The Israeli Journal of Aquaculture - Bamidgeh, IJA_73.2021.1146448, 9 pages
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The inhibitory effects of disinfectants against *Aeromonas salmonicida* at low temperatures

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Keywords: *Aeromonas salmonicida,* temperature, disinfectants, pH, inhibition effect

Abstract

Aeromonas salmonicida is a fish pathogen that has resulted in significant losses to the aquaculture industry worldwide. In the winter of 2018/2019, a serious outbreak of *A. salmonicida* infection of grass carp (*Ctenopharyngodon idella*) occurred in China. Since *C. idella* do not feed at low water temperatures, antibiotics cannot be delivered orally, thus the use of disinfectants is the only viable option to control the disease in the winter months. Therefore, the aim of this study was to elucidate the inhibitory effects of different disinfectants against *A. salmonicida* at various temperatures and pH values. The microdilution method was used to determine the minimum inhibitory concentration (MIC) of five disinfectants against *A. salmonicida* at different temperatures. The results showed that *A. salmonicida* was highly adaptable to temperatures ranging from 8 to 35 °C and pH values from 5 to 9. Moreover, based on the results, benzalkonium bromide (BB) had the strongest inhibitory effect at every temperature. Since *A. salmonicida* has strong environment adaptability, BB should be chosen to control the disease at low temperatures.

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Introduction

Aeromonas salmonicida is a Gram-negative, facultative, anaerobic, non-motile bacterium that causes furunculosis and septicemia in a variety of fish species, resulting in severe economic losses to the aquaculture industry worldwide (Austin & Austin, 1999). Cold-water fish, such as the rainbow trout (*Oncorhynchus mykiss*) (Wiklund & Dalsgaard, 1998), pink salmon (*Oncorhynchus gorbuscha*) (Kimura, 1969), and grayling (*Thymallus thymallus*) (Rintamäki & Valtonen, 1991), are especially susceptible to *A. salmonicida* infection. However, the range of susceptible species is reportedly expanding to include warm-water species due to the ability of *A. salmonicida* to adapt to a wide range of temperatures. Various studies have reported *A*. *salmonicida* infection in a diversity of species, including the common dab (*Limanda limanda*) (Vercauteren et al., 2018), European perch (*Perca fluviatilis*) (Rupp et al., 2019), common carp (*Cyprinus carpio*) (Maurice & Tinman, 2000), Crucian carp (*Carassius carassius*) (Han et al., 2013), and largemouth bronze gudgeon (*Coreius guichenoti*) (Long et al., 2016).

At present, methods used to control *A. salmonicida* include the use of vaccines, immunostimulants, and bacteriophages (Austin & Austin, 2016). However, there is no officially approved *A. salmonicida* vaccine in some Asian countries, including China, which has hampered efforts towards the prevention of outbreaks of *A. salmonicida*-associated diseases. For large-scale disease outbreaks, oral administration of antibiotics is the first-choice treatment strategy because quinolones, florfenicol, sulphonamides, streptomycin, and ciprofloxacin have historically been quite efficacious against *A. salmonicida* (Inglis & Richards, 1991, Kirkan et al., 2003). However, all poikilotherm organisms including fish species, such as carp (*C. carpio*), crucian (*Carassius auratus*), bass (*Lateolabrax japonicus*), and grass carp (*Ctenopharyngodon Idella*) do not feed at low temperatures, thus oral antibiotics are insufficient to control the spread of *A. salmonicida* during the winter months (Woo & Bruno, 2011). Therefore, in addition to strengthening management strategies, the use of an effective disinfectant is one of the few options to control *A*. *salmonicida* infection in order to prevent wide-spread disease outbreaks in cold-water systems. Although the inhibitory effect of most disinfectants strengthens with temperature, the effectiveness of disinfectants at low temperatures in the winter remains unclear.

A dramatic epidemic of *A. salmonicida* infection of *C. idella* emerged throughout China during the winter of 2018/2019. The disease characteristics included skin ulceration as well as bleeding and swelling of internal organs. During the disease outbreak, the water temperature in different ponds ranged from 8 to 18 ºC, while the pH ranged from 6.5 to 8.0. The mortality rate in some provinces was as high as 70%. Farmers attempted to treat the disease by the administration of oral antibiotics, but the water temperature was very low and the *C. idella* did not feed, thus the antibiotics could not reach target organs. Therefore, external use of disinfectants became the only option. Moreover, since the water temperature was generally below 20 ºC, even dropping below 10 ºC during the disease outbreak, the choice of an appropriate disinfectant to achieve an inhibitory effect was problematic. Accordingly, the aim of the present study was to quantify the inhibitory effects of disinfectants against *A. salmonicida* at low temperatures to provide references for the treatment of similar aquatic diseases under similar conditions.

Materials and Methods

Bacterium and reagents

A. salmonicida strain FYp0313 was isolated from diseased *C. idella* and stored in the Fish Disease Lab of Sichuan Agricultural University (Chengdu city, Sichuan province, China).

Trace biochemical reaction tubes were purchased from Qingdao Hope Bio-Technology Co., Ltd. (Qingdao City, Shandong Province, China). Physiological and biochemical tests were performed at 28 °C unless otherwise specified. The bacteria were inoculated into trace biochemical reaction tubes and then cultured at 28 °C for 24–48 h for identification of biochemical characteristics. Representative biochemical tubes are shown in **Table 1**.

Table1 Biochemical characteristics of the isolated strain

Test	***FYp0313***	***A. salmonicida subsp.***				
		salmonicida	***achromogenes***	***masoucida***	***smithia***	***pectinolytica***
Acid from d-glucose	+	+	+	+	[+]	+
ONPG	+	d	d	d	+	+
Acid from maltose	+	+	+	+	-	ND
Arginine double hydrolase	+	+	+	+	[-]	ND
Lysine decarboxylase	+	d	d	d	-	ND
Gas from d-glucose	+	+	-	+	[+]	+
Acid from d-mannitol	+	+	-	+	-	+
Heptapeptide hydrolysis	+	+	-	+	-	ND
Pigment, brown	-	+	-	-	-	+
Acid from d-lactose	-	+	+	+	-	+

Note: "+", >90% or >85% positive; "—", >90% or >85% negative; "[+]", 76%–89% positive; "[-]", 11%–25% positive; "d", 11%–89% positive; and "ND", not determined.

Abbreviation: ONPG, Ortho-nitrophenyl-β-galactoside.

Before formal experiments, we used ten commercial disinfectant stock solutions [bromochlorohydantoin, chlorine dioxide, benzalkonium bromide (BB), diallyl trisulfide, glutaraldehyde (GA), benzalkonium bromide + methylene dithiocyanate (BB+MBT), povidone iodine, methylene dithiocyanate (MBT), formaldehyde (FO), potassium permanganate] in the preliminary experiment to detect the disinfectants effectiveness, and five effective available disinfectants (main ingredients: BB, GA, BB+MBT, MBT, and FO) were selected to quantify the inhibitory capacities against *A. salmonicida* at different temperatures. Detailed information of the five disinfectants below in **Table 2**.

Table 2 Primary information of five disinfectants

Disinfectants	**Concentrations**	**Origin**
BB	*45%*	*Suizhou, China*
GA	*20%*	*Suizhou, China*
BB+MBT	*20% BB, 3.2% MBT*	*Huanggang, China*
MBT	*4%*	*Xi'an, China*
FO	*40%*	*Chengdu, China*

Abbreviations: BB, benzalkonium bromide; BB+MBT, benzalkonium bromide + methylene dithiocyanate; FO, formaldehyde; GA, glutaraldehyde; MBT, methylene dithiocyanate.

Animal infection assay

Healthy *C. idella* (n = 100; mean length, 8 ± 0.5 cm) were purchased from a commercial fish farm (Yingtan, Jiangxi province, China) and acclimated in our laboratory for 2 weeks before experimentation. The aquaria had an uninterrupted oxygen supply to ensure a dissolved oxygen content of more than 5 mg L^{-1}, pH of 6.5–8, and temperature of ~15 ºC. The tank water was aerated and 30% was replaced every day. Fish were fed twice daily with a commercial diet. Of the initial 100 *C. idella*, 30 with bright body colors that were responsive, robust, and healthy were divided into three groups (control group and two experimental groups) of 10 each. Fish in the control group received an intraperitoneal (IP) injection of 0.1 mL of saline (0. 85%), while those in the two experimental group received an IP injection 0.1 mL of 1×10^7 or 1×10^8 purified bacteria, respectively. All fish were examined daily for clinical signs and mortality for 1 week. Bacteria were isolated from the liver, kidney, and spleen of moribund fish and identified. All animal protocols were approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University (permit no. DY-2019202033001).

Growth of A. salmonicida at different temperatures and pH values

The growth of *A. salmonicida* was monitored at various temperatures and pH values, which are key indicators of water quality (Abbink et al., 2012).

A single colony was inoculated to 100 mL of sterile lysogeny broth (LB) liquid medium and incubated at 28 °C for 18 h while rotating at 180 rpm as a stock solution. Then, 0.4

ml of the stock solution were inoculated in 8 mL of sterile LB liquid culture medium and incubated at 8, 18, 28, and 35 °C, respectively. Three replicates were set at each temperature. Samples were collected at 12, 24, 36, 60, and 84 h post inoculation. The optical density at 600 nm (OD_{600}) of each sample was detected using an ultraviolet spectrophotometer.

The prepared LB liquid medium inoculated with 0.4 ml of the stock solution was adjusted to different pH values (i.e., 5, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10, and 10.5) with the use of NaOH and incubated at 28 °C. Samples were collected and the absorbance at OD_{600} was determined at 12, 24, 36, 60, and 84 h.

Minimum inhibitory concentration (MIC) of different disinfectants at different temperatures

At temperatures of 8, 18, and 28 ºC, the MIC of the disinfectants was determined using the microdilution protocol proposed by the Clinical and Laboratory Standards Institute guidelines. The minimum bacteriostatic concentration was defined as the lowest disinfectant concentration that inhibited visible bacterial growth.

Statistical analysis

The results are expressed as the mean ± standard deviation of three replicates. The significance of differences was determined by variance analysis. One-way analysis of variance (ANOVA) was performed to determine whether the differences between groups were significant. All statistical analyses were performed using IBM SPSS Statistics for Windows, version 20.0 (IBM Corporation, Armonk, NY, USA). A probability (p) value of < 0.05 was considered statistically significant. In the figures, significant intra- and intergroup differences are indicated by uppercase and lowercase letters, respectively.

A. salmonicida is pathogenic to C. idella

C. idella were infected with purified *A. salmonicida* by IP injection (n = 20 fish; mean length, 8 ± 0.5 cm). The cumulative mortality percentage of the challenged fish was 80% within 1 week (**Figure 1**). The clinical signs and gross lesions of the infected fish were similar to those observed in naturally infected fish. In contrast, there were no signs of disease or mortality observed in the control group during the experiment (**Figure 1**). The results indicated that *A. salmonicida* strain FYp0313 was pathogenic.

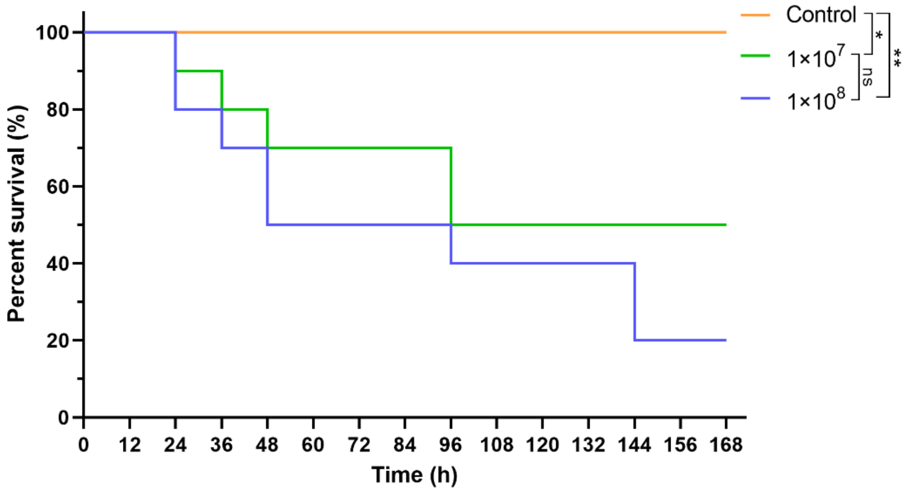

Figure 1 Survival percentage (%) of *C. idella* infected with *A. salmonicida* strain FYp0313 $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. the control group. ns, not significant.

The classification of A. salmonicida strain FYp0313

At present, it is widely believed that *A. salmonicida* includes five subspecies: the typical *A. salmonicida* subsp. *salmonicide* and the atypical *A. salmonicida* subsp. *pectinolytica*, *masoucida*, *achromogenes*, and *smithia (Nash et al., 2006, Dalsgaard et al., 2010)*. Our results showed that the biochemical characteristics of *A. salmonicida* strain FYp0313 were most similar to those of subsp. *masoucida* (similarity, 90%; **Table 2**) *(Mitchell* & *Goodwin, 2006)*. However, the biochemical results of acid from D-lactose differed from the conventional biochemical standards of subsp. *masoucida*, indicating the strain FYp0313 showed some variability. Therefore, the prevention of *A. salmonicida* infection is imperative.

Tolerance of A. salmonicida to different temperatures

Temperature is an important factor in aquaculture. Hence, the growth of *A. salmonicida* was monitored at four temperatures (i.e., 8, 18, 28, and 35ºC). The results showed that as a cold-water pathogen, *A. salmonicida* grew well at a relatively high temperature of 35 °C, as well as lower temperatures of 8 and 18 °C (**Figure 2**), demonstrating that *A. salmonicida* strain FYp0313 could adapt to temperatures ranging from 8 to 35 °C.

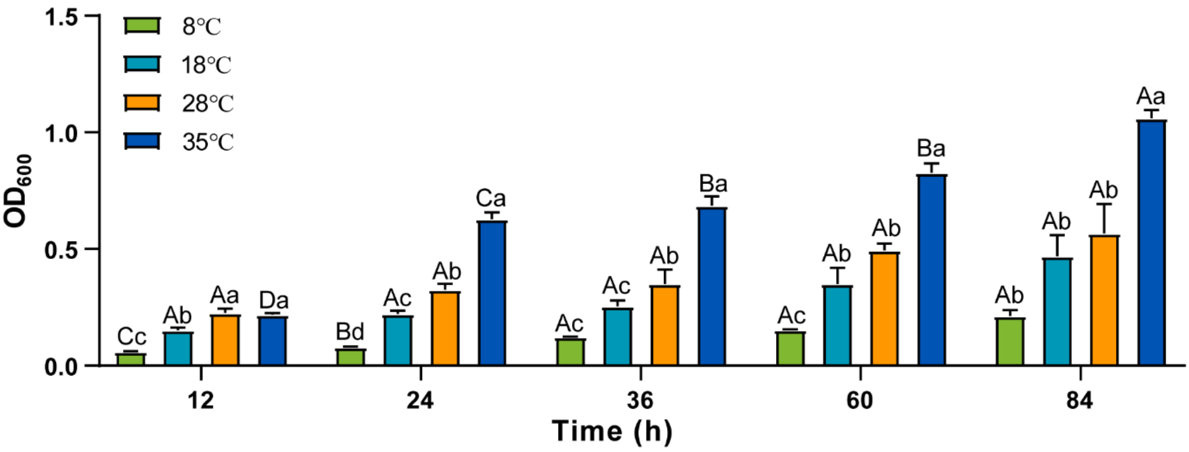

Figure 2 Growth of *A. salmonicida* strain FYp0313 at different temperatures
ANOVA was used to identify significant differences ($p < 0.05$) between and within groups, as indicted by uppercase and lowercase letters, respectively.

Tolerance of A. salmonicida strain FYp0313 to different pH values

As an important water quality index, growth of *A. salmonicida* strain FYp0313 was monitored at different pH values. As shown in **Figure 3**, *A. salmonicida* grew well at pH values from 5 to 9, but had decreased at a pH value > 9 (**Figure 3**). These results indicated that *A. salmonicida* had strong pH tolerance and grew well at a wide range of pH values (5–9).

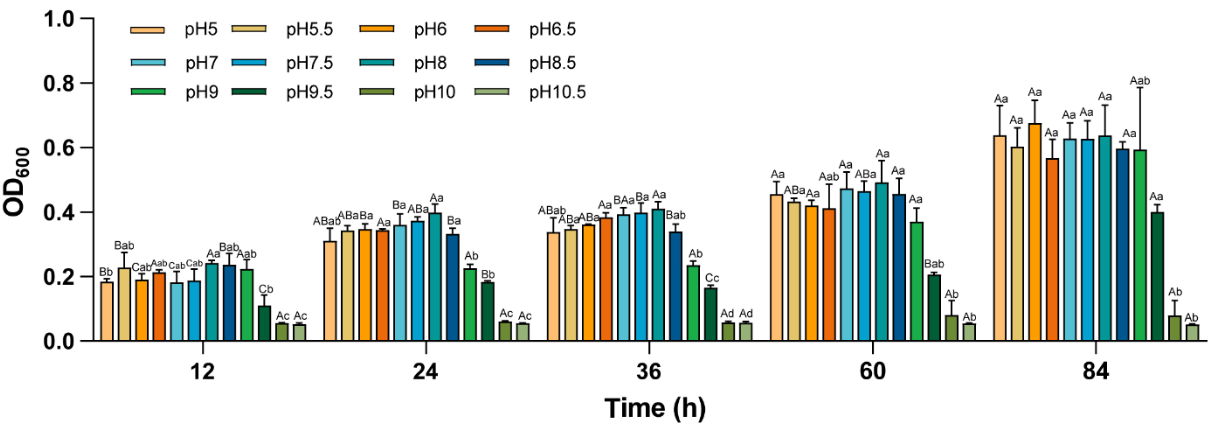

Figure 3 Growth of *A. salmonicida* strain FYp0313 at different pH values
ANOVA was used to assess significant differences ($p < 0.05$) in the growth of *A. salmonicida* strain FYp0313 at different temperatures and pH values between and within groups, which are indicated by uppercase and lowercase letters, respectively.

MIC of different disinfectants at different temperatures

The results of the present study showed that *A. salmonicida* strain FYp0313 adapted to a wide range of temperatures and pH values. However, the sensitivity of this bacterium to various disinfectants at different temperatures and pH values remains unknown. Disinfectants are often used to prevent and treat diseases in aquaculture, especially in situations where antibiotics cannot be orally administered (Bowker et al., 2011, Boyd & Mcnevin, 2014). The inhibitory capacities of disinfectants against *A. salmonicida* strain FYp0313 at low water temperatures remain vague. The inhibitory capacities of five frequently used disinfectants against *A. salmonicida* strain FYp0313 were assessed at four different temperatures (i.e., 8, 18, and 28 °C) to identify those effective at lower water temperatures.

After 24 h of cultivation, the inhibitory effects of different disinfectants against *A. salmonicida* strain FYp0313 were assessed at different temperatures (**Figure 4**). At 28 °C, the inhibitory effect of BB was the strongest among the five disinfectants, followed by MBT and FO. The antibacterial effect of BB+MBT was slightly weaker than that of FO, while that of GA was the weakest. At 18 °C, the inhibitory effect of the five disinfectants was consistent with the results at 28 °C, where BB had the strongest effect and GA had the weakest. At 8 °C, BB had the strongest antibacterial effect, followed by BB+MBT. The antibacterial effects of MBT and FO were slightly lower, while that of GA was the lowest. In summary, at 8, 18, and 28 °C, BB had the strongest inhibitory effect, while GA and FO had the weakest. However, the effect of the same disinfectant also varied at different temperatures. As shown in **Table 3**, the MIC of BB+MBT was 0.33 μL L^{-1} at 28°C and 18°C, but only 0.17 μL L^{-1} at 8 °C, suggesting that these two disinfectants had stronger inhibitory effects at lower temperatures, while BB had the strongest at all four temperatures.

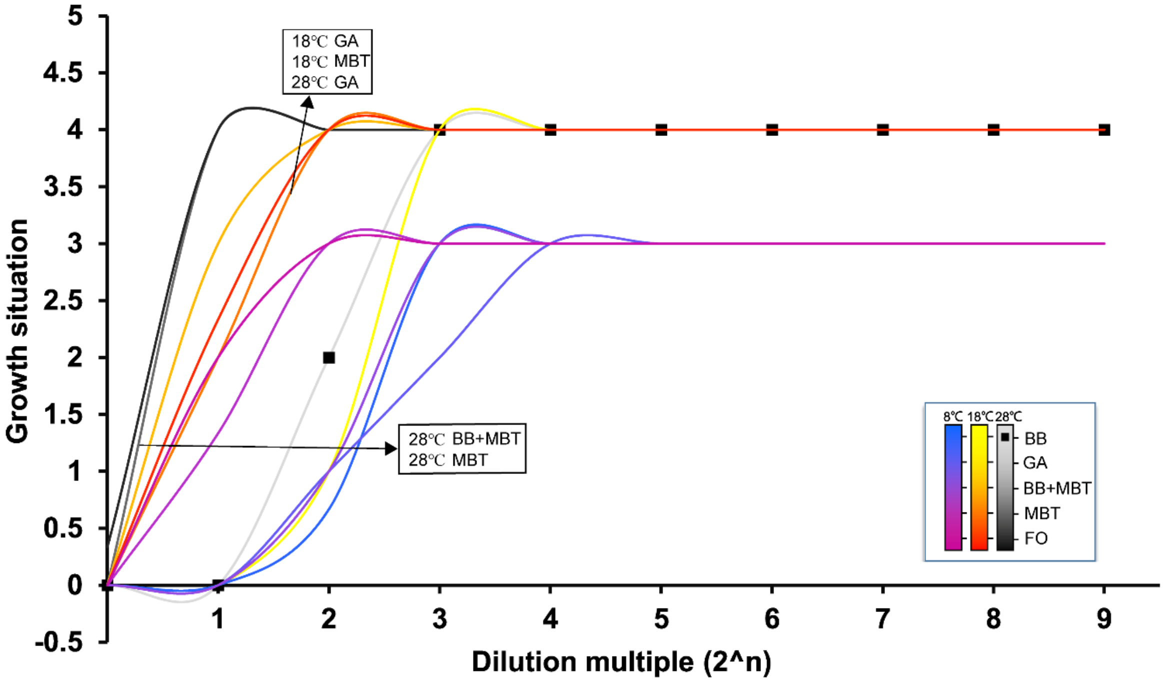

Figure 4 The MIC of different disinfectants against *A. salmonicida* strain FYp0313 at different temperatures. The x-axis represents the dilution multiple, and 2^n represents the double dilution. The y-axis represents the inhibitory effect of different wells after being graded and quantified. The quantify criteria of each well: "0" repents clear and transparent, "1"represents mild turbid, "2"represents moderate turbid, "3" represents severe turbid and "4"represents marked turbid. Different colors indicate different temperatures (orange, 8°C; blue, 18°C; and purple, 28°C). Abbreviations: BB, benzalkonium bromide; BB+MBT, benzalkonium bromide + methylene dithiocyanate; FO, formaldehyde; GA, glutaraldehyde; MBT, methylene dithiocyanate

Table 3 MIC of different disinfectants against *A. salmonicida* at 28 h at different temperatures

Disinfectant	***8℃***		***18℃***		***28℃***	
	MIC (μL /L)	***SD***	***MIC (μL /L)***	***SD***	***MIC (μL /L)***	***SD***
BB	0.083	0.029	0.1	0	0.1	0
GA	2.5	0	5	0	5	0
BB+MBT	0.17	0	0.33	0	0.33	0
MBT	0.2	0	0.2	0	0.2	0
FO	0.2	0	0.2	0	0.2	0

Abbreviations: BB, benzalkonium bromide; BB+MBT, benzalkonium bromide + methylene dithiocyanate; FO, formaldehyde; GA, glutaraldehyde; MBT, methylene dithiocyanate.

Discussion

A. salmonicida strain FYp0313 is a highly pathogenic bacterium. Although *A. salmonicida* is thought to have a predilection for salmonids, many studies have reported that some warm-water fish, such as *Cyprinidae* sp., are also susceptible to *A. salmonicida* infection (A. Plumb & Hanson, 2011, Nikapitiya et al., 2019). Therefore, we speculated that *A. salmonicida* has strong environmental adaptability and can grow and reproduce in harsh environments. Four water temperatures (i.e., 8, 18, 28, and 35 °C) and different pH values (range, 5–10.5) were selected to determine the growth of *A. salmonicida* under different environmental conditions. The results showed that *A. salmonicida* grew well at temperatures ranging from 8 to 35 °C and pH values ranging from 5 to 9, although growth was inhibited at pH values > 9. Hence, which might explain why *A. salmonicida* has a wider host range than previously thought.

In this study, the inhibitory effects of five disinfectants at four different temperatures against *A. salmonicida* were assessed. The five kinds of disinfectants we selected through the preliminary experiment are generally recognized as common disinfectants in production, with relatively low cost and low toxicity. Besides sterilization and bacteriostasis, they also have the effect of improving water quality. The results showed that BB had the strongest inhibitory effect at 8 and 18 °C. At 18 and 28 °C, the inhibitory effect of GA and BB+MBT was decreased, while that of the other disinfectants was unchanged. Therefore, BB should be applied at lower temperatures. So, how to prevent the infection of *A. salmonicida* in aquaculture? Generally, disinfectant such as BB can be used before the early stage of aquaculture, which was contributed to prevent bacterial infections by improving the water environment and inhibiting the growth of bacterial. In addition, to ensure the health of the fish body can prevent secondary infections of bacteria. Due to the differences in the water quality and suspended particles in different breeding ponds, in the actual disinfection process, it is necessary to increase or decrease the dose of disinfectant according to the specific conditions of the pond.

Previous studies have shown that the use of disinfectants in combination could increase or decrease bacteriostatic effectiveness (Alasri et al., 1992, Christen et al., 2017, Masliukov et al., 1992). For example, comparisons of the disinfection efficacy of a mixed-oxidant solution to that of free chlorine found that the use of mixed oxidants may be a more practical and cost-effective approach (Masliukov et al., 1992). In this study, the inhibitory effects of five disinfectants against *A. salmonicida* were evaluated. However, further studies are needed to clarify the bacteriostatic effects of different disinfectant combinations at low water temperatures.

Conclusions

The main problem of this study was to elucidate the inhibitory effects of five disinfectants on *A. salmonicida* at low temperature. From this study, we conclude that BB had good bacteriostatic effect at all temperature. This study provide a base for medication for the prevention and treatment of *A. salmonicida* in aquaculture.

Acknowledgements

We thank all authors for stimulating discussions and support. All authors read and approved the final manuscript. We thank International Science Editing (http://www.internationalscienceediting.com) for editing this manuscript. This research was supported by grants from the double support plan of Sichuan Agricultural University, NO.1921993230; Sichuan key R & D program key technology project, NO.2020YFN0060; Chengdu Science and Technology Bureau key RESEARCH and development support plan, NO.2019-YF05-00835-SN, Chengdu Technology Innovation research and development Project, NO.2019-YF05-02018-SN, Sichuan rice fish comprehensive breeding cooperative promotion project.

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