of PVP-I in indoor aquaculture water

175 In small-scale indoor farming, the dechlorinated tap water is often used as aquaculture water.

176 The results showed that the effects of PVP-I on six *A. hydrophila* isolates was similar in tap

177 water, and with the increase of bacterial content, the concentration of PVP-I for complete

178 sterilization increased accordingly. In tap water with 10^3 CFU/mL bacteria (Fig. 2A), 1.25 ppm

179 of PVP-I (final concentration) could reduce bacterial counts within 12 hours, but did not

180 completely eliminate the *A. hydrophila*. Increasing the concentration of PVP-I to 2.5 ppm, all

181 bacteria were killed within 4 hours. When the bacterial content was 10^4 CFU/mL (Fig. 2B), 10

182 ppm of PVP-I was required to achieve complete disinfection within 2 hours, and lower

183 concentrations (2.5 ppm and 5 ppm) could not kill all bacteria. When the bacterial content was

184 10^5 CFU/mL (Fig. 2C), although 10 ppm of PVP-I could kill more than 99% of the bacteria, to

185 achieve 100% of sterilization 20 ppm of PVP-I was required. When treated with lower

186 concentrations, the amount of culturable bacteria gradually declined to the lowest in 8 hours, and

187 then increased to different levels.

The effect of PVP-I in LB liquid media

189 LB liquid media was used as eutrophic controls. In LB liquid media, neither 500 ppm nor

190 1000 ppm of PVP-I could inhibit the proliferation of *A. hydrophila* at all bacterial concentrations.

191 For example, in LB solution with 10^3, 10^4 and 10^5 CFU/mL bacteria (Fig. 3), after 12 hours of

192 incubation with 500 ppm of PVP-I, the number of bacteria increased to 1800, 6050 and 20000

193 times to the initial number of bacteria, respectively. Increasing the PVP-I concentration to 2000

194 ppm only controlled bacteria at the lowest bacteria concentration (10^3 CFU/mL of LB medium;

195 Fig. 3A). To achieve complete disinfection, 4000 ppm were required and in this concentration all

196 *A. hydrophila* could be killed within 2 hours.

197 **Available iodine measurement**

198   LB medium contained a large amount of organic matter, so it was used to evaluate the effect

199 of organic matter on the available iodine content in this study. The results showed that the

200 concentration of organic matter could significantly affect the available iodine content, and the

201 higher LB concentrations lead to the lower available iodine contents (Fig. 4). In 2 g/L PVP-I

202 solutions, when the concentration of LB was less than 1%, the content of available iodine was

203 equivalent with that in ddH$_2$O. With the increase of LB concentration, the available iodine

204 content decreased rapidly, and when the LB concentration was 20%, the effective iodine content

205 almost reduced to zero.

206 **LC$_{50}$ test**

207   Median lethal dose of PVP-I was calculated using the Probit analysis (Table S1). According to

208 the equation: Probit(p)=-5.214+0.03$\times$lg(dose) (χ^2=6.343), the 24h-LC$_{50}$ of PVP-I to swamp eel

209 was 173.82 ppm. The individuals that survived the LC$_{50}$ test did not die within the month

210 following exposure.

211

212 **Discussion**

213   Disease is a main threat in aquaculture production and disinfection of water bodies has been

214 used as an important measure to prevent waterborne pathogen transmission in aquaculture

215 (Scarfe, Lee & O'Bryen, 2006). The present disinfection tests were mainly carried out in sterile

216 water (Hershberger, Pacheco & Gregg, 2008; Mainous, Smith & Kuhn, 2010), while very few in

217 aquaculture water. However, there is a great differences between sterile water and aquaculture

218 water, and the results obtained in sterile water are not suitable for aquaculture water. The aim of

219 this study was to evaluate the effective concentration of PVP-I against *A. hydrophila* in outdoor

220 aquaculture water and indoor aquaculture water.

221 Our study showed that PVP-I was effective to prevent *A. hydrophila* proliferation in outdoor

222 and indoor aquaculture water and there were water-specific differences in susceptibility. The

223 effective germicidal concentration of PVP-I in outdoor aquaculture water, indoor aquaculture

224 water (tap water) and eutrophic water (LB broth) were 25 ppm, 10 ppm and 4000 ppm,

225 respectively. These water-specific differences in bactericidal effect might be mainly caused by

226 organic matter. It had been proved by serially diluted LB solution, in which the higher ratio of

227 organic matter (LB medium) lead to the lower concentration of available iodine, and 20% LB

228 was sufficient to neutralize all the free iodine in 2000 ppm PVP-I solutions. This result was also

229 supported by many reports which indicated that the efficacy of PVP-I declined when organic

230 matter (e.g. blood, fish mucus, amino acids, or simple aromatic compounds) was present

231 (Truesdale & Luther, 1995; Yoneyama et al., 2006). Similar results also had been observed in

232 seawater that the content of molecular iodine reduced within hours when added to seawater

233 (Truesdale, Luther & Canosa-Masb, 1995). Considering the differences in different aquaculture

234 water, it was necessary to determine the effective disinfectant concentration before use. It should

235 be also noted that, in addition to the effects of a small amount of organic matter in tap water, the

236 inorganic matter, such as the hardness of the water, had been proved to affect the available iodine

237 content by influencing pH (Amend, 1974).

238 Disinfection can be used not only as a preventive measure in ponds or for equipments, but also

239 as a remedial measure after the outbreak of the disease. The majority of commercially available

240 disinfectants are very effective when used at high concentrations within short contact time.

241 However, for fish, prolonged exposure to high concentrations of disinfectants might be

242 dangerous (Bergmann, Monro & Kempter, 2017). Especially for extensive outdoor aquaculture,

243 it is impossible to change the water after disinfection. Therefore, it was necessary to determine

244 the toxicity of disinfectants to fish (LeValley, 1982). In this study, the 24h-LC_{50} for *M. albus* was

245 173.82 ppm, which was much higher than the effective germicidal concentration in aquaculture

246 water, suggesting that PVP-I could be used safely as a disinfectant for the culture of *M. albus*.

247 On the other hand, the commercial standard procedure for PVP-I is to pour directly into the

248 water with the final concentration of 0.075-0.093 ppm for prevention and 0.093-0.125 ppm for

249 treatment (Wang et al., 2015). Our data showed that these recommended concentrations were far

250 less than the effective concentrations and would not have any germicidal effect on *A. hydrophila*.

251 Compared with higher PVP-I concentration, the lower PVP-I concentrations reduced the

252 toxicity to *M. albus*, but reduced the bactericidal effect. For example, in outdoor aquaculture

253 water, indoor aquaculture water (tap water) and LB medium, when treated with non-lethal

254 concentration, the number of cultivable bacteria decreased first, then increased during 12 hours.

255 Similar phenomenon was also reported in seawater when treated with some disinfectants (sodium

256 hypochlorite, bleaching powder, formalin) for 12 hours (Wang et al., 2015). The increase of

257 bacteria in later period might be due to the decrease of available iodine content and the

258 proliferation of surviving bacteria. On the one hand, the content of available iodine decreased

259 significantly with the passage of time (Chang et al., 2015); on the other hand, the organic matter

260 in the solution would neutralize free iodine (Takeda et al., 2016). Meanwhile, the organic matter

261 in water samples could supply nutrients for the proliferation of the surviving culturable bacteria.

262 Our results supported this hypothesis; the earliest rise and largest increase of the number of *A.*

263 *hydrophila* happened in LB broth which contains the most abundant organic matter, followed by

264 outdoor aquaculture water and tap water. Moreover, Kersters et al (1996) reported that *A.*

265 *hydrophila* was able to grow and proliferation in nutrient-poor filtered and autoclaved tap water,

266 which made it hard to control the proliferation of *A. hydrophila* effectively.

267 It should be noted that we did not consider the biofilm formation and viable but non-culturable

268 (VBNC) state of *A. hydrophila*. It was reported that the biofilms of *A. hydrophila* was more

269 resistant to disinfectants than planktonic cells (Jahid & Ha, 2014). Although there was no work

270 about the relationship between the VBNC of *A. hydrophila* and disinfection, it has been

271 demonstrated that stressed and starved *A. hydrophila* could enter a VBNC state (Pianetti et al.,

272   2008; Rahman, Suzuki & Kawai, 2001). Disinfectants, such as hypochlorous acid, similarly have

273   induced *Escherichia coli* and *Salmonella typhimurium* into the VBNC state (Oliver, Dagher &

274   Linden, 2005). In this study, when PVP-I concentrations were lower than the effective

275   sterilization concentration, the bacteria might not be completely killed and some of them went

276   into the VBNC state. So the early decline in the number of bacteria might be caused by death

277   cells and VBNC state cells together. As for the subsequent increase in the number of bacteria in

278   outdoor aquaculture water, tap water and LB medium, whether it was associated with the

279   resuscitation and growth of some mildly injured VBNC cells, further study was needed. But,

280   there was some evidence that VBNC bacteria could resuscitate and proliferate under certain

281   conditions (Dukan, Levi & Touati, 1997). Moreover, the use of disinfectant might result in the

282   development of resistant strains. Nuñez reported the resistant gram-negative rods strains for

283   chlorhexidine, such as *A. hydrophila*, *Shigella flexneri* (Nuñez & Moretto, 2007). In addition,

284   resistant *Staphylococcus* sp. for PVPI were also isolated from hospital wastewater (Nuñez &

285   Moretto, 2007) and some instances have been described that iodophors had been found to be

286   contaminated with *Pseudomonas* sp (Anderson et al., 1984). However, there has been no report

287   of *A. hydrophila* tolerant to PVPI so far.

288   This study confirmed the effect of organic matter on PVP-I sterilization and suggested that

289   aquatic organic matter should be considered when PVP-I was used in aquaculture. The results

290   showed the great bactericidal activities of the PVP-I in different aquaculture waters, and we

291   recommended 25 ppm for outdoor aquaculture and 20 ppm for indoor treatment, which were safe

292   and effective for normal swamp eel farming.

293

294   Conclusions

295   In conclusion, the organic matter in aquaculture water has negative influence on the

296   bactericidal effectiveness of PVP-I. The minimum effective bactericidal concentration of PVP-I

297   in indoor aquaculture water and outdoor aquaculture water was 10 ppm and 20 ppm, respectively.

298   The results suggest PVP-I could help prevent the transmission of *A. hydrophila* in swamp eel

299   aquaculture.

300

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Figure 1

Bactericidal efficacy of different povidone-iodine concentrations in four outdoor aquaculture water WA (A), WB (B), WC (C) and WD (D).

At each time point, the capital letters above the columns show extremely significant differences (P<0.01); the little letters above the columns show significant differences (P<0.05). The y-axis indicates the percent survival [percent survival = (CFU of treatments/CFU of controls)×100 %]. Data are shown as means ± SEM (n=3).
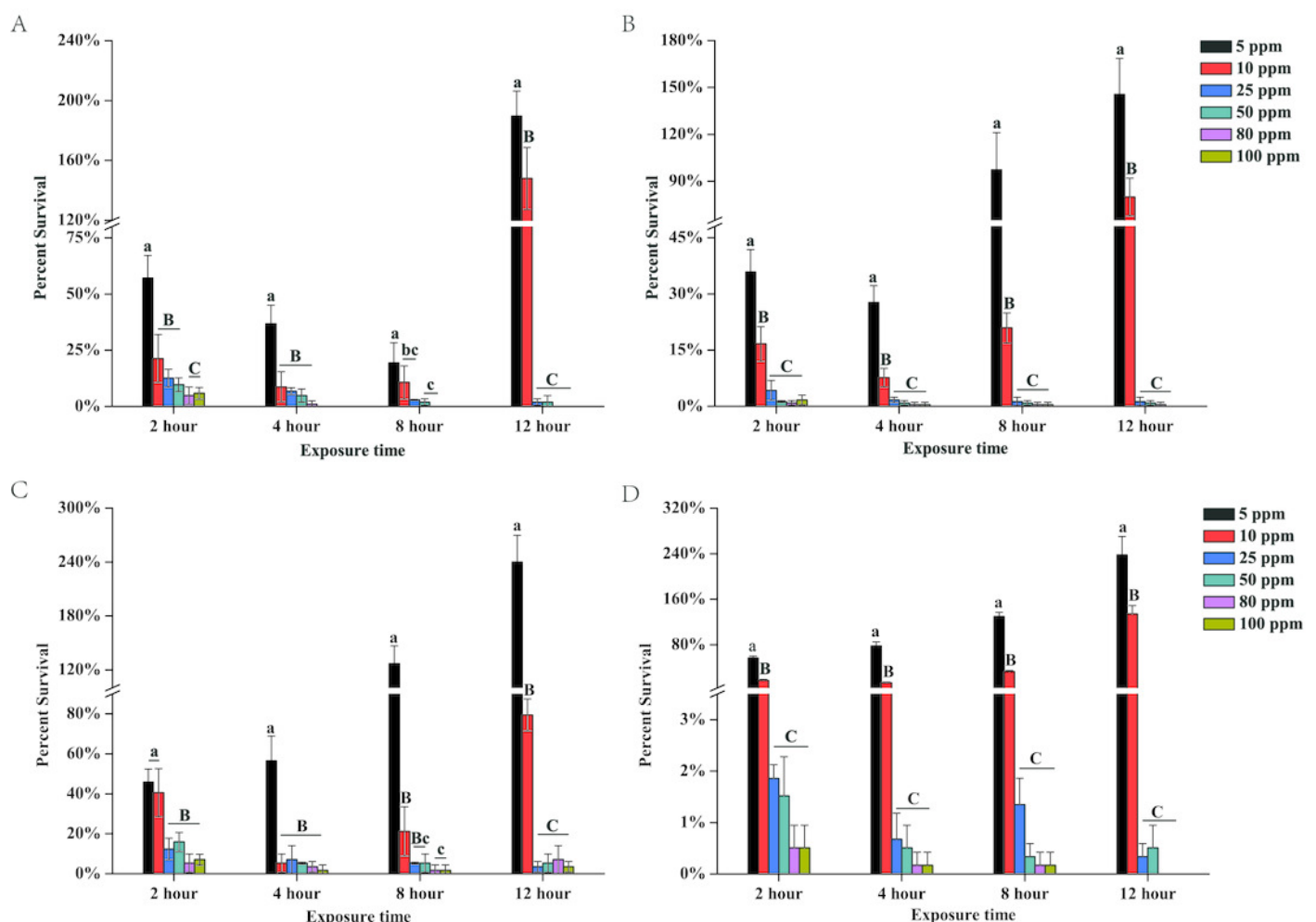
Figure 2

Bactericidal efficacy of povidone-iodine against six Aeromonas hydrophila isolates (Ah 1-6) in indoor aquaculture water (tap water).

The test bacterial concentration were 10^3 CFU/mL (A), 10^4 CFU/mL (B) and 10^5 CFU/mL (C).

The y-axis indicates the percent survival. Data are shown as means ± SEM (n=3).
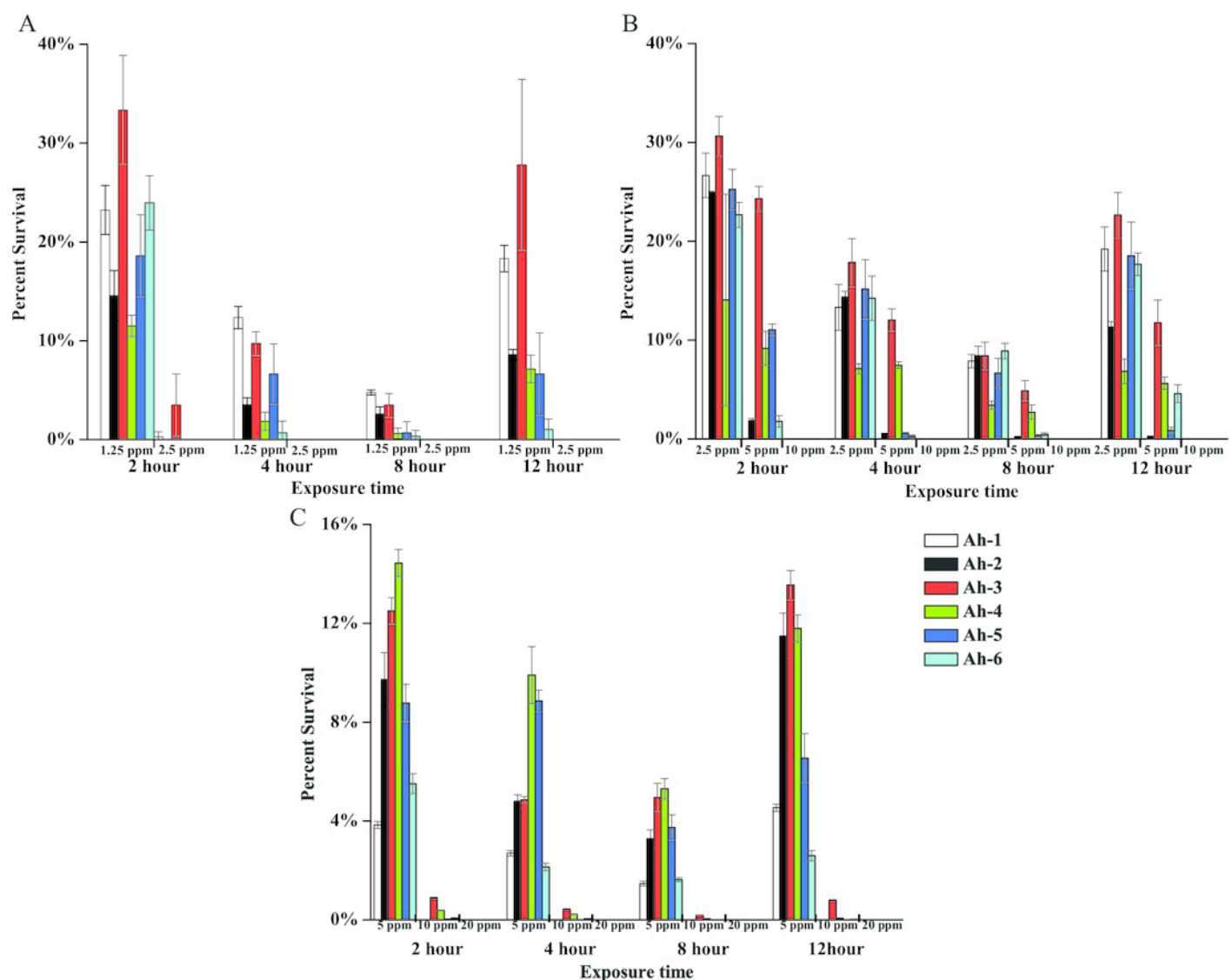
Figure 3

Bactericidal efficacy of povidone-iodine against *Aeromonas hydrophila* isolate (Ah 1) in Luria-Bertani broth. The tested bacterial concentration were 10^3 CFU/mL (A), 10^4 CFU/mL (B) and 10^5 CFU/mL (C).

At each time point, the capital letters above the columns show extremely significant differences ($P<0.01$), the little letters above the columns show significant differences ($P<0.05$). The y-axis indicates the non-percent survival (non-percent survival = CFU of treatments/CFU of controls). Data are shown as means ± SEM (n=3).
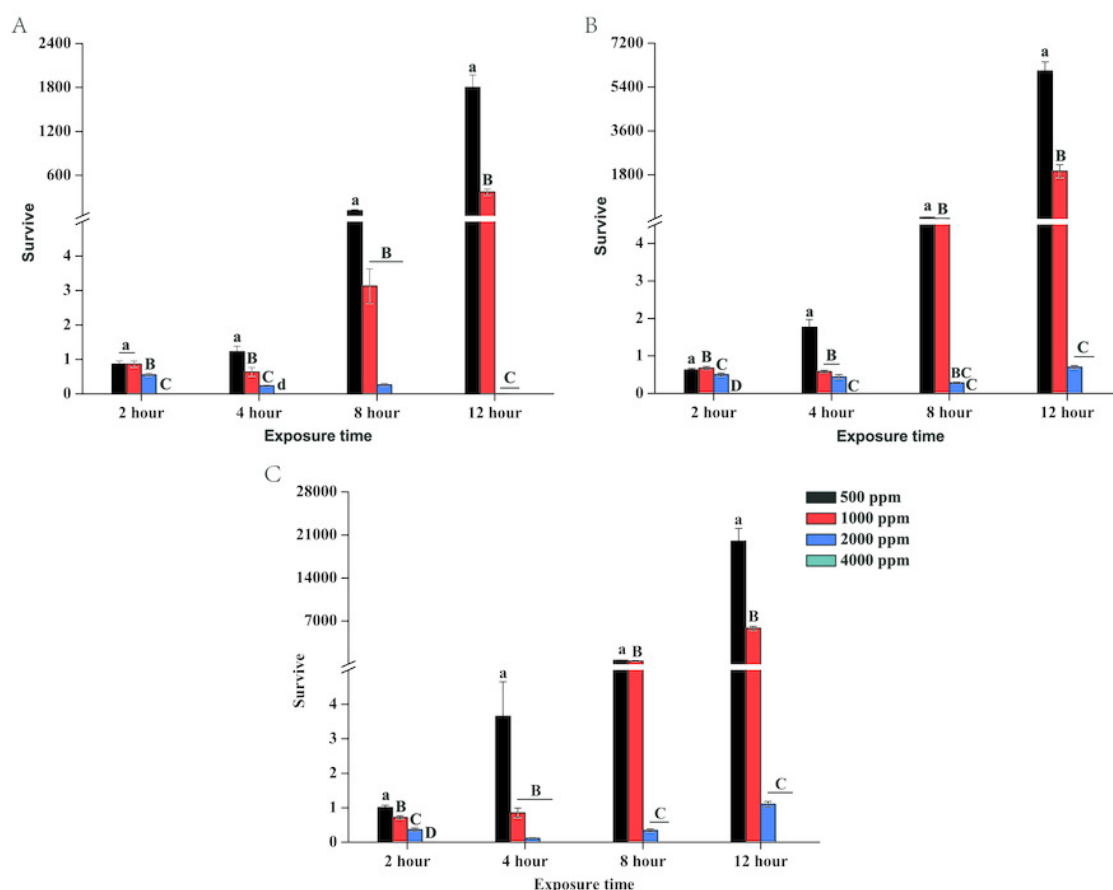
Figure 4

Effect of different LB concentrations on the effective iodine content of povidone-iodine.

Data are shown as means ± SEM (n=3).