Demonstration application of pure chlorine dioxide in disease control and water quality control of Litopenaeus vannamei culture

In this project Cleanbay Inc. is used to develop and produce a series of high-purity chlorine dioxide production equipment, and site is used to prepare high-purity chlorine dioxide disinfectant. Pure chlorine dioxide is internationally recognized as a broad-spectrum, efficient and safe fourth generation of AI class disinfectant, which can kill bacteria, spores, fungi, mycobacteria, various viruses, decompose algae, oxidize and decompose reducing pollutants in water. As a strong oxidant, through the reaction with a variety of inorganic and organic substances in the water, it can control the number of microorganisms in the water, and play a role in the prevention and control of aquaculture diseases and water quality. The experiment proved that pure chlorine dioxide had a strong killing effect on common pathogenic bacteria in aquaculture.

Table 1 minimum bactericidal concentration (MBC) of pure chlorine dioxide preparation to fish pathogens.

Types of pathogens	Strain	Minimum bactericidal concentration (mg/L)
Flavobacterium columnare	SC - 2	0.03
	GCR - 9061.	0.03
Aeromonas hydrophilia	BS - 6	0.04
	ZHB - 7	0.04

Vibrio anguillarum	J - O - 1	0.03
	K - 3	0.03
Edwarda tarda	EF - 1	0.03
	Et - 12	0.03

In conclusion, based on the original culture technology and demonstration application of vannamei prawn, the project integrates the mature pure chlorine dioxide water quality control and disease prevention and control technology to establish a new healthy culture technology system of vannamei prawn in the project area.

1.Technology Studies

1.1 Study on the disinfection effect of pure chlorine dioxide in the waterfront culture pool.

Test method: the water of prawn culture pond was collected and stored in Plastic Aquarium. Three concentrations of disinfectant were used respectively. Each concentration group was set with three parallel, the first group was 0.2mg/L, the second group was 0.5mg/L, the third group was 1.0mg/L. Another 3 control groups were set up.

After an interval of 40 minutes, it was quantitatively absorbed from the water and coated in a dish containing BHIA medium under sterile conditions. Each aquarium is coated with 3 Petri dishes. After 24 hours incubation in a constant temperature incubator at 25 ℃, observe and count the number of colonies in the dish.

The results showed that 0.2mg/L pure chlorine dioxide could kill

99% of bacterial colonies in aquaculture water.

Table 2 Relationship between pure chlorine dioxide concentration (mg/L) and bacterial colony number (CFU/ml)

Project	Concentration of pure chlorine dioxide (mg/L) and number of bacterial colonies (cfu/ml)		
	0.2 mg/L	0.5 mg/L	1.0 mg/L
The control group	6.3×10^{8} CFU /mL		
Pure chlorine dioxide	3.4×10^{6}	4.6×10^{5}	8.2×10^{4}
Sterilization rate	99.5%	99.9%	99.99%

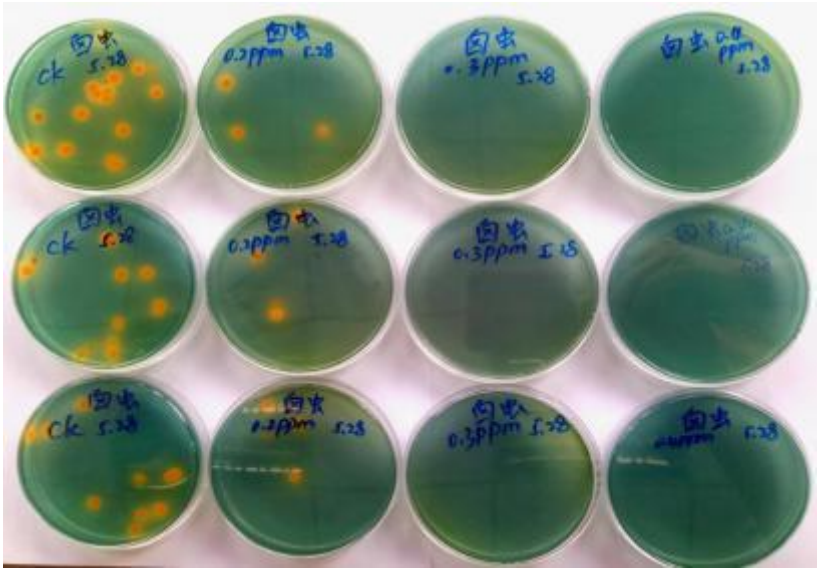
1.2 Disinfection effect of pure chlorine dioxide on Vibrio in brine shrimp culture water.

Test method: The concentration of chlorine dioxide in the water body of Artemia culture was 0.2mg/L, 0.3mg/L and 0.4mg/L respectively. The control group did not add pure chlorine dioxide. Disinfect for 40min. Under sterile condition, TCBS plate was coated and cultured at 25℃ for 24-48h, counting the number of colonies in the plate.

The results showed that after disinfection with 0.2mg/l pure chlorine dioxide for 40min, the killing rate of Vibrio in brine shrimp culture water reached 72%; after disinfection with 0.3mg/l pure chlorine dioxide for 40min, the killing rate of Vibrio reached 100%.

Table 3 concentration of pure chlorine dioxide (mg / L) and quantity of Vibrio in brine shrimp culture water.

Disinfection concentration	Colony count	Killing rate	note
The control group	14	-	unsterilised
0.2 mg/L	4	71.4%	
0.3 mg/L	0	100%	
0.4 mg/L	0	100%	



1.3 Study on the toxicity of pure chlorine dioxide to the shrimp fry of Litopenaeus vannamei.

Test method: The size of shrimp fry is 1.2-1.4cm. The experiment was carried out in a glass beaker with a capacity of 500mL, in which 10 shrimps were cultured. The test temperature shall be controlled between 24-25℃. Five concentration gradients of pure chlorine dioxide in the experimental group were 2.5mg/L, 2.0mg/L, 1.5mg/L, 1.0mg/L and 0.5mg/L, respectively. Three parallel groups were set for each concentration. The total number of dead shrimps was counted every 24 hours.

If the shrimp seedlings in the control group died, the Abbott formula was used for correction. Calculate the safe concentration according to the following formula:

$$\text{Safe concentration} = \frac{48\ hC_{50} \times 0.3}{\left(24\ hLC_{50} / 48\ hLC_{50}\right)^2}$$

The results showed that the 96(h) safe concentration of pure chlorine dioxide was 2.14mg/L.

Table 4 mortality of Penaeus vannamei fry in different concentrations of pure chlorine dioxide solution.

Project	Concentration (mg/L)	Shrimp fry (tail)	Mortality rate (%)			
			24 (h)	48 (h)	72 (h)	96 (h)
Experimental group	0.5	30	0	0	0	3.3
	1.0	30	0	0	0	0
	1.5	30	0	10	10	13.3
	2.0	30	10	13.3	16.7	16.7
	2.5	30	10	10	13.3	13.3
The control group		30	0	0	0	3.3

1.4 Study on the inhibitory effect of pure chlorine dioxide on Vibrio in industrial culture of Litopenaeus vannamei.

Test method: Before putting shrimp seedlings, 100mg/L pure chlorine dioxide liquid was used to spray and disinfect the surface of cement pool in the factory Culture Workshop. The concentration of pure chlorine dioxide in the water reached 1mg/L after adding the pure chlorine dioxide liquid into the aquaculture water body; the number of

vibrios in the water body was detected by TCBS plate counting method from 280cfu/ml to 15cfu/ml. In the process of culture, every 7-10 days, pure chlorine dioxide liquid is injected into the culture water, so that the concentration of pure chlorine dioxide in the water reaches 0.5mg/L.

Results: after 10 days of culture, the number of vibrios in the water was 5.41×10^3cfu/ml by TCBS plate counting method. The concentration of pure chlorine dioxide in aquaculture water was 0.5mg/L when pure chlorine dioxide was injected. After disinfection, TCBS plate counting method was used to detect the number of vibrios in the water body, which decreased to 4.8×10^2CFU/ml, and the killing rate of vibrios reached 91%. It can effectively control the number of vibrios and ensure the healthy growth environment of prawn.

Table 5 Effect of pure chlorine dioxide on inhibition of industrial Vibrio culture in shrimp culture.

	Water disinfection concentration (mg/L)	Quantity of vibrio in water before disinfection (cfu/mL)	The number of vibrio in the water after disinfection (cfu/mL)	Killing rate (%)	note
Put before seedling	1	2.80×10^2	15	94.6	
In the breeding	0.5	5.41×10^3	4.8×10^2	91.1	Sterilize every 7-10d

1.5 Study on the effect of pure chlorine dioxide on the growth of Litopenaeus vannamei.

Artemia is the most commonly used live food for aquaculture in hatcheries of marine fishes and crustaceans. Artemia is very important for the early cultivation and maturity of crustaceans. The Artemia fed Penaeus vannamei grows fast and has strong resistance to adversity. Therefore, Artemia is widely used as the bait of shrimp larvae in the industrial culture of Penaeus vannamei. But the untreated Artemia can become the carrier of bacteria or virus. A large number of vibrios were found on the surface of Artemia by TCBS plate counting method, the number was 3.74×10^4CFU/g. Without disinfection, feeding shrimp with Artemia will cause serious risk of disease.

Test method: A 150cm diameter and 80cm height bucket was selected as the experimental container, and 1 m^3 sea water was added into the bucket, and microporous oxygen was used. Two buckets were selected as the experimental group and one bucket as the control group. Add about 400 shrimps in each bucket. In the experimental group, 1mg/L pure chlorine dioxide was used for disinfection in the water before the seedlings were released, and 0.5mg/L pure chlorine dioxide was used for disinfection after 10 days of culture. The control group was not disinfected.

Feed prawn No.0 three times a day after stocking. The feeding frequency and feeding amount of the experimental group and the control group were identical. The control group was fed with fresh ice Artemia.

The experimental group was disinfected with 100 mg/L pure chlorine dioxide for 0.5 h, then rinsed with purified water and fed with prawn.

Results: In the control group, prawn vannamei developed a slow defecation phenomenon after feeding Artemia for three days; in the test group, prawn vannamei had a normal diet, no abnormal state, and grew fast. After feeding Artemia for 15 days, 10 prawns were randomly collected from the experimental group and the control group, and the body length of prawns was measured. No matter the minimum body length, the maximum body length and the average body length of Litopenaeus vannamei in the experimental group were better than those in the control group. The average body length of test group 2 was 29% higher than that of control group.

Table 6 Effect of pure chlorine dioxide disinfection on the growth of Litopenaeus vannamei.

	Minimum body length /cm	Maximum body length /cm	Average body length /cm
The control group	2.7	3.5	3.1
Experimental group 1	3.2	4.3	3.9
Experimental group 2	3.5	4.7	4.0

The amount of vibrios on the surface of Artemia can be effectively reduced by using the concentration to soak and disinfect the Artemia for 30 minutes. The results are shown in Table 7: the killing rate of vibrios on the surface of 100 mg/L pure chlorine dioxide liquid Artemia is 98.7%.

Table 7 efficacy of pure chlorine dioxide in disinfection of Vibrio on the surface of Artemia.

Disinfection concentration (mg/L)	Duration of disinfection (min)	The number of vibrio on the body surface of halogen insect before disinfection (cfu/g)	The number of vibrio on the body surface of halogen insect after disinfection (cfu/g)	Killing rate (%)
100	30	3.74×10^4	4.8×10^2	98.7

The whole intestine of Litopenaeus vannamei was obtained by dissection. After crushing the intestine of each sample, grind and shake it in 10mL sterile water. 0.1mL was coated on TCBS plate, cultured at 35℃ for 24-48h, and colony count was carried out. Results as shown in Table 8: compared with the control group, the number of vibrios in the intestine of Penaeus vannamei in the test group decreased by 41.7% and 50.3% respectively, with significant difference.

Table 8 quantity of vibrios in prawn after disinfection with pure chlorine dioxide.

	CFU/ vibrio colony (shrimp intestinal tract)	The average	Reduce the ratio of
The control group	2.78×10^3	2.88×10^3	-
	2.98×10^3		
Group 1	1.55×10^3	1.68×10^3	41.7%
	1.80×10^3		
Group 2	1.59×10^3	1.43×10^3	50.3%
	1.27×10^3		

1.6 Effect of different disinfection products on three halogenated

methane in aquaculture water.

Chlorine preparation has always occupied an important position in the disinfectants for aquaculture, mainly including bleaching powder, trichloroisocyanuric acid, etc. in addition, there is also a large amount of brominated disinfectants (such as BROMOCHLOROHYDANTOIN, etc.) which belong to halogen disinfectants. The sterilization mechanism is to denature the protein of bacteria and other microorganisms through chlorination, so as to eliminate pathogenic microorganisms. Objective. In the process of disinfection of these two kinds of disinfectants, halogens will react with the organic compounds in the water body to generate volatile halogenated hydrocarbons, which include chloroform ($CHCl_3$), bromodichloromethane ($CHBrCl_2$), dibromo chloromethane ($CHBr_2Cl$) and bromoform ($CHBr_3$), which are collectively called total trihalomethane (THMs).

THMs is a colorless, transparent and volatile liquid with special sweetness and slightly soluble in water. THMs has a strong anesthetic effect on animals, mainly on the central nervous system of animals, causing liver and kidney damage. There is epidemiological evidence that THMs is a carcinogen of animals, which is harmful to the health of animals. THMs can be rapidly absorbed in the digestive tract of animals. It only takes about 2 hours from human body fat to body fluid. It is transformed into carbon monoxide in the body, which increases the

content of carboxyhemoglobin in the blood, causes poisoning symptoms in the human body, and causes vomiting, dyspepsia, anorexia, weakness, nausea, neurosis, insomnia, depression, insanity, psychosis, etc. It has been proved that THMs have mutagenicity and/or carcinogenicity, and some have teratogenic and/or neurotoxic effects, which can cause liver, kidney and intestinal tumors.

Chlorine dioxide is recognized as an AI level safety disinfectant by the United Nations Health Organization. Its disinfection process is oxidation rather than chlorination. Therefore, even if there are organic substances in the water body, there will be no THMs after disinfection with high-purity chlorine dioxide. This may be why more and more countries in the world, especially developed countries, have adopted high-purity chlorine dioxide as disinfectant for drinking water. In order to prove that pure chlorine dioxide and other chlorine containing disinfectants produce different amounts of THMs in aquaculture water, our company specially selects three disinfection products, namely, bleaching powder, trichloroisocyanuric acid and kelinbao pure chlorine dioxide to disinfect the fish pond water, and the comparative study on the influence of trihalomethane content in the water.

Test method:

1.6.1 Type and concentration of disinfection products:

(a) bleaching powder: effective chlorine content 2mg/L;

(b) trichloroisocyanuric acid: effective chlorine content 0.3mg/L;

(c) kelinbao pure chlorine dioxide disinfectant: chlorine dioxide content 0.3mg/L.

1.6.2 Trihalomethane detection method: Determination of volatile organic compounds in water by Purge and trap/gas chromatography-mass spectrometry.

1.6.3 Entrusted testing agency: Hengli Testing Co., Ltd.

The results showed that the content of trichloromethane in the water treated with bleaching powder and trichloroisocyanuric acid was significantly higher than that in the water before treatment, which increased from 36.8 μg/L before treatment to 50.2μg/L and 46.8μg/L, with an increase of 36.4% and 27.2%, respectively. However, the content of trichloromethane in the water treated with Kelinbao pure chlorine dioxide disinfectant had no significant change compared with that before treatment. To the extent permitted.

Table 9 trihalomethane content in water after disinfection by different disinfectants.

The sample	The test items		Test results	growth
Pre-treatment sample	THM	chloroform	36.8 mu g/L	-
		monobromodichloro	Did not check out	-
		dibromochlorometha	Did not check out	-
		Three methyl	Did not check out	-
Bleach treatment (2.0 mg/L)	THM	chloroform	50.2 ug/L	36.4%
		monobromodichloro	Did not check out	-
		dibromochlorometha	Did not check out	-
		Three methyl	Did not check out	-
Treatment with	THM	chloroform	46.8 ug/L	27.2%

trichloroisocyanuric acid		monobromodichloro	Did not check out	-
		dibromochlorometha	Did not check out	-
		Three methyl	Did not check out	-
Treatment with pure chlorine dioxide	THM	chloroform	38.3 ug/L	4.1%
		monobromodichloro	Did not check out	-
		dibromochlorometha	Did not check out	-
		Three methyl	Did not check out	-

The above experiments show that the disinfection of aquaculture water with pure chlorine dioxide can effectively inhibit the formation of chloroform and can be widely used in aquaculture.

2. Demonstration application

2.1 Culture of Penaeus vannamei in a high water level pond, with an area of 4667m^2.

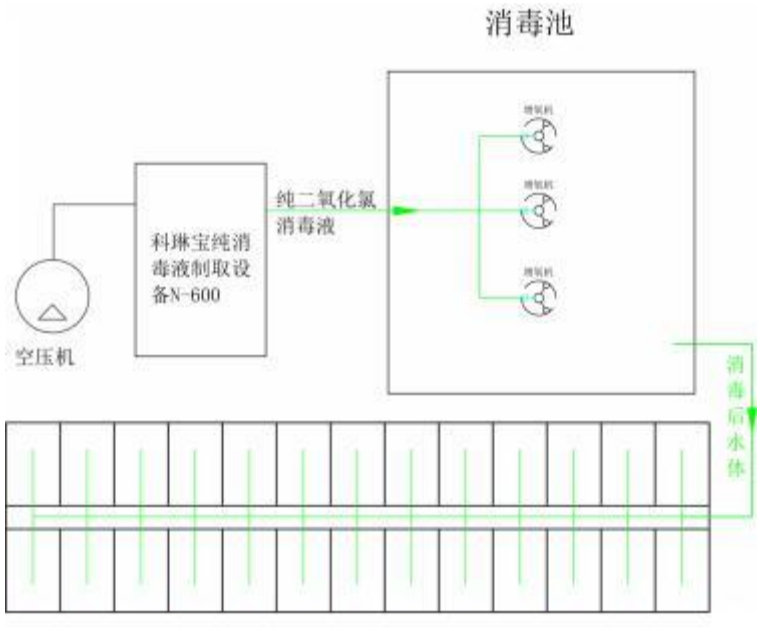


Fig. 2 Schematic diagram of shrimp culture and disinfection project in high water level culture pond.

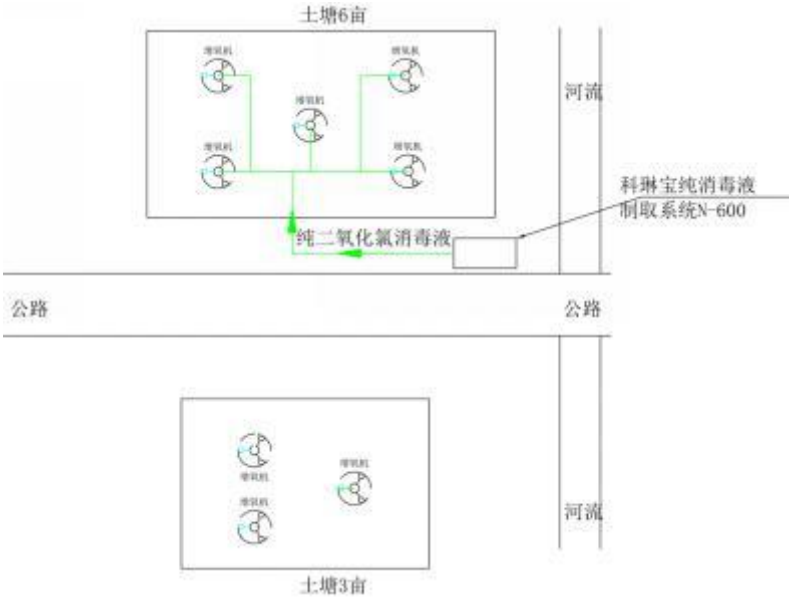
On May 15, pure chlorine dioxide was used to disinfect the seawater

of the disinfection pool, and the disinfection concentration of pure chlorine dioxide was 0.3mg/L-0.4mg/L. During the breeding process, the date and concentration of disinfection operation are as follows:

Table 10 disinfection operation date and concentration

The date	Area (m^2)	Duration (h)	Concentration of pure chlorine dioxide (mg/L)	The depth of the water (m)	Concentration of disinfectant in water (mg/L)	Traffic $(m/h)^3$	note
2018/05/15	4667	5	150	3	0.3	5	Disinfect pool
2018/06/08	4667	5	150	3	0.3	5	Disinfect pool
2018/06/22	4667	7	150	3	0.4	5	Disinfect pool
2018/08/05	4667	7	150	3	0.4	5	Disinfect pool
2018/08/26	4667	7	150	3	0.4	5	Disinfect pool

2.2 The cultivation area of shrimp pond is 4000m^2, and another 2000m^2 shrimp pond is set as the control.



On May 3, pure chlorine dioxide was used to disinfect the seawater in the disinfection pool. The concentration of pure chlorine dioxide was 0.3mg/L-0.8mg/L. During the breeding process, the date and concentration of disinfection operation are as follows:

Table 11 disinfection operation date and concentration

The date	Area (m^2)	Duration (h)	Concentration of pure chlorine dioxide (mg/L)	The depth of the water (m)	Concentration of disinfectant in water (mg/L)	Traffic $(m/h)^3$	note
2018/05/03	4000	6	130	1.5	0.3	3	South Area
2018/05/23	4000	6	130	1.5	0.4	3	South Area
2018/06/13	4000	6	130	1.5	0.4	3	South Area
2018/06/28	4000	6	130	1.5	0.4	3	South Area

2018/07/06	4000	10	180	2	0.8	3	South Area
2018/07/21	4000	6	130	1.5	0.4	3	South Area
2018/08/04	4000	6	130	1.5	0.4	3	South Area
2018/08/25	4000	6	130	1.5	0.4	3	South Area

2.3 The cultivation mode of the earth pond in the cultivation base is 1667m^2, and another 2000m^2 shrimp pond is set as the control.

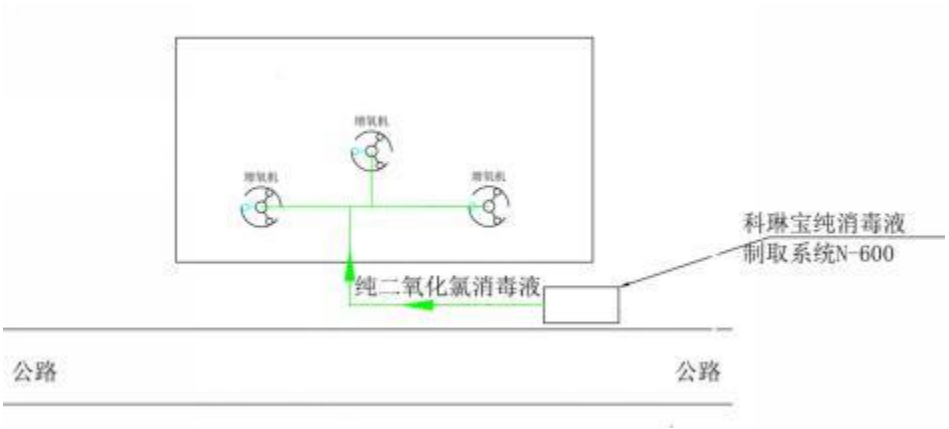


Fig. 4 Schematic diagram of prawn culture and disinfection project in the earth pond.

On June 1st, pure chlorine dioxide was used to disinfect the seawater in the disinfection pool. The concentration of pure chlorine dioxide was 0.3mg/L-0.5mg/L. During the breeding process, the date and concentration of disinfection operation are as follows:

Table 12 disinfection operation date and concentration

The date	Area (m^2)	Duration (h)	Concentration of pure	The depth	Concentration of disinfectant	Traffic $(m/h)^3$

			chlorine dioxide (mg/L)	of the water (m)	in water (mg/L)	
2018/06/01	1667	1.2	120	0.9	0.4	3
2018/06/12	1667	1.2	120	1.1	0.4	3
2018/06/25	1667	3	140	1.4	0.4	3.5
2018/07/10	1667	3.5	160	1.4	0.4	4
2018/07/23	1667	4	160	1.4	0.5	4
2018/08/04	1667	4	150	1.4	0.5	4
2018/08/15	1667	4.5	150	1.4	0.5	4
2018/08/26	1667	4	150	1.4	0.4	3

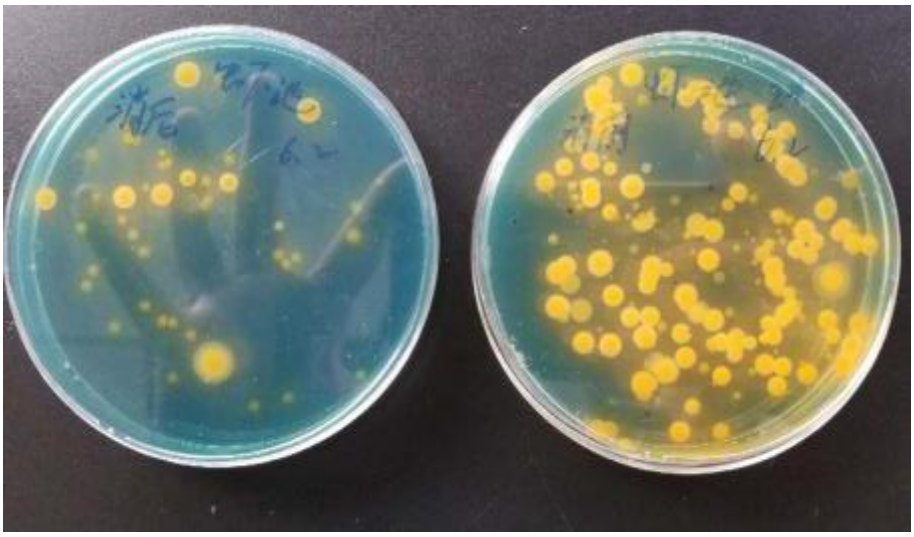


Fig. 5 Schematic diagram of Vibrio colony before and after disinfection.

Table 13 comparison between disinfection time and Vibrio quantity.

Project		Number of vibrio colonies (cfu/ml)	Sterilization rate of vibrio (%)
Before disinfection		1.62×10^3	-
After disinfection	0 days	4.40×10^2	72.84%
	2 days	7.35×10^2	-
	5 days	1.46×10^3	-
	8 days	2.07×10^3	-
	11 days	3.40×10^3	-

	15 days	2.86 x 10^3	-

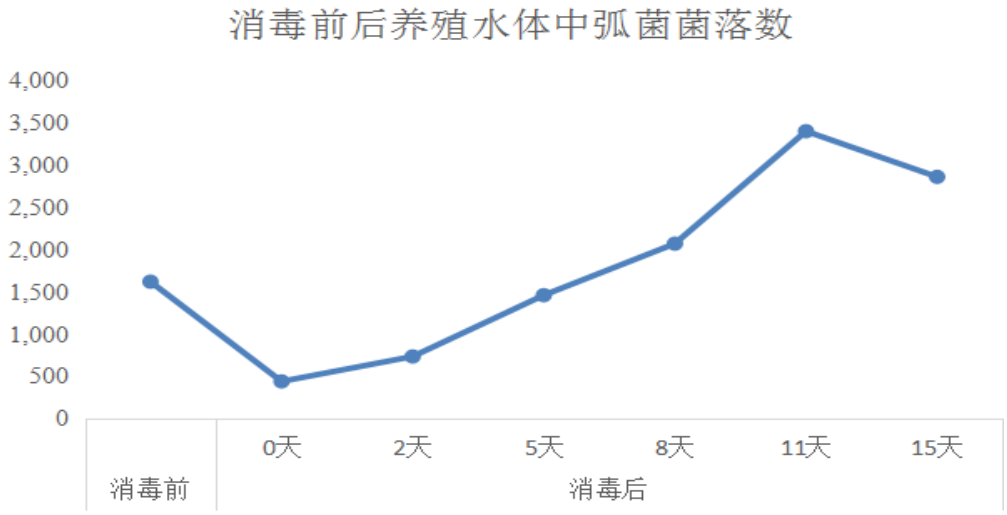


Fig. 6 number of Vibrio colonies in cultured water before and after disinfection.

2.4 There are 4 small arch sheds (shed No. A6, a7, B6, B7) with cultivation area of 467m^2, and another 5 small arch sheds (shed No. A1-A5) with cultivation area of 467m^2 as the control group.

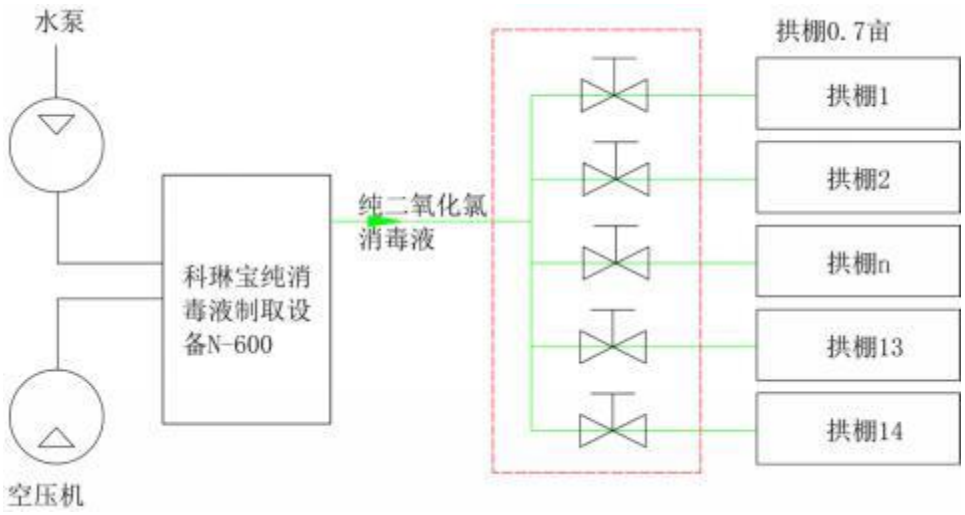


Figure 7 Schematic diagram of shrimp culture and disinfection project in small arch shed.

On May 4, pure chlorine dioxide was used to disinfect the seawater in the disinfection pool. The concentration of pure chlorine dioxide was 0.3mg/L-0.4mg/L.During the breeding process,the date and concentration

of disinfection operation are as follows:

Table 14 disinfection operation date and concentration

The date	Area (m^2)	The depth of the water (m)	Concentration of disinfectant in water (mg/L)	Traffic $(m/h)^3$	Remarks
2018/05/04	1867 (467m^2 per arch shed, 4 in total)	0.8	0.5	2.2	
2018/05/21		0.8	0.5	2.2	
2018/06/09		0.8	0.5	2.2	
2018/06/25		0.8	0.5	2.2	

Arched shed culture was early in seedling casting, and was fully fished in early July. The specific yield is as follows:

Table 15 comparison of production between control group and test group.

Number		Output (Kg)	Average yield (Kg)	Yield increase (%)
The control group	A1	220	274.40	
	A2	305		
	A3	247		
	A4	320		
	A5	280		
Experimental group	A6	368	335.75	22.36%
	A7	328		
	B6	345		
	B7	302		

2.5 Industrial culture of Penaeus vannamei.

100 mg / L pure chlorine dioxide was used to soak and disinfect Artemia for 30 minutes to kill Vibrio on the body surface of Artemia and prevent shrimp from infecting hepatopancreatic necrosis.

Table 16 concentration of Artemia disinfection.

Date	Operating area	Operating concentration (mg/L)	note
2018/05/31	The whole farm	110	Disinfection of Artemia with pure chlorine dioxide
2018/06/14		120	
2018/08/07		110	
2018/08/20		100	

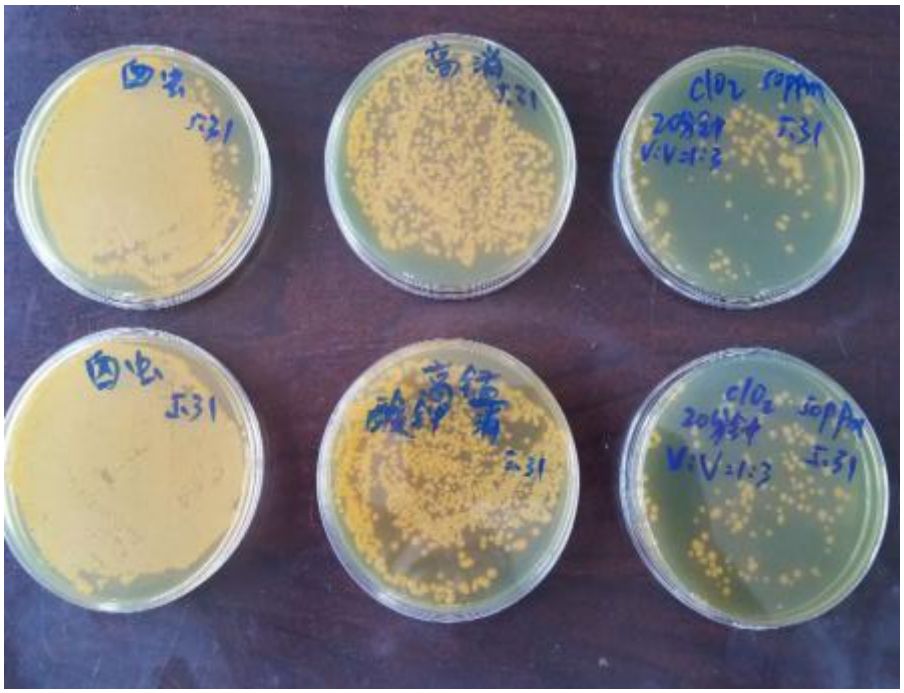


Figure 8 schematic diagram of disinfection effect of potassium permanganate and pure chlorine dioxide on Artemia.

2.6 The cultivation area of shrimp pond is 2667m^2 and 4000m^2 respectively, and two 2667m^2 shrimp ponds are set as the control group.

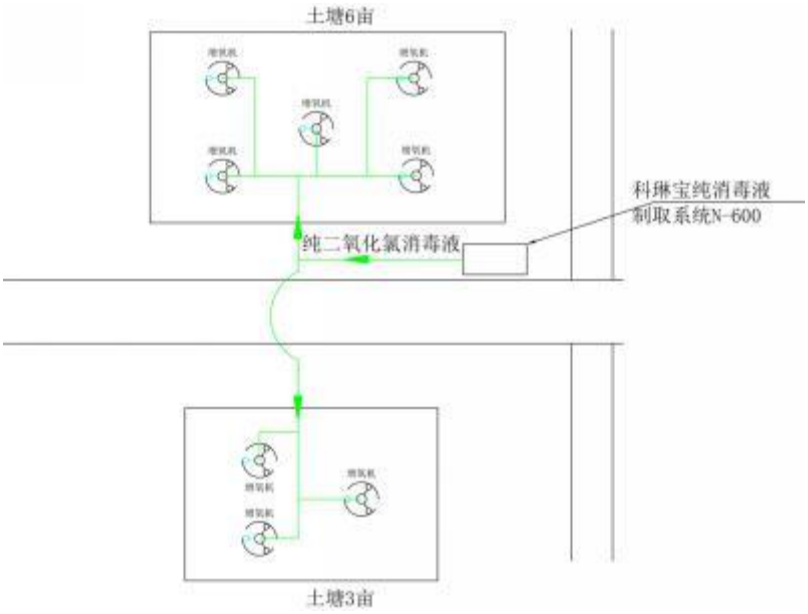


Fig. 9 Schematic diagram of prawn culture and disinfection project in the earth pond.

On June 15, pure chlorine dioxide was used to disinfect prawn pond. The concentration of pure chlorine dioxide was 0.4-0.5 mg/L. After that, the water body will be disinfected every 10-15 days.

Table 17 date and concentration of disinfection operation.

The date of	area (m^2)	The length (h)	Operating concentration (mg/L)	The depth of the water (m)	Disinfection water concentration (mg/L)	traffic (m^3/h)
2018/06/15	2667	1.5	130	0.7	0.4	3.5
2018/07/08	2667	3.4	160	1.3	0.6	4
	4000	5	160	1.3	0.6	4
2018/07/20	2667	3.8	150	1.3	0.5	3
	4000	5.8	150	1.3	0.5	3
2018/07/30	2667	3.8	150	1.3	0.5	3
	4000	5.8	150	1.3	0.5	3
2018/08/11	2667	3.8	150	1.3	0.5	3
	4000	5.8	150	1.3	0.5	3
2018/08/22	2667	3.8	150	1.3	0.5	3
	4000	5.8	150	1.3	0.5	3
2018/09/03	2667	3.8	150	1.3	0.5	3
	4000	5.8	150	1.3	0.5	3

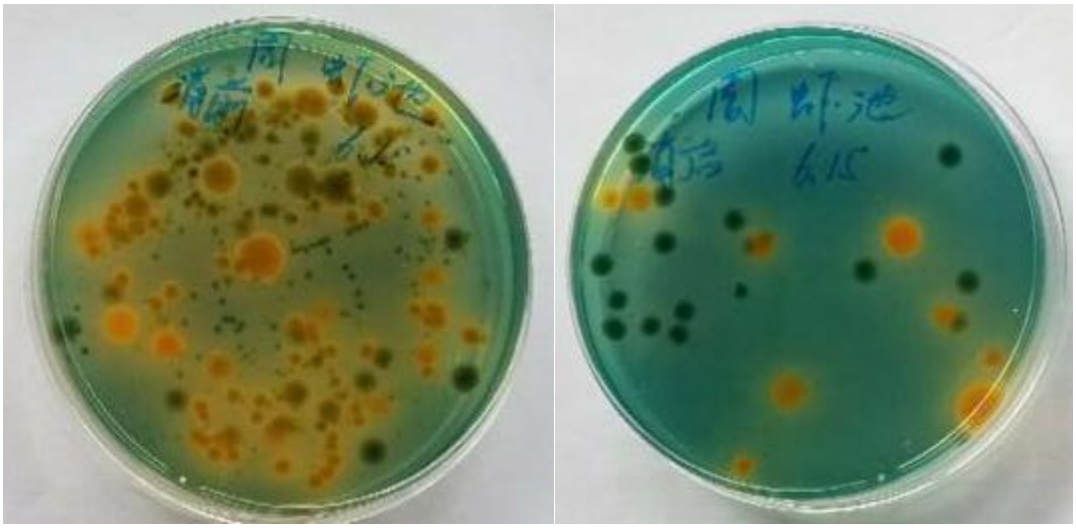
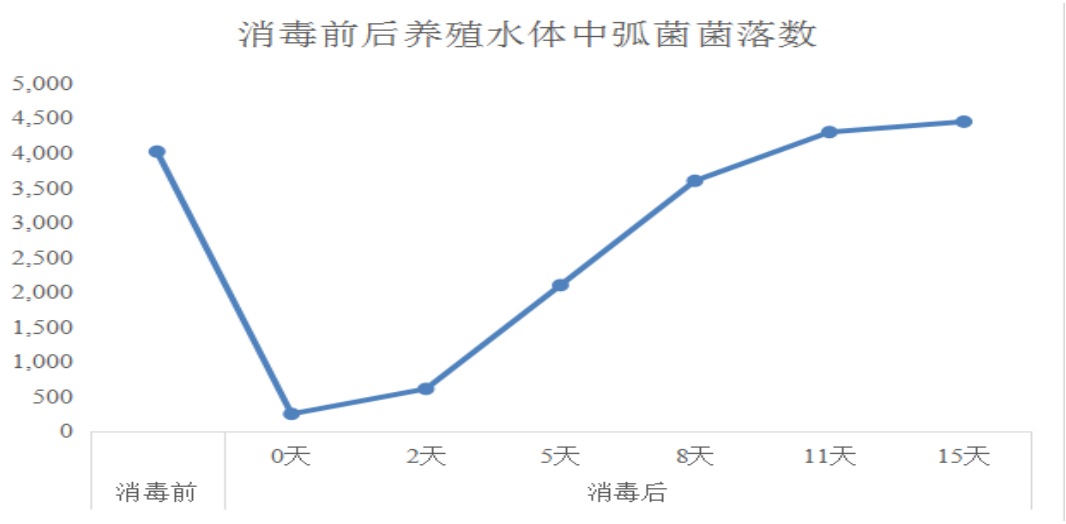


Figure 10 schematic diagram of Vibrio colony number before and after disinfection.

Table 18 comparison of the relationship between the duration of disinfection and the number of vibrios.

Project		Number of vibrio colonies (cfu/ml)	Sterilization rate of Vibrio(%)
Before disinfection		4.02×10^3	-
After disinfection	0 days	2.50×10^2	93.78%
	2 days	6.10×10^2	-
	5 days	2.13×10^3	-
	8 days	3.60×10^3	-
	11 days	4.30×10^3	-
	15 days	4.45×10^3	-



3. Project benefit

From 2017 to 2018, the project covers an area of 330000m^2. The total output of the project area is 3,200,000Kg of Penaeus vannamei, realizing an additional benefit of $4,500,000 dollar. Compared with the control, the survival rate of pond culture in the demonstration area increased by 28%, small arch shed culture by 36%, and factory culture by 13%.