Development of Indoor Recirculating Culture Systems for Intensive Shrimp Production in China

Production System Design and Best Management Alternatives/Experiment/09BMA05UM

Part 1. Objectives 1–3

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ABSTRACT

Indoor recirculating ponds, traditional ponds and eco-culture ponds were evaluated for water quality and *Litopenaeus vannamei* culture in 2010 and 2011. pH changed more dramatically in outdoor ponds. Nutrient content such as TN, TP, NO_2-N, NO_3-N, TAN and PO_4-P were lowest in eco-culture ponds, then in indoor RAS, and highest in traditional outdoor ponds. Nutrient budget analysis showed that both nitrogen and phosphorus gain to ponds occurred mostly from feed (nitrogen: 94.95% in indoor RAS, 85.21% in traditional ponds and 88.06% in eco-culture ponds; phosphorus: 97.06%, 92.34% and 94.87%, respectively), while 34.24% of nitrogen and 16.84% of phosphorus were retained in shrimp from indoor RAS, 31.02% N and 13.21% P were retained in shrimp from traditional outdoor ponds, and 35.73% and 15.25% for eco-culture ponds. Phosphorus was more important than nitrogen causing algae blooms during shrimp culture. Good water quality is essential, but not the only factor that affects shrimp production.

INTRODUCTION

Shrimp is a favored aquatic product around the world since it has high protein, low fat and is rich in nutrition. Marine shrimp has a wide range of adaptability to salinity and can be cultured in salt, brackish, or fresh water. China's shrimp farming industry has made remarkable achievements in the last two decades. In 1993, shrimp farming was severely damaged by outbreak of epidemic diseases, but it recovered, and shrimp production increased significantly. This increase was caused by several new technologies, such as the replacement of cultured species, structural transformation of the pond, and disinfection of rearing water.

In recent years, culturing shrimp has been widely developed in Shanghai and adjacent provinces. Traditional aquaculture models are still being applied throughout China, which means high density, high input, high yield, and high water exchange rate, with much drug use and high consumption of energy. These traditional models will not prevent outbreak of diseases and cause water pollution by discharging of wastewater rich in nitrogen, phosphorus, and organic matter to surrounding rivers or lakes. The damaged water ecosystems then cause further disease outbreaks, which in turn threaten human health and food safety.

Intensive fish farming has a long history in many countries (Sung-Koo et al., 2000). Production by a Danish aquaculture company was from 100-300 kg/m^3. Although there is noticeable gap between China and other developed countries in facilities and techniques (Chen 1998), indoor intensive aquaculture has

also been explored around China (Ying, 2001). Water treatment equipment and technologies have been developed and successful systems have been introduced to culturists. Indoor intensive aquaculture technologies have been used in culturing abalone, Atlantic turbot *(Scophthalmus maximus)*, and flounder in Shandong, Liaoning, and other provinces (Chang-fa, 2002).

Indoor intensive aquaculture has been developed worldwide. Recycling intensive shrimp farming has succeeded in Hawaii, Florida, Texas, and other places, and may produce approximately 5-10 kg shrimp per cubic meter of water in three months. In indoor intensive shrimp aquaculture, the most important thing is to control water quality and micro-organisms within desirable levels.

Considering the cost of infrastructure construction, relatively high electricity use, and need for highly educated or at least high skilled farmers, people in China are uncertain whether to develop indoor RAS or outdoor eco-culture systems. In this study, three different systems for *Litopenaeus vannamei* culture were studied to discuss their feasibility and effect.

METHODS

The research was originally designed to be conducted at Shanghai Bluesea Aquatech Co., Ltd, Fengxian District, Shanghai, China. In 2010, the infrastructure construction for greenhouse was delayed due to local policy for land use planning, so our study on an intensive indoor recirculating system (Figure 1) for *Litopenaeus vannamei* culture was carried out at Langxia's Special Cultivation Co., Jinshan District, Shanghai. At the same time, 22 outdoor ponds with traditional culture technologies (Figure 2) and 3 eco-culture ponds with special water quality control technology (Figure 3) were monitored for survival rate of shrimp and water quality. According to the results of shrimp production in 2010, we continued the study only on 17 eco-culture ponds in Bluesea Co. in 2011.

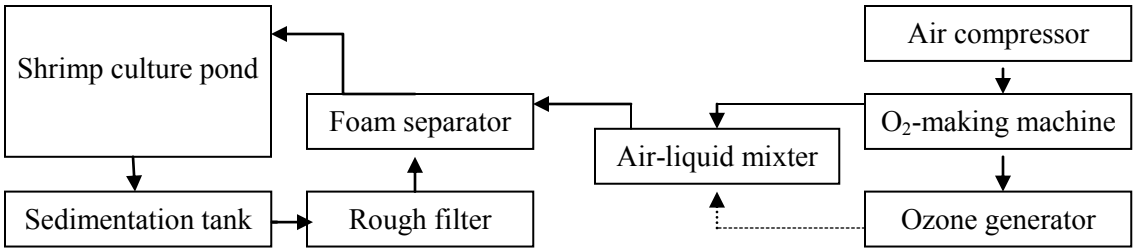

Figure 1. Flow diagram of our intensive indoor recirculating system.

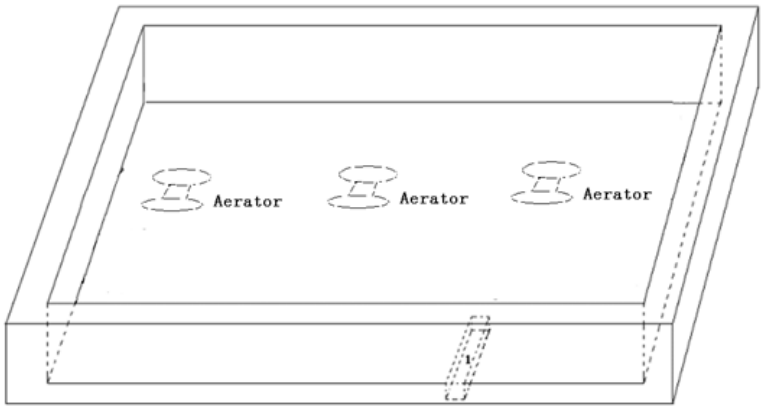

Figure 2. Schematic of a traditional outdoor pond.

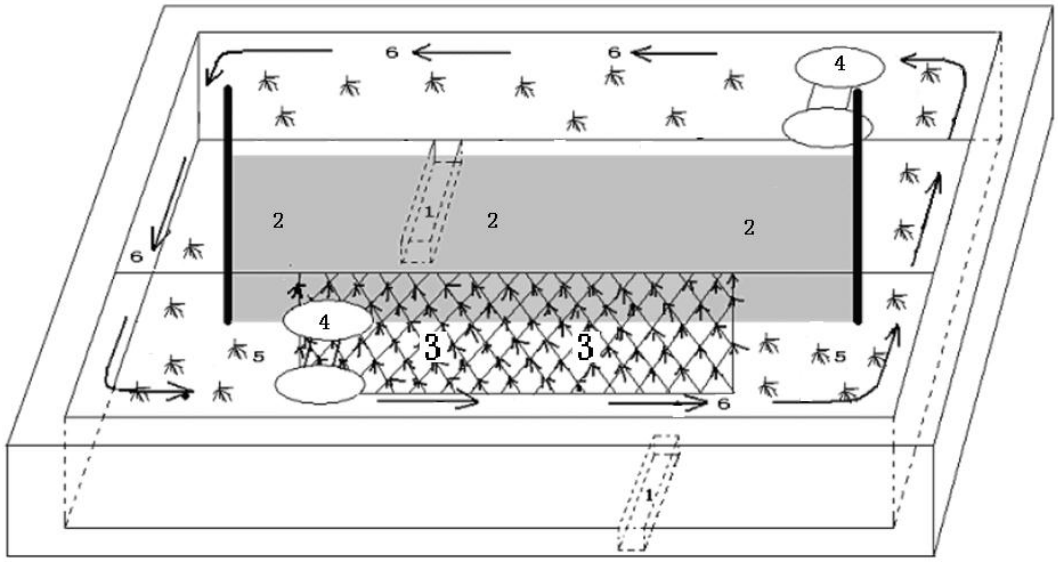

Figure 3. Schematic of our outdoor eco-culture pond. Characteristics included: 1-discharge ditch; 2-canvas divider; 3-water purifying net; 4-aerator; 5-aquatic plants; 6-water flow.

The research was accomplished from 2010 to 2011. Table 1 shows the basic information on facilities and shrimp culture methods for the experimental ponds. Commercial feed was used for the whole culture period. In 2010, all post-larvae of *Litopenaeus vannamei* were bought from Hainan. In 2011, shrimp in 12 ponds were from Hainan Province, while shrimp for the other 5 ponds were nursed by Shanghai Bluesea Aquatech Co., Ltd.

Table 1. Basic conditions and shrimp culture methods for the experimental ponds.

Research year	**Pond type**	**Location**	**Ponds number**	**Pond size**	**Facilities**	**Shrimp density**	Remarks
2010	Indoor recirculating system	Jinshan District	2	700m^2	Settling tank, rough filter, foam separator combined sometimes with ozone treatment constituted the water treatment unit. In each pond, there was a canvas divider (37.65m×1.70m), 6 aerators and several water purifying nets.	342 ind/m^2	One crop June-Sept.
2010	Outdoor traditional pond	Fengxian District	11 ponds A 11 ponds B	A:2855±167m^2 B: 3864±171m^2	Three aerators of 1.5 KW were used in each pond.	85.5±6.0 ind/m^2	Two crops May- July July-Sept.
2010	Outdoor eco-culture pond	Fengxian District	3	550 m^2	Each pond has a piece of canvas, 2 aerators, several water purifying nets, air stripping tubes and aquatic plants	200 ind/m^2	One crop July-Sept.
2011	Outdoor eco-culture pond	Fengxian District	17	550 m^2	Each pond has a piece of canvas, 2 aerators, several water purifying nets and air stripping tubes and aquatic plants	112.5 ind/m^2	One crop July-Sept.

Water samples were collected at the depth of 0.5m, and parameters including pH, dissolved oxygen (DO), Transparency (SD), suspended solid (SS), total organic carbon (TOC), biological oxygen demand (BOD_5), chemical oxygen demand (COD_{Mn}), nitrite (NO_2-N), total ammonia (TAN), nitrate-nitrogen (NO_3-N), total nitrogen (TN), phosphate phosphorus (PO_4-P), and total phosphorus (TP) were measured. Table 2 shows the analytic methods used for these water qualities.

Table 2. Water quality parameters and methods.

Parameters	Method	Parameters	Method
SD	Secchi disc	NO_3-N	DIONEX IC1500
pH	WTW pH330	NO_2-N	DIONEX IC1500
DO	YSI ProPlus	TAN	Nessler's reagent spectrophotometry
SS	Gravimetric method	TN	Alkaline potassium persulfate digestion-UV spectrophotometric method
TOC	TOC-VCPH	TP	Ammonium molybdate spectrophotometric method after alkaline potassium persulfate digestion
COD_{Mn}	Alkaline Permanganate method	PO_4-P	Ammonium molybdate spectrophotometric method
BOD_5	HACH BODTrak™	Chl.a	Acetone extraction spectrophotometric method

In the indoor recirculating system, sediment samples were collected from the drain that was in the middle of the eco-culture pond. In outdoor ponds, sediment was collected from the pond bottom after harvest. TN and TP were analyzed after samples had been mixed and dried. Feed samples were also analyzed for TN and TP.

In 2010, shrimp samples were taken from 22 outdoor ponds with traditional culture technologies and 3 eco-culture ponds every two weeks. The white spot syndrome virus (WWSV), taura syndrome virus (TSV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) were monitored. At the same time, the total number of culturable heterotrophic bacteria, and vibrio in pond water were analyzed.

RESULTS AND DISCUSSION

Outdoor ponds had a wider range of pH values and reached higher pH levels compared to indoor ponds (Tables 3-5). DO was not measurably different among the three pond different types.

Technologies used in indoor RAS and eco-culture ponds showed obvious effects on control of water quality. Overall, the concentrations of TAN, NO_2-N, NO_3-N, TN, PO_4-P, and TP in eco-culture ponds were much lower than those in traditional ponds. The indoor recirculating system also had lower concentrations than outdoor traditional ponds, although type b (larger outdoor ponds) had relatively similar water quality to the indoor system.

Both the indoor RAS and eco-culture ponds used aerators and canvas to manage water flow, and provided water purifying nets for attachment of microorganisms. Due to the relatively weak light conditions, aquatic plants did not grow well in the indoor RAS, so they were removed from the ponds soon after culture began. In outdoor eco-culture ponds, plants grew quite well and absorbed inorganic nutrients which caused lower levels of dissolved nitrogen and phosphorus compared to indoor RAS.

In eco-culture ponds, no water was discharged directly to the adjacent environment during the culture period and new water was added only to make up for evaporation loss. In the traditional outdoor ponds, water was not usually discharged if the water quality was controllable. However, in 2010, the water quality in type-a ponds decreased dramatically after the first crop of shrimp, so all water was exchanged.

Table 3. Water quality measurements for the indoor recirculating ponds.

Variable	Pond 1		Pond 2	
	Range	$\bar{x} \pm s$	Range	$\bar{x} \pm s$
pH	7.50-8.36	7.92±0.33	7.50-8.59	7.96±0.38
DO (mg/L)	3.70-8.00	6.43±1.21	3.78-8.42	6.37±1.49
TAN (mg/L)	0.231-1.119	0.517±0.263	0.219-1.281	0.558±0.326
NO_2-N (mg/L)	ND-2.626	0.396±0.842	0.054-0.785	0.318±0.273
NO_3-N (mg/L)	2.532-19.663	12.229±6.495	2.372-22.249	13.357±7.695
COD_{Mn} (mg/L)	6.45-16.98	9.54±3.49	7.84-22.04	11.03±4.44

Table 4. Water quality measurements for the outdoor traditional ponds.

Pond size	Variable	Range	$\bar{x} \pm SD$	Variable	Range	$\bar{x} \pm SD$
A	temperature(℃)	18.5-30.6	26.18±3.40	NO_2-N(mg/L)	0.134-6.652	0.64±0.74
B		18.0-30.7	26.20±3.46		0.025-3.556	0.13±0.44
A	SD (cm)	10-90	27.31±13.58	NO_3-N(mg/L)	0.100-8.293	1.26±1.20
B		15-65	26.23±12.00		0.056-8.032	0.89±1.18
A	SS (mg/L)	4-208	53.80±31.71	TAN (mg/L)	0.049-3.390	0.82±0.71
B		6-156	59.98±32.05		0.098-1.588	0.43±0.26
A	pH	7.32-9.60	8.05±0.50	TN (mg/L)	1.023-6.493	2.81±1.12
B		7.31-10.11	8.34±0.43		0.111-7.471	2.63±1.90
A	DO (mg/L)	2.23-14.76	5.79±2.10	TP (mg/L)	0.030-0.849	0.37±0.14
B		3.83-15.26	7.85±2.15		0.068-1.731	0.40±0.32
A	COD_{Mn} (mg/L)	6.42-31.21	15.19±5.50	PO_4-P (mg/L)	0.004-0.391	0.09±0.08
B		7.17-44.41	17.62±9.48		0.004-0.242	0.04±0.04
A	Chl.a (mg/L)	0.003-0.29	0.10±0.07			
B		0.003-0.511	0.16±0.13			

Table 5. Water quality measurements for the outdoor eco-culture ponds.

Variable	2010 (pond number=3)		2011 (pond number=17)	
	Range	$\bar{x} \pm s$	Range	$\bar{x} \pm s$
DO(mg/L)	3.20-11.70	5.23±1.68	3.60-12.10	5.58 ±1.98
temperature(℃)	18.8-30.7	25.6±4.32	26.00-31.70	29.07 ±1.51
pH	7.84-8.63	8.23±0.37	7.60-9.80	8.38± 0.46
COD_{Mn} (mg/L)	10.88-15.67	13.27±3.12	7.88-40.18	18.36± 6.98
Chl.a (mg/L)	0.03-0.81	0.23±0.11	ND-0.60	0.12 ±0.11
TAN (mg/L)	0.04-0.70	0.30±0.26	0.13-4.08	0.55 ±0.62
NO_2-N (mg/L)	0.02-0.461	0.13±0.08	ND-0.20	0.01 ±0.03
NO_3-N (mg/L)	1.01-2.67	1.64±0.57	ND-0.70	0.25 ±0.11
TN (mg/L)	0.43-5.15	2.57±1.56	0.58-6.79	2.41 ±1.31
PO_4-P (mg/L)	0.006-0.24	0.13±0.06	ND-0.70	0.05 ±0.08
TP (mg/L)	0.04-0.371	0.18±0.14	0.03-0.80	0.22 ±0.13

The amount of culturable heterotrophic bacteria in traditional ponds water was higher than that in eco-culture ponds. And no *vibrio* was detected in eco-culture ponds while *vibrio* was detected in 52% water samples from traditional ponds even if there showed no obvious disease outbreak. The three kinds of specific virus including WWSV, TSV and IHHNV were not detected in shrimps of the two different types of ponds (Table 6). It seemed that good water quality in eco-culture ponds would affect the amount of bacteria thus lower the probability of bacterial infection.

Table.6 Monitoring results of culturable heterotrophic bacteria and vibrio in water and virus analysis results in two types of culture ponds in 2010.

Pond type	culturable heterotrophic bacteria (cfu/mL)	Vibrio (cfu/mL)	WWSV	TSV	IHHNV
Traditional ponds (n=22)	1.3×10^2-1.9×10^6	ND -1.4×10^4	ND	ND	ND
Eco-culture ponds (n=3)	$1.4\times10^2\pm6.7\times10^4$	ND	ND	ND	ND

ND: not detected

In 2010, shrimp production per crop in traditional ponds was lower than in indoor RAS or eco-culture ponds (Table 7). Good water quality seemed valuable in improving shrimp production. But in 2011, there was a disease outbreak in ponds with shrimp from Hainan, which spread to the other ponds. Virus monitoring showed that WWSV were found in infected shrimp. By September 26, there were only two ponds with shrimp that were still healthy, so production values could not be obtained but were obviously likely to be quite low. Water quality appeared to be quite good even when disease occurred (Table 5). SPF shrimp and disease prevention should be given a higher priority in these grow-out systems.

Table 7. Shrimp production in different experimented ponds in 2010.

Pond type	Production per crop (kg/m^2)
Indoor RAS	0.80-1.40
Traditional ponds	0.29-0.44 (Pond A) , 0.20-0.23 (Pond B)
Eco-culture ponds	0.80-0.92

The nutrient budget was analyzed in indoor recirculating systems in 2010 (Figure 4). Feed input 94.95% of total nitrogen and 97.06% of phosphorus, with only 4.75% of nitrogen and 2.93% of phosphorus originating from water. Since shrimp juveniles were so small, they accounted for only 0.30% and 0.01% of the input. After 100 days of culture, 34.24% of input was bound shrimp tissue, 53.84% was in the water, and 0.825% was in organisms attached to purifying nets. Shrimp retained only 16.84% of the phosphorus while the unaccounted proportion was quite high at 34.53%.

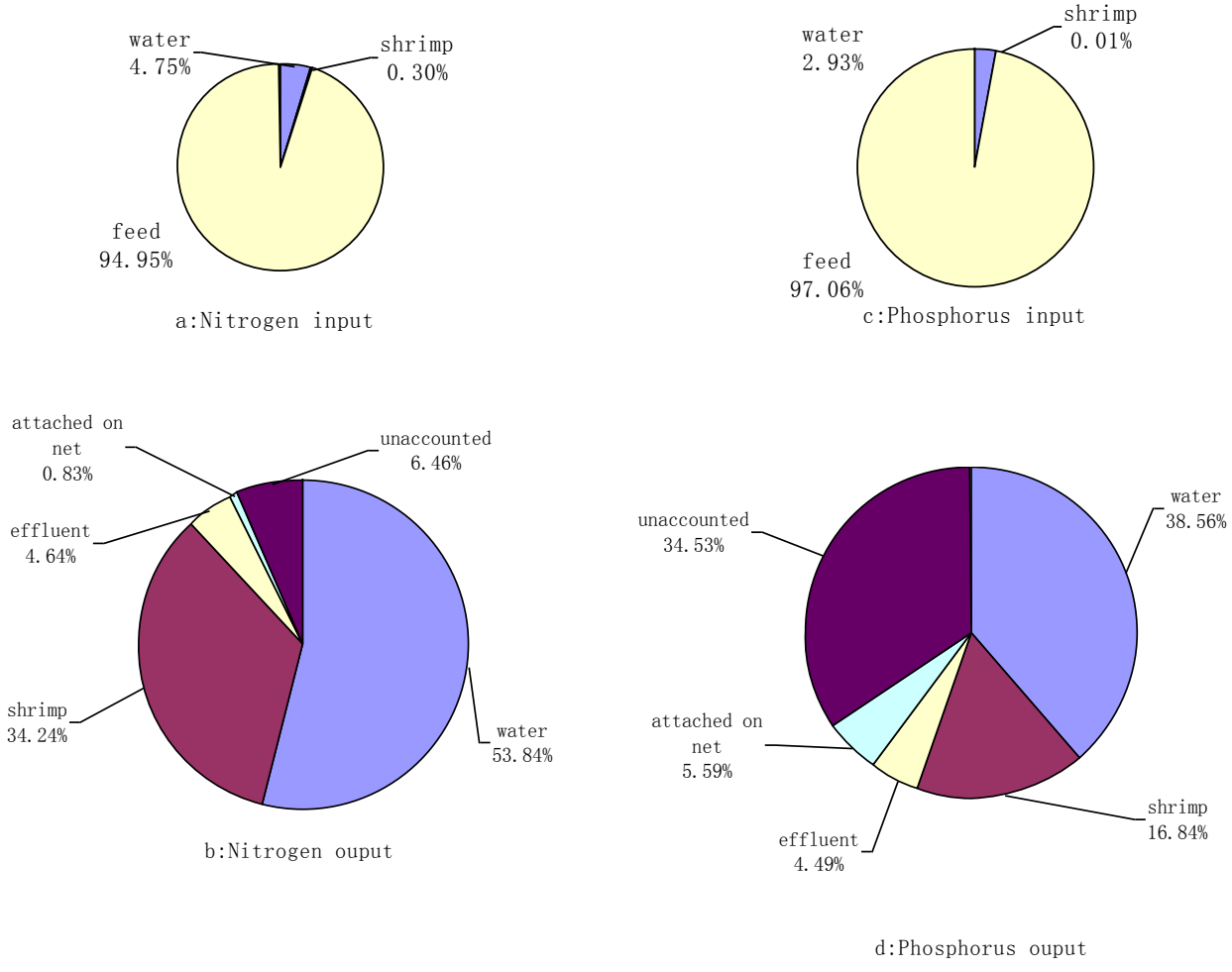

Figure 4. Nutrient budget for the indoor recirculating system.

Feed nutrient input was higher than optimum (Dhirendra et al., 2003). In Dhirendra's research over 90 days in closed systems, shrimp feed accounted for 76-92% of input nitrogen and 70-91% of phosphorus, while major sinks of nutrients were in the sediment (14-53% nitrogen and 12-29% phosphorus). In that study, the drained water at harvest contained 14-28% of input nitrogen and 12-29% of phosphorus.

Li et al. (2007) found that an intensive *Litopenaeus vannamei* culture had 84.3%-98.3% of nitrogen and 93.2%-97.3% of phosphorus inputs from feed. The major outputs of nitrogen and phosphorus were sediment (30.9%-43.9% and 51.5%-60.7%) and water exchange (27.5%-36.3% and 8.4%-23.9%). Their nutrient budget showed that only 14.5%-28.7% of nitrogen and 7.4% and 16.5% of phosphorus were transformed into harvested shrimp, which was a much lower retention rate than that in our RAS system.

Nitrogen and phosphorus inputs showed similar patterns in both traditional ponds and eco-culture ponds (Table 8). Nitrogen input to ponds was mostly from feed (85.21% and 88.06%), then water (14.68% and 11.85%). Nitrogen retention in the two types of ponds was different. Since aquatic plants were grown in eco-culture ponds, 7.89% of nitrogen was incorporated into plants, and the percent of nitrogen retained in water and sediment was less than that in traditional ponds. Phosphorus input also came mostly from feed (92.34% and 94.89%) and phosphorus retention was mainly in sediment (48.78% and 38.12%).

Table 8. Nutrient budgets for traditional ponds and eco-culture ponds.

		Traditional ponds	Eco-culture ponds
Nitrogen input (%)	Feed	85.21	88.06
	Water	14.68	11.85
	Shrimp	0.11	0.09
Nitrogen output (%)	Shrimp	31.02	35.73
	Water	19.45	11.32
	Sediment	33.24	27.09
	Aquatic plant	-	7.89
	Unaccounted	16.29	17.97
Phosphorus input (%)	Feed	92.34	94.89
	Water	7.57	5.04
	Shrimp	0.09	0.07
Phosphorus output (%)	Shrimp	13.21	15.25
	Water	11.36	5.69
	Sediment	46.78	38.12
	Aquatic plant	-	6.22
	Unaccounted	28.65	34.72

In 2010, algae blooms broke out in two traditional ponds, so we tried to determine the relationship between chlorophyll-a and other water quality parameters. Chlorophyll-a had highly significant positive correlations to SS, COD_{Mn}, TN and TP. A significant positive correlation also existed between chlorophyll-a and DO. Highly significant negative correlations were found between chlorophyll-a and SD

and between chlorophyll-a and PO_4-P. No significant correlations were found between chlorophyll-a and water temperature, pH, NO_2^--N, NO_3^--N and NH_3-N. According to the methods for selecting independent variables in multiple linear regression analysis, four water quality parameters including COD_{Mn}, TN, PO_4-P, and TP were used to determine the stepwise regression model which was

$$\text{Chl.a} = -0.03457 + 0.0217\,\text{TN} + 0.0007\,\text{COD}_{\text{Mn}} - 0.49\,\text{PO}_4\text{-P} + 0.338\,\text{TP},\ (r = 0.6896)$$

The effects of these four factors to chlorophyll-a were tested using partial regression coefficients. The most influential water quality parameter to chlorophyll-a was TN, and then TP, PO_4-P, and COD_{Mn} in turn.

Using the multiple regression formula, we calculated chlorophyll-a and compared it with measured values (Figure 5). The results matched reasonably well, especially when the concentration of chlorophyll-a was high.

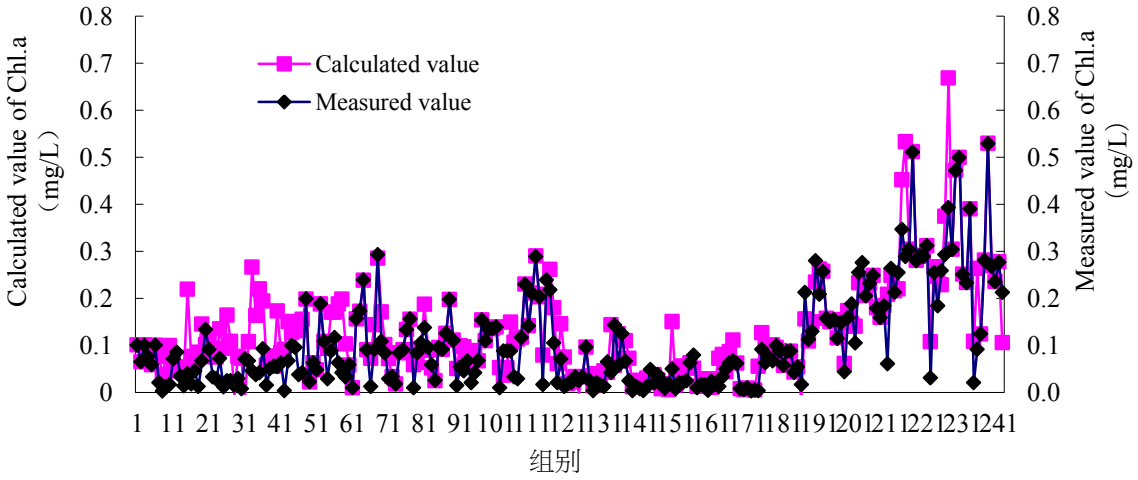

Figure 5. Comparison between calculated and measured chlorophyll-a in traditional ponds in 2010.

We did same statistical analysis on data from 17 eco-culture ponds in 2011 and following equation was obtained:

$$\text{Chl.a} = 0.21809 + 0.00590\text{COD}_{\text{Mn}} - 0{,}0092\text{t} + 0.340\text{TP} - 0.32\text{PO}_4 - \text{P}\ \ (\text{r} = 0.7032)$$

Compared to traditional ponds, TN was not included in the regression for eco-culture ponds. Combined technologies of purifying nets and aquatic plants probably played important roles in reducing nitrogen content of the water, resulting in low correlation between TN and chlorophyll-a. Phosphorus had a closer relationship with chlorophyll-a, so it is essential to keep phosphorus content low in water. According to the budget, over 90% of P came from feed but less than 16% of P was converted to shrimp. Improving utilization rate of feed P is important not only in cost savings, but also in controlling pond algae blooms.

ANTICIPATED BENEFITS

Recently, farmers have been encouraged by local government to culture shrimp or other aquatic animals with standardized methods, which mean acceptable pond types, preferred feed, SPF shrimp, suggested water quality control technologies, and permitted chemical use. But aquaculture is a complicated industry and successful production depends on not only technology, but also on management experience, shrimp quality, and even weather. In this study, we compared three types of shrimp culture models, analyzed water quality and production differences, evaluated the nutrient budgets and relationship between chlorophyll-a and other parameters. Combined water quality control technologies can be extended to other farmers. Some farmers have already begun to use purifying nets, bottom aeration in their ponds, and aquatic plants or aquatic vegetables to remove nutrients.

In short-term training to farmers, we taught them how to monitor water quality and showed them the basic technology for eco-culture of shrimp. Further research is still needed, particularly related to disease control.

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Part 2. Objective 4
Effects of *Microcystis Aeruginosa* on Juvenile Survivial and on Enzyme Activites of Adult Crayfish (*Procambarus Clarkii)*

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ABSTRACT

In this paper, larvae of the crayfish *Procambarus clarkii* were exposed to different concentrations of the algae *M. aeruginosa* (1.0×10^6, 5.0×10^6, 1.0×10^7, and 2.0×10^7 cells/mL) to investigate the algae's impact on larvae survival and hepatopancreatic ultrastructure. At the same time, adult crayfish were exposed to the same algae concentrations and the total hemocyte count (THC), serum hemocyanin content, and superoxide dismutase (SOD), peroxidase (POD), phenoloxidase (PO), and Na^+/K^+-ATPase activities in gill filaments were evaluated. The results showed that: (1) *M. aeruginosa* significantly reduced larval survival rate, such that, when exposed to 1.0×10^7 cells/mL algae, the survival rate was significantly lower than controls by day 19 ($p<0.05$) and, at the end of the 30 d exposure, survival rate was 48%. (2) Microscopic observations showed that the larval hepatopancreas became darkened and, under transmission electron microscopy, the tissue's cells were observed to be damaged. (3) At the highest algae density, the crayfish exhibited a stress reaction in which their THCs increased significantly and stayed high after being exposed for 1 d. There were no significant differences in hemocyanin content at the beginning of algae exposure, but it was significantly decreased by 5 d ($p<0.05$). Serum SOD activity was inhibited after 1 d of algae exposure and appeared elevated to a higher level by 5 d. POD and PO activities showed fluctuating trends, and Na^+/K^+-ATPase activity in gill filaments dropped significantly after 1 d ($p<0.01$), then increased, and finally remained at a higher level. These results indicated that *M. aeruginosa* exerted a strong negative impact on juvenile crayfish survival and affected the immunity status of adult crayfish, which may cause decreased adult growth from the elevated stress.

INTRODUCTION

Procambarus clarkii is a crayfish that exists in fresh water, such as channels and ponds, and is widely cultivated, particularly in Hubei and Jiangsu provinces, because of their delicious taste and tolerance for poor environmental conditions. However, cyanobacteria blooms occur frequently due to water eutrophication (Zha et al., 2004, 2007; Zhang et al., 2001), which has drawn academic attention to the blooms and their potentially harmful metabolic products. Research in the past ten years has shown that *Microcystis aeruginosa* in natural water and its toxin microcystin have toxicological effects (Reimkainen et al., 2001; Andersen et al., 1993; Zurawell et al., 2004; Orr et al., 2001; Park et al., 2001; Zimba et al., 2006) on organisms, and cyanobacteria blooms can alter water quality, while the toxin itself can accumulate in aquatic organisms and move up the food chain, causing severe damage to aquaculture and human health. However, the impact of *M. aeruginosa* on *P. clarkii* has received little study and been rarely reported.

In this paper, larvae and adult of crayfish *P. clarkii* were exposed to different *M. aeruginosa* concentrations to investigate the impacts of the algae on larval survival as well as total serum hemocyte density (THCs), serum hemocyanin content, superoxide dismutase (SOD), peroxidase (POD), phenoloxidase (PO), and Na^+/K^+-ATPase activities in gill filaments of adults. Moreover, the effect of *M.*

aeruginosa on larval body color and hepatopancreas ultrastructure were examined by visual and transmission electron microscopy (TEM), with the goal to observe the chronic toxicological mechanism of this algae on crayfish and to provide some useful information to aid healthy cultivation of crayfish.

MATERIAL AND METHODS

The larvae of *P. clarkii* (body length: 12.09 ±0.98 mm, body weight: 99.20 ±13.80 mg) were contributed by the Aquatic Life Vegetative Reproduction Laboratory of Shanghai Ocean University, and adult crayfish (body length: 57.52 ±3.84 mm, body weight: 16.90 ±2.10 g) from Shanghai Mudflat Resource Development Institute. Experimental crayfish were raised in fresh water after it was sterilized with 5% salt solution and full aeration, after which, healthy individuals of similar size were selected randomly for experimentation. *M. aeruginosa* was contributed by the Institute of Hydrobiology, Chinese Academy of Sciences, and maintained in culture solution BG-11 for 15 d, at 20 ±1℃, with a 12/12 light/dark cycle.

In accordance with the usual algae density in *M. aeruginosa* blooms (Lu et al., 2005; Vasconcelos et al., 2001) three concentrations were selected: 1.0×10^6, 5.0×10^6, and 1.0×10^7cells/mL, to examine the effect of *M. aeruginosa* on juvenile *P. clarkii* survival. A control group was maintained in fresh water previously sterilized by boiling. All treatments were triplicated. The experimental vessel was a square plastic 5 L casing with 4 L of water. Healthy crayfish larvae were divided randomly into groups of 20 per casing, fed daily at a fixed time, and the water renewed every two d to maintain a steady algae density. The surviving larvae were counted daily with death based on lack of appendage movement.

Healthy *P. clarkii* larvae were cultivated with *M. aeruginosa* at 5.0×10^6, 1.0×10^7, and 2.0×10^7 cells/mL for 7 d and their hepatopancreas removed after the larvae were washed in sterile water. The collected tissue was fixed in 2.5% glutaraldehyde and 1% osmic acid, and dehydrated in ethanol. Next, tissue was embedded in epoxy resin EPON812, microtomed into ultrathin sections with a LKB NOVA slicing machine (LKB Nova, Bromma, Sweden), dyed twice with uranyl acetate and lead citrate, and observed by transmitting electron microscope (TEM, H-700, Hitachi, Ltd., Japan) with an accelerating voltage of 75 kV.

Triplicate groups of 60 randomly selected healthy *P. clarkii* adults were cultivated with *M. aeruginosa* at 1.0×10^6, 5.0×10^6, 1.0×10^7 cells/mL, a control group in sterilized fresh water. Each group was cultivated in a 200 L plastic storage box and evaluated for THC and serum hemocyanin content, as well as SOD, POD, PO, and Na+/K+-ATPase activities of their gill filaments. THC was assessed using the method of Yao et al. (2007), PO activity with the method of Ashida (1971), and hemocyanin content using the combined methods of Johnson et al. (1984) and Zhang et al. (2005). Also, in consideration of the strong hemocyanin absorption peak at 280 and 334 nm, the spectrophotometer was adjusted to measure optical density at both OD_{280} and OD_{334}. An examination reagent box from Nanjing Jiancheng Company was used to measure the SOD, POD, and Na+/K+-ATPase.

RESULTS

Juvenile crayfish survival was significantly correlated with the *M. aeruginosa* concentration. There was no reduction in survival when algae density was 1.0×10^6 cells/mL; young crayfish began to die after 25 d of exposure, and at the end of cultivation (30 d), survival rate was 95%, with no significant difference from the control. However, when algae density was 5.0×10^6 cells/mL, survival rate at 26 d was only 68.33%, significantly lower than the control ($p<0.05$). In addition, when algae density reached 1.0×10^7 cells/mL, survival was only 61.67% after 19 d of exposure, also significantly lower than the control ($p<0.05$), and at the end of this exposure, survival rate was only 48.33% (Figure 1).

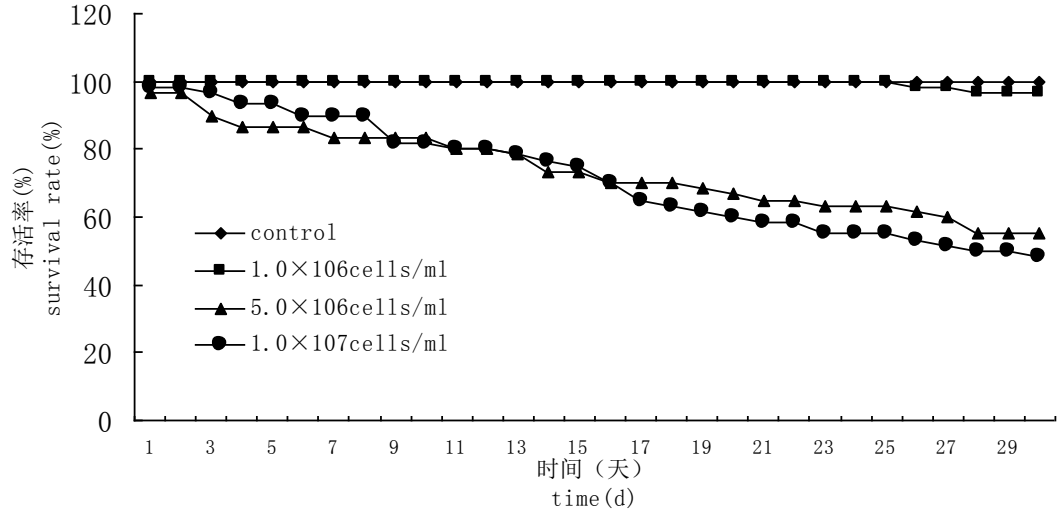

Figure 1. Effect of different densities of *M. aeruginosa* on survival rate of *P. clarkii* larvae.

Observation by anatomical lens revealed that, compared to controls, *P. clarkii* larvae showed differences in testa spots and body color when cultivated in different densities of *M. aeruginosa*. Specifically, testa spots and body color were darker in *M. aeruginosa* at density of 1.0×10^7 and 2.0×10^7 cells/mL. Closer observation of the carapace revealed that the hepatopancreas was swollen to varying degrees (Figure 2).

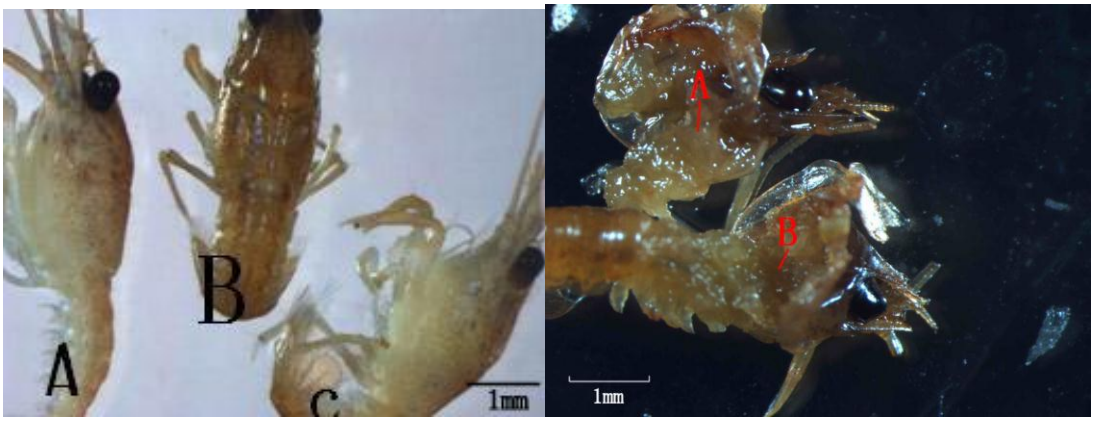

Figure 2. Effect of *M. aeruginosa* on body color of crayfish larvae. Left, morphology of whole body; right, hepatopancreas; A, 5.0×10^6 cells/mL; B, 1.0×10^7 cells/mL; and C, control.

TEM images of larval hepatopancreas revealed damaged hepatopancreatic cells in crayfish cultivated at 1.0×10^7 and 2.0×10^7 cells/mL algae (Figure 3), including damage to cell chromatin around the nuclear membranes, nucleolus margination, large cytoplasmic vacuoles, and organella damage; while hepatopancreatic cells of the control group were structurally complete, with evenly distributed chromatin, and lipid droplets similar to a yolk or fat granule. The group reared in the lowest algae density also had relatively complete cell membranes in their hepatopancreas, with no obvious damage.

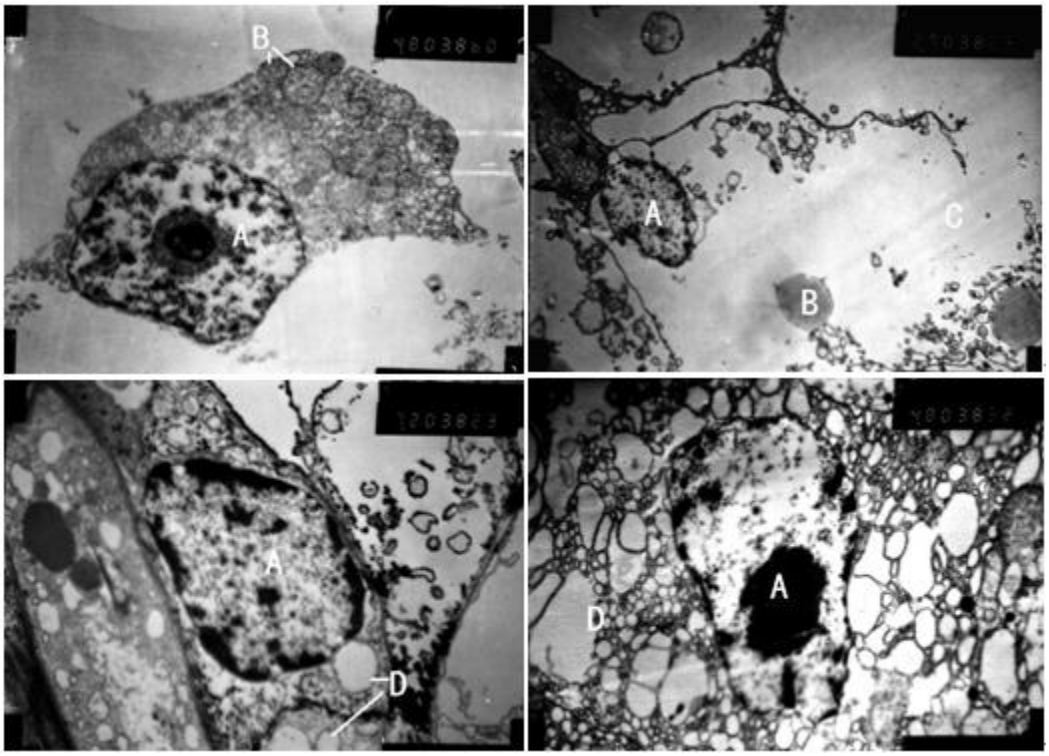

Figure 3. Ultrastructure of crayfish larvae hepatopancreas. A, cell nucleus; B, cellular organelle/lipid droplet; C, cell membrane breakage site; D, vacuoles between cytoplasm.

The THCs of adult *P. clarkii* were increasingly affected with increasing *M. aeruginosa* density (Figure 4); while the THCs of controls were basically the same as the 1.1×10^6 cells/mL treatment group. After exposure to algae-laden water for 24 h, adult crayfish THCs in each experimental group rose, with higher density groups at 5.0×10^6 and 1.0×10^7 cells/mL showing significant differences compared to the control ($p<0.01$). The lowest density group also rose significantly in THC after three days of exposure. After exposure to *M. aeruginosa*, the THC of all experimental groups was sustained at relatively high values.

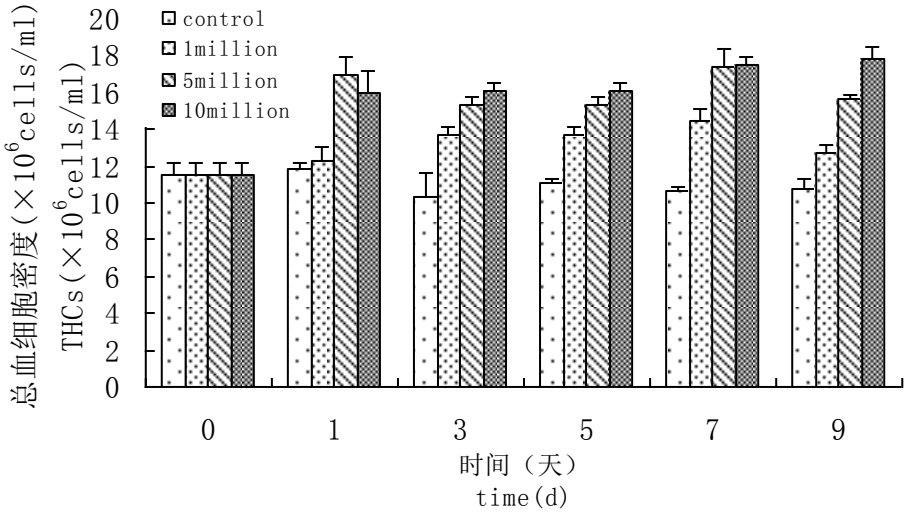

Figure 4. Effect of *M. aeruginosa* density on adult crayfish THCs.

Evaluation of the effect of *M. aeruginosa* density on *P. clarkii* hemocyanin revealed that the serum hemocyanin concentration experienced no significant variation after 3 d of algae exposure but decreased significantly after 5 d (Figure 5), with the hemocyanin concentration of the two higher algae density groups significantly lower than the control ($p<0.01$). Soon afterwards, hemocyanin returned to a concentration similar to controls in all groups. Moreover, the hemocyanin spectrophotometer measurements at 280 and 334 nm were similar.

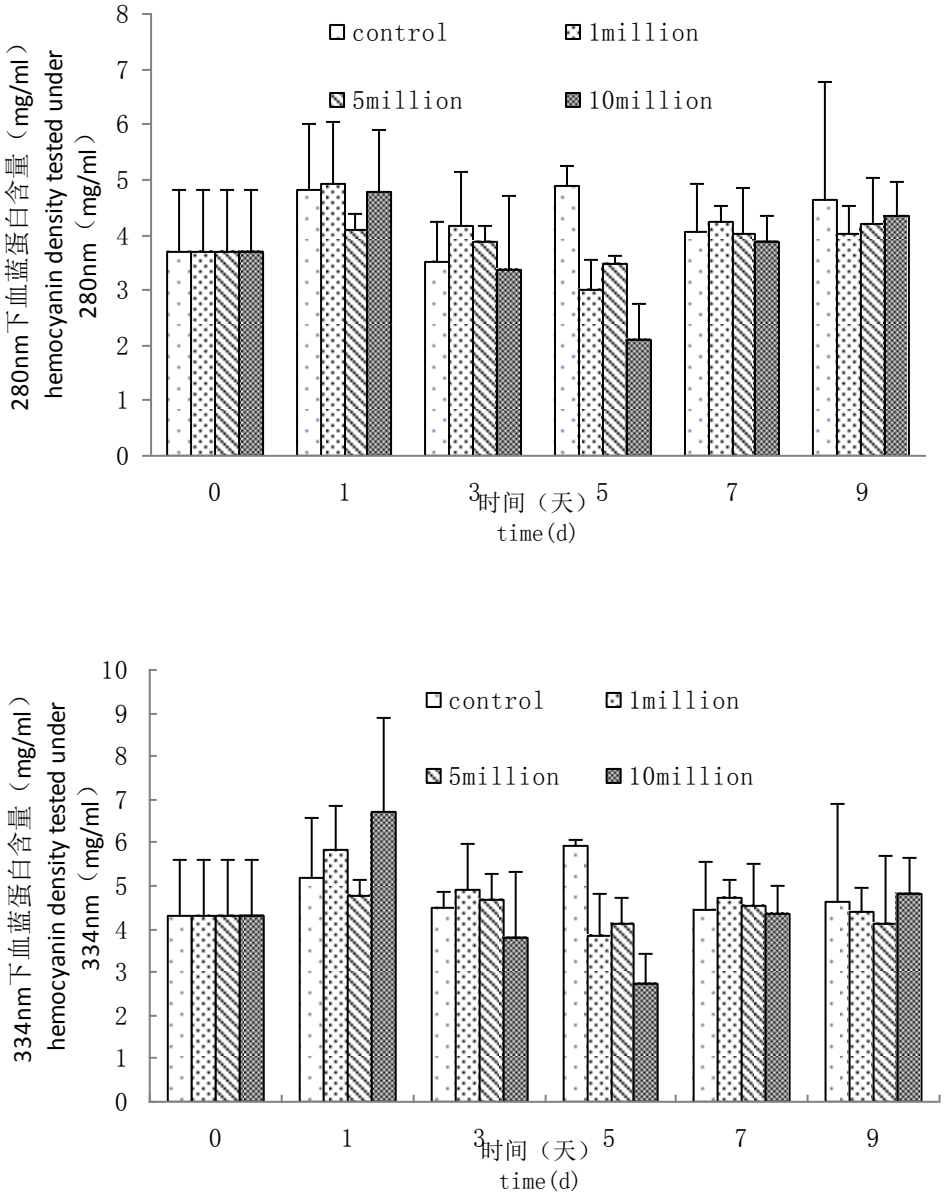

Figure 5. Effect of *M. aeruginosa* density on adult crayfish hemocyanin. Top, 280 nm; bottom, 334 nm.

Evaluation of the effect of *M. aeruginosa* density on adult *P. clarkii* PO activity revealed that after exposure to algae at 1.0×10^7 cells/mL for 1 d, PO activity in the serum increased significantly ($p<0.01$),

but soon afterwards, began to decrease and was finally restored to initial values (Figure 6). In addition, PO activity in groups with 1.0×10^6 and 5.0×10^6 cells/mL were not significantly different from the control.

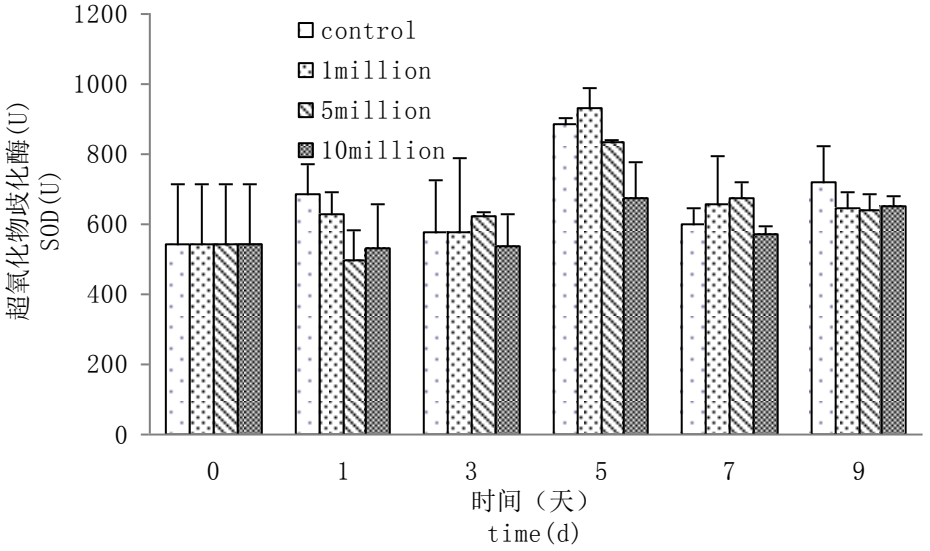

Figure 6. Effect of *M. aeruginosa* density on adult *P. clarkii* PO activity.

M. aeruginosa in lower concentrations, 1.0×10^6 and 5.0×10^6 cells/mL, produced no significant effect on *P. clarkii* SOD activity, but at the highest concentration, SOD activity was significantly restrained (Figure 7). SOD activity in the highest algae group decreased slightly after 1 d of exposure and dropped significantly after 5 d of exposure ($p<0.01$).

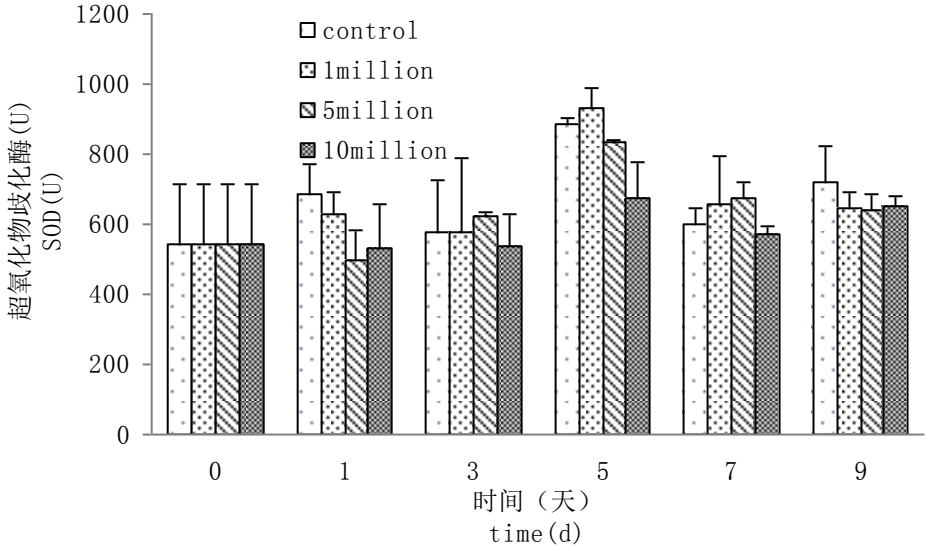

Figure 7. Effect of *M. aeruginosa* density on adult crayfish SOD activity.

In contrast, after exposure to *M. aeruginosa*, POD activity in adult crayfish demonstrated a tendency of rising and then falling. In the high algae group, POD activity rose significantly after 1 d of exposure

($p<0.05$), and in the two lower algae groups, POD activity increased slightly after 3 d and, soon afterwards, POD activity of all experimental groups returned to the level of the control (Figure 8).

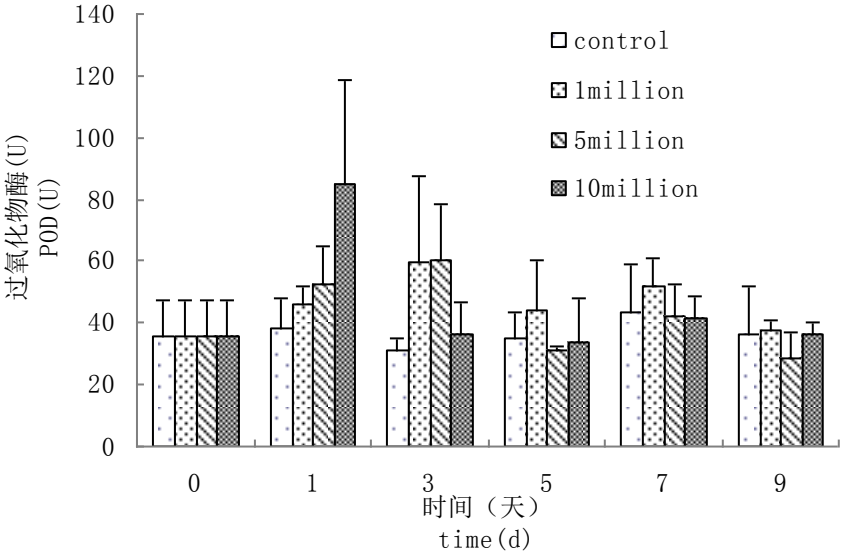

Figure 8. Effect of *M. aeruginosa* density on adult crayfish POD activity.

The effect of *M. aeruginosa* exposure on the serum Na^+/K^+-ATPase activity of adult *P. clarkii* showed a tendency of declining and then rising (Figure 9). Specifically, Na^+/K^+-ATPase activities in all 3 algae groups declined significantly after 1 d of exposure, returned to their initial values after 3 d, strengthened gradually until reaching a peak at 7 d, and then decreased again after 9 d of exposure.

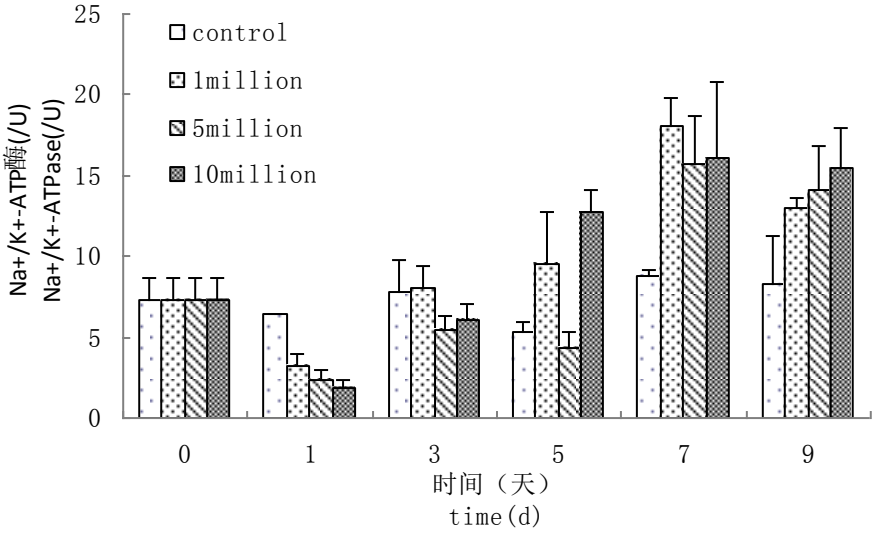

Figure 9. Effect of *M. aeruginosa* density on adult crayfish Na^+/K^+-ATPase activity.

DISCUSSION

Survival And Health Status Of *P. Clarkii* Larvae Were Influenced by *M. aeruginosa* in the culture water, which corresponded to the fact that cyanobacteria blooms in water bodies cause death in different organisms (Babica et al., 2006; Zimba et al., 2001; Xie et al., 2004; Obernum et al., 1999; Yokoyama and Park, 2002, 2003), and indicated that even crayfish with strong environmental tolerances were affected by *M. aeruginosa*. Microcystin is a type of hepatotoxin, which can result in liver swelling and congestion, increasing the liver to body weight ratio and causing a series of enzymological changes. Moreover, this toxin can induce apoptosis in various cells (Amorim and Vasconcelos, 1999; Fu et al., 2004; Lankoff, 2004) and cause framework damage to cell ultrastructures (Beasley et al., 2000). In this study, the larval hepatopancreas was found to experience swelling to different degrees and hepatopancreas necrocytosis after algal exposure for 7 d, but no apoptotic body or cell nucleus breakage was observed in the process. Therefore, the larval hepatopancreas was strongly affected by high concentration of microcystin, resulting in large quantities of necrocytosis. However, further research is required to understand whether *M. aeruginosa* at low density can also induce hepatopancreatic apoptosis.

Among crustaceans, hemocyte count and PO activity have direct correlations with immunity. Blood lymphocytes hold a central position in the immune system and THC can reflect, to some extent, the immunological stress, or physical condition of an organism and has been adopted as one of the indicators for measuring immunity level of crustaceans (Huang et al., 2007). *M. aeruginosa* caused THCs to rise in *P. clarkia* during our exposures, indicating that the algae stimulated their immune system to generate more blood lymphocytes in an effort to resist the adverse environment. PO is the most important antioxidizing element in crustacean body fluid and is the first defensive line against external damage, yielding PO activity as a common indicator of immunity level. PO activity strengthened significantly after algal exposure in this study, which was similar to results reported by Huang et al. (2007) and Lei et al. (2001) that PO activity strengthened with different infection status. As the respiratory pigment of crustaceans, hemocyanin is dispersed in the hemolymph, performing oxygen transport and participating in immune defense, exhibiting functions similar to phenoloxidase under the influence of certain substances (Adachi et al., 2003). Yoganandhan et al. (2003) found that the hemocyanin ratio in *Penaeus indicus* declined significantly after WSSV infection, which agrees with the results in our study, and this may indicate that the algae caused hemocyanin decomposition and reduced oxygen transport, and damage to the crayfish immune system. When algal density reached a higher level, it rapidly stimulated the crayfish immune system, generating large numbers of blood lymphocytes and releasing phenoloxidase as well as similar immunological factors to participate in the immune response.

Antioxidases, such as SOD and POD, are important in metabolic detoxification in organisms. SOD is a crucial enzyme in balancing oxidation and antioxidation, and variation in its activity directly reflects the ability to eliminate free radicals in the organism itself. Research has demonstrated that SOD activity declines significantly when aquatic organisms are in an adverse environment (Hu et al., 2009; Zhang et al., 2007).

In this study, the SOD activity of *P. clarkii* was also found to decline significantly after exposure to *M. aeruginosa*, which was similar to a report by Sun and Ding (1999) on the effect of ammonia nitrogen on the premonition of *Penaeus chinesis*. POD performs the function of catalytic decomposition of oxides or peroxides, as well as oxygenolysis of toxins (Ashida and Soderhall, 1984; Soderhall and Cerenius, 1998). In the current work, serum POD activity of crayfish rose significantly after exposure to high-density algae for 1 d, demonstrating that oxides increased significantly in the crayfish with algae exposure and that POD was generated in large amounts by the organisms and could have helped eliminate excessive internal oxides. The changes of antioxidase activity observed here may have been caused by the *M. aeruginosa* generated microcystin MC-LR, which produced damage after crossing the cell membrane. Research has indicated that, after entering cells, algae toxins can produce oxidative damage, lipid peroxidation, DNA chain breakage, and oxidative modification of proteins (Ding et al., 2000; Chen et al., 2006; Fan et al., 2008), which are features that appear common to the toxicological mechanisms of *M. aeruginosa*.

For crustaceans, Na^+/K^+-ATPase is an important osmoregulatory enzyme, mainly scattered in the gill, hepatopancreas, and antennal gland, and its activity is an important measure of an organism's physiological metabolism and growth status (Pan and Liu, 2005). In the present study, Na^+/K^+-ATPase activity changes observed in gill filaments were similar to results of Zhao et al. (2006) on the effect of salt concentration on Na^+/K^+-ATPase activity in *Acipenser schrenckii* gill tissue. One possible explanation for this effect is that gill tissue had direct access to the algae in the external environment and experienced a stress response when Na^+/K^+-ATPase activity declined rapidly. However, after residing in an adverse environment for some time, the crayfish entered into an active adjustment stage in which Na^+/K^+-ATPase activity increased significantly, regulating the cell osmotic pressure and performing the functions of respiration, excretion, osmoregulation, and disease defense. Consequently, the crayfish successfully adapted to the external environment.

The stress response in organisms is an active fight against adverse environmental conditions and when abrupt changes in environmental factors exceed the crustaceans' endurance, metabolic disorders, osmoregulation dysfunction, immunity decline, or even death can result (Guo et al., 2007). When the external environmental factors are within their endurance levels, they can adapt with a buffering stress response. The experimental results here confirmed that: the significant death rate in juvenile *P. clarkii* was caused only when the *M. aeruginosa* density reached 1.0×10^7 cells/mL, beyond the endurance of the juveniles. However, the adult enzyme activities experienced increases and decreases at a variety of exposure densities, finally stabilizing and demonstrating that when the algae density reached 1.0×10^7 cells/mL, adults managed to show great tolerance, gradually adapting to the external environment after a clear stress response stage.

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