การตอบสนองทางสรีรวิทยาของกุ้งกุลาดำ *Penaeus monodon*

ต่อไนเทรตในน้ำทะเล

นาย สรายุทธ อ่อนสนิท

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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PHYSIOLOGICAL RESPONSES OF BLACK TIGER SHRIMP *Penaeus monodon* TO NITRATE IN SEAWATER

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Marine Science

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สรายุทธ อ่อนสนิท : การตอบสนองทางสรีรวิทยาของกุ้งกุลาดำ *Penaeus monodon* ต่อไนเทรตในน้ำทะเล. (PHYSIOLOGICAL RESPONSES OF BLACK TIGER SHRIMP *Penaeus monodon* TO NITRATE IN SEAWATER) อ.ที่ปรึกษา : ศ.ดร. เปี่ยมศักดิ์ เมนะเศวต, อ.ที่ปรึกษาร่วม : ดร. สรวิศ เผ่าทองศุข 93 หน้า. ISBN 974-17-5118-4.

ศึกษาการตอบสนองทางสรีรวิทยาของกุ้งกุลาดำ (*Penaeus monodon*) ต่อไนเตรทที่ระดับความเข้มข้นต่างๆ ในน้ำทะเล ในการทดลองส่วนแรกได้ทดลองหาค่าพิษเฉียบพลันของไนเทรตที่ระดับความเข้มข้นระหว่าง 2 ถึง 4449 มก. ไนเทรต-ไนโตรเจนต่อลิตร โดยใช้กุ้งกุลาดำน้ำหนักเฉลี่ย 17.37±2.7 กรัม ที่ความเค็ม 30 ส่วนในพัน พบว่าค่าความเป็นพิษเฉียบพลันที่ทำให้กุ้งกุลาดำตายร้อยละ 50 ที่เวลา 96 ชั่วโมงมีค่าเท่ากับ 2636 มก.ไนเทรต-ไนโตรเจนต่อลิตร เมื่อเสร็จสิ้นการทดลองได้เก็บเลือดกุ้งกุลาดำที่รอดจากการทดลองมาวิเคราะห์การตอบสนองทางสรีรวิทยาได้แก่ ปริมาณเม็ดเลือดรวม ปริมาณโปรตีน ปริมาณกลูโคสในเลือด (ตัวบ่งชี้ความเครียด) ความว่องไวของเอนไซม์ฟีนอลออกซิเดส (ตัวบ่งชี้ระบบภูมิคุ้มกัน) ปริมาณแอมโมเนีย ไนไตรท์ และไนเทรตในเลือด พบว่าปริมาณเม็ดเลือดรวมมีค่าสูงที่ระดับไนเทรตต่ำกว่าค่า LC_{50} (2636 มก.ไนเทรต -ไนโตรเจน) และมีความแตกต่างกันอย่างมีนัยสำคัญยิ่งทางสถิติ (P<0.01) กับความเข้มข้นของไนเทรต ส่วนค่าความว่องไวของเอนไซม์ฟีนอลออกซิเดส ปริมาณโปรตีนและปริมาณกลูโคสในเลือดมีค่าความแปรปรวนสูง ปริมาณแอมโมเนียมในเลือดกุ้งไม่มีความสัมพันธ์กับปริมาณแอมโมเนียมในน้ำทะเลโดยตรวจพบในช่วงความเข้มข้นระหว่าง 12-22 มก.แอมโมเนียม-ไนโตรเจนต่อลิตร ในขณะที่ความเข้มข้นของไนเทรตและไนไตรท์ในเลือดมีความสัมพันธ์เชิงบวกอย่างมีนัยสำคัญทางสถิติ (P<0.05) กับปริมาณไนเทรตและไนไตรท์ในน้ำทะเล แม้ว่าความเข้มข้นของไนเตรทในน้ำจะมีค่าสูงกว่า 2000 มก.ไนเทรต-ไนโตรเจนต่อลิตร

ในการทดลองส่วนที่สองได้ทดลองเลี้ยงกุ้งกุลาดำน้ำหนักเฉลี่ยเริ่มต้น 24 กรัม จำนวน 50 ตัวในบ่อรูปกลม พื้นที่ 7 $ม^2$. เป็นเวลา 216 วัน โดยแบ่งเป็นบ่อชุดควบคุมที่ไม่มีระบบบำบัดไนเทรต ซึ่งมีความเข้มข้นของไนเทรตสะสม 86 มก.ไนเทรต-ไนโตรเจนต่อลิตร และบ่อชุดทดลองมีการติดตั้งระบบบำบัดไนเทรตทำให้สามารถควบคุมความเข้มข้นของไนเทรตให้ต่ำกว่า 25 มก.ไนเทรต-ไนโตรเจนต่อลิตร เมื่อสิ้นสุดการทดลองพบว่า กุ้งมีอัตรารอดเท่ากับร้อยละ 38 และ 54 และมีอัตราการเติบโตเฉลี่ยเท่ากับ 0.18 และ 0.16 กรัมต่อวัน สำหรับกุ้งในบ่อชุดควบคุมและชุดทดลองตามลำดับ ในการวิเคราะห์ตอบสนองทางสรีรวิทยาได้เก็บเลือดกุ้งทุก 10 วันพบว่า ปริมาณเม็ดเลือดรวมของกุ้งในสองบ่อไม่แตกต่างกันทางสถิติ ปริมาณโปรตีน กลูโคส และความว่องไวของเอนไซม์ฟีนอลออกซิเดส มีความแตกต่างกันทางสถิติในบางช่วงเวลาทดลองและมีความแปรปรวนค่อนข้างสูงของค่าที่ตรวจวัดได้ สำหรับแอมโมเนียมในเลือดไม่มีความสัมพันธ์กันทางสถิติกับแอมโมเนียมในน้ำทะเล แต่ไนไตรท์และไนเทรตในเลือดมีความสัมพันธ์เชิงบวกอย่างมีนัยสำคัญยิ่งทางสถิติ (P<0.01) กับความเข้มข้นของไนไตรท์และไนเทรตในน้ำทะเล

ภาควิชา วิทยาศาสตร์ทางทะเล ลายมือชื่อนิสิต.....

สาขาวิชา วิทยาศาสตร์ทางทะเล ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา 2546 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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KEY WORD: *Penaeus monodon* / NITRATE / TOXICITY / HAEMOLYMPH / STRESS

SARAYUT ONSANIT : PHYSIOLOGICAL RESPONSES OF BLACK TIGER SHRIMP *Penaeus momodon* TO NITRATE IN SEAWATER. THESIS ADVISOR : PROF. PIAMSAK MENASVETA, Ph.D. THESIS COADVISOR : SORAWIT POWTONGSOOK, Ph.D. 93 pp. ISBN 974-17-5118-4.

This study investigated the physiological responses of black tiger shrimp (*Penaeus monodon*) to various nitrate concentration in seawater. In the first section, the acute toxicity of nitrate was studied using 17.37$\pm$2.7 g shrimps exposed to nitrate concentrations ranged from 2 to 4449 mgNO_3-N/L in 30 psu salinity. It was found that median lethal concentration (LC_{50}) at 96-hr was 2636 mgNO_3-N/L. Haemolymph samples were collected from survived shrimps for the determination of total haemocyte counts (THCs), protein, glucose (stress response), phenoloxidase activity (immune response), ammonium, nitrite, and nitrate. The results showed that high THCs was found in shrimp exposed to nitrate concentration below the LC_{50} (2636 mg-N/L) with significant difference (P<0.01 by ANOVA) compared to each nitrate concentration. On the other hand, phenoloxidase activity, haemolymph protein, and haemolymph glucose showed high fluctuation. Analysis of nitrogenous compounds in haemolymph showed that haemolymph ammonium was constant within the range between 12-22 mg-N/L while haemolymph nitrite and nitrate had significant correlation (P<0.05 by ANOVA) with nitrate concentrations in seawater even at nitrate concentration more than 2000 mg-N/L.

In the second section, 50 shrimps with initial weight of 24 g were cultured with two closed recirculating seawater systems, each consisted of 7 m^2 round pond (3m in diameter) and nitrification biofilter tank. The concentration of nitrate in the treatment pond was controlled by nitrate treatment system (the tubular denitrification reactor) attached to nitrification tank while control pond was without nitrate treatment. The results showed that, after 216 days rearing, concentration of nitrate in control pond was accumulated to 86 mg-N/L while nitrate in treatment pond was less than 25 mg-N/L. Survival rate of shrimp in control and treatment ponds were 38% and 54%, respectively. Physiological responses indicated by total haemocyte, haemolymph protein, glucose and phenoloxidase activity were fluctuate with high standard deviation. Highly significant correlation between haemolymph nitrate and nitrate concentration in the water was found, in which the concentration of nitrate in haemolymph was almost equal to the concentration of nitrate in the seawater.

Department MARINE SCIENCE Student's signature.....

Field of study MARINE SCIENCE Advisor's signature.....

Academic year 2003 Co-advisor's signature.....

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TABLE OF CONTENTS

TABLE OF CONTENTS (cont.)

TABLE OF CONTENTS (cont.)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (cont.)

LIST OF FIGURES (cont.)

Abbreviations

ANOVA	analysis of variance
BTB	bromthymol blue
CAC	Ca-codylic acid
$CaCl_2$	Calcium chloride
CRSS	the closed recirculating seawater system
CO_3	carbonate
cm	centimeter
DN	denitrification
DO	dissolved oxygen
FIA	flow injection analysis
g	gram
HC	hyaline cell
HCl	hydrochloric acid
HCO_3	bicarbonate
HLS	haemocyte lysate supernatant
hr	hour
H_2CO_3	carbonic acid
H_2SO_4	sulfuric acid
L	liter
LC_{50}	median lethal concentration
LGC	large granular cell
LPS	lipopolysaccharide
L-DOPA	L-dihydroxyphenylalanine
m	meter
M	molar
mg	milligram
mg%	milligram percent
ml	mililiter
min	minute
mM	milimolar
mm^3	cubic millimeter
m^2	square meter
N	nitrogen

Abbreviations (cont.)

$NaNO_2$	sodium nitrite
$NaNO_3$	sodium nitrate
$Na_2Fe\ (CN)_5\ NO.2H_2O$	sodium nitroprusside
NH_3	ammonia
NH_4^+	ammonium ion
NNED	N-(1-Naphthyl)-Ethylenediamine dihydrochloride
nm	nanometer
No.	number
NO_2	nitrite
NO_3	nitrate
$(NH_4)_2SO_4$	ammonium sulphate
O_2	oxygen
O_3	ozone
OCR	oxygen consumption rate
PO	phenoloxidase
proPO	prophenoloxidase
psu	per salinity unit
PTFE	polytetrafluroethylene
rpm	rotation per minute
R^2	r square
SGC	semi-granular cell
TA	total ammonia
THCs	total haemocyte counts
UV	ultraviolet
v/v	volume/volume
ΔOD	delta optical density
µg	microgram
µL	microliter
%	percent
^oC	degree Celsius

CHAPTER I

INTRODUCTION

In Thailand, cultivation of black tiger shrimp (*Penaeus monodon*) is mostly depend on shrimp broodstock that captured from the sea. Declining of good quality broodstock probably cause a serious problem to the future of shrimp business. Recently, an attempt has been made to produce shrimp broodstock from the culture pond, especially with the closed recirculating seawater system (CRSS). The use of CRSS is, therefore, one of the most appropriate way to achieve "specific pathogen-free" broodstock production in a "biosecure" system.

The aim of using closed recirculating seawater system is to prevent diseases (virus, bacteria, protozoa etc.) transmission through water exchange and maintain good water quality within the safety range. Most CRSS use nitrification process to reduce ammonia and nitrite which are highly toxic nitrogenous substances into nitrate. With nitrification biofilter, water in CRSS can be reused for months without water exchanged. However, as nitrate is the end product of nitrification process, an accumulation of nitrate in the water is then found and water exchange is recommended when nitrate in the water reach 50 mg NO_3-N/L.

Although toxicity of nitrate is much lower than ammonia and nitrite, it is generally known that high concentration of nitrate is also toxic to animal. Hence the build up of nitrate in CRSS could possibly affect shrimp especially during long culture period. The aim of this study was to determine the toxicity of nitrate in both short term (acute effect) and long term (chronic effect) to *P. monodon*. The experiment consisted of two sections. The first section was a determination of lethal concentration of nitrate to *P. monodon* and the second section was the monitoring of the physiological response of *P. monodon* to nitrate in the water along 216 days of cultivation in CRSS. In both sections, physiological responses of shrimp were monitored: oxygen consumption rate, stress parameters (haemolymph protein, haemolymph glucose, and total haemocyte counts) and parameter related with immune system (phenoloxidase activity).

Objectives

1. To evaluate the acute toxicity of nitrate to *P. monodon* by determining the lethal concentration and physiological responses.

2. To compare the physiological responses among *P. monodon* cultured under low and high nitrate condition in the closed recirculating seawater system.

Expected Result

1. Obtain more knowledge in the physiological responses of black tiger shrimp to nitrate in the water.

2. The results could be used for improving regulation processes and control of the closed recirculating seawater system for long term rearing of shrimp broodstock.

CHAPTER II

LITERATURE REVIEW

2.1 Roles of nitrate in natural environment

Nitrate ion (NO_3^-) plays an important role in nitrogen cycle in natural environment. In general, nitrate is one of the dissolved inorganic nitrogen compounds concurrently found with ammonia (NH_3 or NH_4^+) and nitrite (NO_2^-). Although there were many inorganic nitrogen compounds that related with the nitrogen cycle, most of the reactive compounds were mainly within three forms: ammonia, nitrite and nitrate. Natural sources of nitrate include igneous rocks and volcanic activity, ground water, mineralization of native soil organic nitrogen and debris of plant and animal. Many human activities cause nitrate contamination in natural water resources. For example, the release of agriculture fertilizer from land into the river or sea can cause the eutrophication problem.

Nitrification process is the principle source of nitrate in aquatic environment (Canadian Council of Ministers of The Environment, 2003). Wastewater containing high nitrate concentration from intensive aquaculture ponds is one of the important environmental problems found in many parts of the world. In aquaculture pond, animal excretion and decomposition of uneaten feed and other organic compounds cause ammonia accumulation. As ammonia is toxic to animals, high concentration of ammonia in aquaculture pond must be avoided. Nitrogen from feed and organic decay from aquatic animal in well oxygenated water may build up to a concentration of 0.32 mM (4.5 mg-N/L) in commercial penaeid shrimp rearing system by nitrification process (Muir *et al*., 1991). In the earth pond, nitrate is actively taken up by aquatic plants and algae.

2.2 The closed recirculating seawater system (CRSS) for aquaculture

One of the limiting factors of shrimp production in Thailand is the insufficiency of broodstock capture from the sea. To diminish this problem, rearing broodstock in secured pond must be accomplished. The broodstock rearing system so called “biosecure” or “specific pathogen-free” (MPEDA/NACA, 2003) is conducted in the closed recirculating seawater system (CRSS). The aim of CRSS is to prevent disease transmission through

water changing and control water quality within the safety range. Seawater in CRSS is usually treated by nitrification process in which ammonia is converted to nitrite and nitrate respectively. Nitrate, the final inorganic nitrogen produced after nitrification treatment, has less toxicity than ammonia and nitrite. Therefore, nitrification treatment can prolong the water changing cycle until the concentration of nitrate accumulated in the pond reach more than 50 mg-N/L (Hanlon and Forsythe, 1985). Grguric and Coston (1998) found that nitrate concentration in seawater aquarium build up to 135 mg-N/L after 5 year of operation. As a result of nitrification biofiltration, nitrate accumulation up to 310 mg-N/L was reported in CRSS for shrimp broodstock. The closed, recirculating seawater system with denitrification was therefore designed to control and reduce nitrate concentration for long term rearing system without seawater exchange (Menasveta *et al*., 2001).

2.3 Toxicity of nitrate to animals

The results from nitrate toxicity study in human showed that nitrate can undergo endogenous reduction to nitrite, and nitrosation of nitrites can form N-nitroso compounds which are potent carcinogens (Parslow *et al*, 1997; Gulis *et al*., 2002). They suggested that N-nitroso compounds are related with non-Hodgkin lymphoma, cancer of the digestive and urinary tracts and may be a precursor of chemicals which are toxic to pancreas. For aquatic animal, nitrate is taken up from the high nitrate environment or via food chain transfer (Cheng *et al*., 2002). Nitrate toxicity as indicated by LC_{50} in various animals is shown in Table 1.

Table 1. The LC_{50} (median lethal concentration) of nitrate-N on several species of vertebrates and invertebrates.

Species	Condition	LC_{50} (mg/l)	Reference
Vertebrates			
Frog, *Pseudacris regilla* (embryo)	Freshwater	10 day 578	Schuytema and Nebeker (1999)
Frog, *Xenopus laevis* (embryo)	Freshwater	10 day 438-871	Schuytema and Nebeker (1999)
Channel catfish, *Ictaturas punctatus*	freshwater	96-h 6200	Colt and Tchobanoglous (1976)
Guppy fry	Freshwater	72-h 199	Rubin and Elmaraghy (1977)
Heteromycteris capensis	34.4-35.7psu	24-h 5050	Brownell (1980)
Gaidropsarus capensis	34.4-35.7psu	24-h >4000	Brownell (1980)
Diplodus sargus	34.4-35.7psu	24-h 3560	Brownell (1980)
Lithognathus mormyrus	34.4-35.7psu	24-h 3450	Brownell (1980)
Guadalupe bass, *Micropterus treculi*	Freshwater	96-h 1261	Tomasso and Carmichael (1986)
Carp, *Catla catla*	Freshwater	24-h 1565	Tilak *et al.* (2002)
Invertebrates			
Macrobrachium rosenbergii	1 psu	3-4 week 160	Wickins (1976)
Penaeid shrimps	28 psu	48-h 4300	Wickins (1976)
Cheumatopsyche pettiti (early instar)	Freshwater	96-h 113.5	Camargo and Ward (1992)
Cheumatopsyche pettiti (last instar)	Freshwater	96-h 165.5	Camargo and Ward (1992)
Hydropsyche occidentalis (early instar)	Freshwater	96-h 97.3	Camargo and Ward (1992)
Hydropsyche occidentalis (last instar)	Freshwater	96-h 109.0	Camargo and Ward (1992)
Penaeus paulensis (broodstock)	32 psu	96-h 2171	Cavalli *et al*. (1996)
Abalone, *Haliotis tuberculata*	Seawater	Safe level 100-250	Basuyaux and Mathieu (1999)
Urchin, *Paracentrotus lividus*	Seawater	Safe level 100	Basuyaux and Mathieu (1999)
P. monodon (juveniles)	15 psu	96-h 1449	Tsai and Chen (2002)
P. monodon (juveniles)	25 psu	96-h 1575	Tsai and Chen (2002)
P. monodon (juveniles)	35 psu	96-h 2316	Tsai and Chen (2002)

2.4 Determination of toxicity

Determination of the toxicity of chemical compounds to living animals can be examined by several ways, primarily from molecular to cellular level, depend on the objective of the study. With environmental toxicology studies, however, dose of the toxic substance is implied from a measuring of concentration within the medium such as air, water, or sediment, associated with exposure time. Toxicity bioassay are usually performed by exposing a representative population of organisms to range of chemical concentrations and recording physiological responses such as mortality, or graded effects such as growth or reproductive performance. The end point may be any quantifiable response that can be related to chemical dose or exposure and may include changes in enzyme activity, tissue chemistry, pathology, or even behavioral changes (Wright and Welbourn, 2002).

2.5 Physiological responses of aquatic animals to nitrate

Newman (2000) stated that stress response is “a measurable alteration of a physiological steady-state that is induced by an environmental change and that renders the individual more vulnerable to further environment change.” With this experiment, sodium nitrate ($NaNO_3$) was used as a stressor to *P. monodon*

In amphibians, after *Pseudacris regilla* and *Xenopus laevis* embryos were exposed to sodium nitrate, nine percent of survived *P. regilla* embryos exposed to 979.2 mgNO_3-N/L exhibited cardiac and abdominal edema and lordosis (Schuytema and Nebeker, 1999). In fish, Shimura *et al*. (2002) reported that *Oryzias latipes* exposed to 75 mgNO_3-N/L had lower fertilization rate, delay hatching time and reduce hatching rate of egg laid by adults. The decrease of growth rate of juveniles and lower number of egg laid by fish was found in juvenile fishes exposed to nitrate at the concentration as low as 50 mgNO_3-N/L. Moreover, they suggested that the effect of nitrate on the reproductive system may be initiated by low concentration such as 30 mgNO_3-N/L.

Invertebrate response to stress such as environmental change and water quality can be indicated by several physiological parameters. With this study, total haemocyte counts, haemolymph protein, haemolymph glucose and phenoloxidase activity, which mostly related with immune response, were among the main parameters to be used. The information concerning the mentioned parameters is described as following:

2.5.1 Total Haemocyte Counts (THCs)

In abalone, *Haliotis diversicolor supertexa,* total haemocyte number was reported to be correlated with immune defense system (Cheng *et al*., 2004). In crustacean, most of the haemocyte cells (80%) are hyaline cells (HC). HC is a small cell containing large nucleus. Other haemocyte cells are semi-granular cells (SGC) and large granular cells (LGC) which represent 10-13% and 4-10% of the total haemocyte cells, respectively. The total number of haemocyte cells is used as stress indicators in many studies.

For example, in crustaceans, THCs was used to study the effects of stress to the western rock lobsters, *Panulirus cygnus,* under the simulating condition of shrimp transportation from farm to market (Jussila, 2000). Increase in total haemocyte was found in this lobster when it was exposed in the air with vigorous vibration (*Jussila et al.,*

1999). Other stress conditions that were reported to reduce THCs including low salinity exposure, eyestalk ablation or spermatophore exploitation in shrimp *Farfantepenaeus paulensis* (Perazzolo *et al*. (2002), exposed to the harbour dredge spoils in common shrimp *Crangon crangon* (Smith *et al*., 1995), intermolting stages of *Penaeus stylirostris* (Moullac *et al*., 1997). On the other hand, no significant difference in THCs was reported in *Litopenaeus vannamei* exposed to 0-21.6 mgNH_4-N/L for 7 day (Chen *et al*., 1994). Generally, normal THCs of *P. monodon* as reported in Supamattaya *et al*., (2000a) was 6.49$\pm$2.33 x10^4cell/mm^3 and 3.07$\pm$1.91 x10^4cell/mm^3 for shrimp in grow-out pond and in laboratory condition respectively.

2.5.2 Haemolymph protein

Stress of *Penaeus vannamei* and *Penaeus stylirostris* caused by different salt concentrations has been studied using the determination of total protein in blood serum (Rodriguez, 1981). It was found that *P. vannamei* was more tolerant to salinity despite the fact that *P. stylirostris* naturally occurs in coastal areas where conditions are virtually marine with little freshwater influence. Significant decrease in total blood protein was observed in *P. sytlirostris* at a salinity of 50 psu associated with an extremely high osmotic pressure. In *Palaemon elegans* (Rathke), Taylor *et al*. (1987) detected the increase in total protein concentration after exposing with hyper and hyposaline conditions. Haemolymph protein was also found related to molting and starvation (Paterson *et al*., 1999; Cheng *et al*., 2001; Bursey and Lane, 1971; Chen and Cheng, 1993) and different stages of ovarian development (Lee and Chang, 1997; Marangos *et al*., 1988).

Response of haemolymph protein to nitrite and nitrate was studied in *Marsupenaeus japonicus* (Cheng and Chen, 2002b). They found that haemolymph protein in shrimp exposed to 5.03 and 20.40 mgNO_2-N/L (71.03 and 65.81 mg.protein/ml respectively) was lower than control shrimp without nitrite (79.84 mg.protein/ml). While haemolymph protein of shrimp exposed to 104.48 mgNO_3-N/L decreased to 74.54 mg.protein/ml. Moreover, change in haemolymph protein as cause by ambient nitrite was also reported in *Penaeus japonicus* (Chen and Cheng, 1995).

2.5.3 Haemolymph glucose

Haemolymph glucose was used as an indicator to stress in aquatic animals. The study in Gilthead sea bream (*Sparus aurata*) showed that the concentration of plasma glucose in this fish increased when fishes were kept at high density (Barton *et al*., 2004). Knoph and Thorud (1996) found that Atlantic salmon exposed to high ammonia (225 μgNH_3-N /L) had higher plasma glucose concentration than the fish that exposed to low ammonia (22 μgNH_3-N /L). Decrease in haemolymph glucose was found in rainbow trouth exposed to 5 psu NaCl, in which the energy requirement was elevated due to osmoregulatory processes (Krumschnabel and Lackner, 1993).

In crustaceans, haemolymph glucose of the river crab (*Potamonautes warreni*) increased from 8.52$\pm$7.53 mg % to 173.80$\pm$77.29 mg % after 6 hours anoxia condition (Van Aardt, 1988). Chinese freshwater crab, *Eriocheir sinensis* exposed to hypoxia condition exhibited the rapid increase in blood glucose and following with slowly decline to the original concentration (Zou *et al*., 1996). In contrast, blood glucose concentrations of the estuarine crab, *Chasmagnathus granulate*, decreased to 0.00 mg % after the reduction of salinity to freshwater for 72 hours and restored to normal concentration (7.47 ± 1.53 mg %) after 168 hours (Santos and Nery, 1987). Spaargaren and Haefner (1987) found that haemolymph glucose of brown shrimp, *Crangon crangon*, decreased with increasing size. The highest glucose concentration was found in male and the lowest was found in non-ovigerous females, and intermediate in ovigerous females.

Moreover, haemolymph glucose concentration was studied versus oxygen consumption in shrimps (*P. aztecus* and *Sicyonia brevirostris*) portunid crab (*Callinectes similes, Portunus spinicarpus* and *P. gibbesii*) and the calappids (*Calappa sulcata*; *Hepatus ephelyticus*). It was found that the rates of oxygen consumption versus glucoses concentration were greater in shrimps than in portunids and calappids. Glucose concentration was considered as the main energy source for crustacean muscle and proportion of glucose concentration in relation to oxygen consumption (100 %) varies among species, with lowest values for shrimps (55 %), against the values recorded in portunid (126 %) and calappid crabs (235-423 %), indicating the amount of glucose required for chitin synthesis of species with different morphophysiological designs and activity pattern (Rosas *et al*., 1992). Baseline of haemolymph glucose concentrations in black tiger shrimp (*P. monodon*) reported in (Supamattaya *et al*., 2000a; Supamattaya *et al*., 2000b) was 32.91$\pm$27.95 mg % and 53.87$\pm$54.84 mg % for shrimp in farm and in

laboratory conditions respectively. They also suggested that the glucose concentration did not depend on sex and size of shrimps.

2.5.4 Phenoloxidase (PO) *activity*

Activity of phenoloxidase enzyme has been used to indicate the immunological response of crustaceans to bacterial infection. More than 90% of phenoloxidase activity was found in haemocytes and the rest was in the serum. Phenoloxidase activity was studied in various crustacean species such as the tropical rock lobster, *Panulirus ornatus* (Norton *et al*., 1999), giant freshwater prawn, *M. rosenbergii* (Sung *et al*., 2003), white shrimp *Litopenaeus vannamei* (Liu and Chen, 2004), common shrimp, *Crangon crangon* (Smith *et al*., 1995) and *P. paulensis* (Perazzolo and Barracco, 1997). In general, trypsin and lipopolysaccharides were able to increase the enzyme activity. The phenoloxidase activity of *P. californiensis* was studied using L-DOPA as the substrate (Galván *et al*., 1999). Baseline of the phenoloxidase activity of *P. monodon* under farm and laboratory conditions were 335.18$\pm$106.29 and 217.84+161.99 unit/min/mg protein respectively (Supamattaya *et al*., 2000a; Supamattaya *et al*., 2000b).

2.5.5 Haemolymph inorganic nitrogenous compound

In aquatic animals, nitrogenous waste derived from cellular metabolism is usually excreted as urea and ammonia. The excretion is diffusion process in which high concentrations of urea and ammonia in the body fluid diffuses to the surrounding water that contain lower concentration. In shrimp *P. paulensis* , sudden change in ammonia concentration in the water from 7, 20, or 50 mgNH_4-N/L to 20 mgNH_4-N/L caused a transient increase in haemolymph ammonia-N concentration. A similar transient increase in haemolymph ammonia occurred during recovery period (Schmitt and Santos, 1999). Schmitt and Uglow (1997) studied the effects of ambient total ammonia (TA= NH_3+NH_4^+) in the water on TA in haemolymph of the prawn, *Nephrops norvegicus*. They found that concentration of haemolymph TA increased rapidly in the first 2 hours but tend to drop thereafter. Original concentration of haemolymph TA was restored within 6 hours after transferring prawns from 28 mg-TA/L to <0.01 mg-TA/L. In addition, sudden exposing to 7, 14, 28, and 56 mg-TA/L induced haemolymph TA concentration were 50, 30, 33 and 36% of ambient TA concentration respectively. After transferring the shrimp back to <1 µmol TA/L seawater, TA excretion rates were higher

than those of control prawns and the absolute amounts of TA excreted were considerably higher than the calculated TA accumulation in the haemolymph. Another study was done by Chen and Kou (1993) with *P. monodon* exposed to 10, 50, and 100 mg/L ambient ammonia-N in 34 psu at pH 8.2. They found that haemolymph ammonia of shrimp exposed to 10, 50, and 100 mg/L was significantly (P<0.05) higher than the controls after 1.5 hour exposure. Since the normal concentration of haemolymph ammonia was 12.33±2.18 mg/L, increase of haemolymph ammonia up to 20 mg ammonia-N/L could induce weakness or eventually death to shrimps. In contrasts, haemolymph ammonia of *P. japonicus* was inversely related to ambient nitrite and exposure time (Cheng and Chen, 2001).

For haemolymph nitrite, several studies with *P. monodon* and *P. japonicus* found the positive linear correlation between haemolymph nitrite and nitrite concentration in the water (Cheng and Chen, 2000; Cheng and Chen, 2001; Chen and Cheng, 1995; Cheng and Chen, 2002a). The study in *M. rosenbergii* by Chen and Lee (1997) also found the similar results. In detail, haemolymph nitrite of *P. monodon* exposed to the water containing 2, 4, 8, and 20 mg/L nitrite-N was 2.229, 6.849, 9.311 and 25.704 µg/ml respectively. Cheng and Chen (2000) suggested that when *P. monodon* was exposed to ambient nitrite, nitrite was immediately incorporated into the haemolymph via brachial chloride uptake, and later accumulated in the tissue.

The study in giant clams; *Tridacna gigas,* found that nitrate could be rapidly accumulated in the haemolymph (Shepherd *et al*., 1999). In *P. monodon* exposed to combined solution of 20 mgNO_2-N/L and 100 mgNO_3-N/L, haemolymph nitrate were 6.3 mgNO_3-N/L higher than the ambient nitrate concentrations. This was concluded that incorporation of nitrite is converted to nitrate in haemolymph (Cheng and Chen, 2002a; Cheng and Chen, 2002c). In freshwater crayfish, *Astacus astacus,* haemolymph nitrate was also found increase in parallel with ambient nitrate. This was suggested as a result of passive diffusion between water and body fluids (Jensen, 1996).

2.5.6 Oxygen consumption rate

The transport of oxygen is essential for efficient aerobic metabolism in most animals. Haemocyanin, the binuclear type 3 copper proteins, is the main component of oxygen transport mechanism in many arthropods and mollusks (Van Holde *et al*., 2001).

Oxygen consumption is the physiological parameter undergo significantly change with body weight, temperature, as well as with certain physiological activities (Alikhan, 1983). Contamination of toxic substances in the water may effect to aquatic animals and can be detected with the change in oxygen consumption rate. For example, oxygen consumption rate of crab, *Sesarma quadratum* (Fabricius), was found negatively correlation to the sublethal concentrations of copper chloride and chlorine (Valarmathi and Azariah, 2002). In *P. chinensis*, oxygen consumption of the shrimp increased when they were exposed to increasing ambient ammonia (Chen and Nan, 1993). In contrast, the rise of oxygen consumption could be consequence of the stress caused by the confinement conditions in a limited space and/or the isolation since it is species behavior (Esteve and Patti, 2004).

CHAPTER III

MATERIALS AND METHODS

This study consisted of two experiments. The first experiment was the toxicity of nitrate to shrimp (acute toxicity). The second experiment was the physiological study in shrimp exposed to different nitrate concentrations in the closed recirculating seawater system (long term toxicity).

3.1 Acute toxicity study

Black tiger shrimp (*Penaeus monodon*), 10.9±0.6 cm in length and 17.4±2.7 g in weight, were collected from shrimp pond in Pathum Thani province and kept in cement tanks containing seawater (30 psu) at the Marine Biotechnology Research Unit, Chulalongkorn University, prior to use. The acute toxicity test of nitrate to shrimp, estimated by median lethal concentration (LC_{50}) at 96 hour, was conducted in fiber glass tanks containing 25 L of 30 psu seawater with various nitrate concentrations (0-4500 mg NO_3-N/L) made up with sodium nitrate. The study was arranged in to three trials with different nitrate concentrations in each trial (see Table 2).

Table 2. Sodium nitrate prepared in each stock nitrate concentration in 100 liter tanks for daily exchange of acute nitrate toxicity test.

Nitrate concentration (mg NO_3-N/L)	**Sodium Nitrate (g/100 liter)**
0	0
500	303.32
1000	606.64
1500	909.96
2000	1213.28
2250	1364.94
2500	1516.60
2750	1668.25
3000	1819.91
3250	1971.57
3500	2123.23
4000	2426.55
4500	2729.87

Before the experiment, shrimps were starved for 24 hours before being transferred into the experiment tanks. Each experiment tank was separated into 6 cells using plastic net (Figure 1). This reduced the loss of shrimp due to cannibalism behavior.

Figure 1. Experimental tank for nitrate toxicity test was separated into 6 cells using plastic net in order to prevent cannibalism behavior.

During Experiment, water was changed everyday with fresh seawater containing the same nitrate concentration in order to reduce the accumulation of ammonia excreted by shrimps. 25 ml of water from each experiment tank was taken for ammonia, nitrite and nitrate determination every day. The detail of water analysis is described in section 3.5. Mortality of shrimps was recorded every 4 hours and the LC_{50} at 96 hour was calculated with Probit Analysis (Raymond, 1985) (Appendix A).

At the end of each trial, haemolymph of survive shrimps after exposed with various nitrate concentrations was collected using small (No. 26) needle syringe, mixed 25 µL haemolymph with 25 µL anticoagulant in tube I and 100 µL haemolymph with 100 µL anticoagulant in tube II (10% Sodium citrate, pH 7.2), and kept refrigerated ($0^{o}C$) prior to analysis. The haemolymph analysis included total haemocyte counts (THCs), haemolymph protein, haemolymph glucose, phenoloxidase activity, haemolymph ammonium-N, haemolymph nitrite-N, and haemolymph nitrate-N. Detail of haemolymph analysis is described in section 3.3.

3.2 Long term toxicity test

In this experiment, shrimps were cultured in the closed recirculating seawater systems (CRSS). Diagram and photograph of CRSS and nitrate treatment system are shown in Figures 2 and 3, respectively. Two CRSS were used for long term nitrate toxicity study, both systems consisted of 7 m^2 round pond and water treatment tank containing 7,000 L of 30 psu seawater. One of the CRSS was attached with tubular denitrification treatment system for nitrate removal and therefore assigned as treatment set. Fifty black tiger shrimps collected from shrimp pond in Pathum Thani Province (11.6±1.4 cm in length and 24.4±6.6 g in weight) were grown in both CRSS for 216 days. With nitrate removal system, nitrate in the treatment CRSS was regulated within the range of 15.24-25.27 mg-N/L while nitrate concentration in control CRSS was accumulated to more than 50 mg-N/L at the end of 216 day experiment.

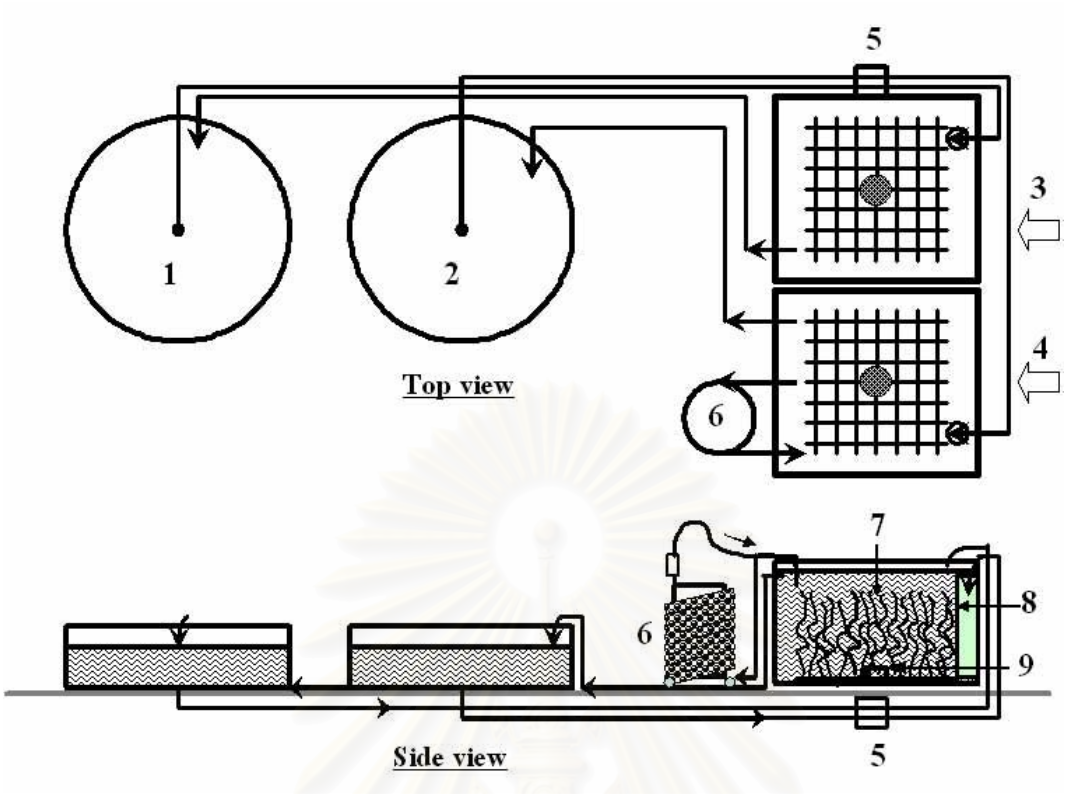

Figure 2. Diagrams illustrates the closed, recirculating seawater systems (CRSS) used in long term toxicity experiment. CRSS consisted of: 1 Circular rearing pond (control); 2, circular rearing pond (treatment); 3, nitrification tank (control); 4, nitrification tank (treatment); 5, water filtering system; 6, tubular denitrification reactor (treatment); 7, nitrification biofilter substrate (BIO-POLYMA^{TM}); 8, water sink-flow column; 9, air diffuser (Modified from Menasveta *et al*., 2001).

(a) **(b)**

Figure 3. The closed recirculating seawater system at the Marine biotechnology research unit, Chulalongkorn University. (a) two round ponds for shrimp culture, (b) tubular denitrification reactor for nitrate removal in treatment CRSS.

Physiological study

During long-term toxicity study, haemolymph was collected every 10 days from 10 shrimps in each pond throughout 216 days culture period. Haemolymph analysis included THCs, haemolymph protein, haemolymph glucose, phenoloxidase activity, haemolymph ammonium-N, haemolymph nitrite-N, and haemolymph nitrate-N. Growth and survival rate of shrimps were monitored every 30 days by measuring length and weight of all shrimps in both ponds. Moreover, comparison of oxygen consumption rate (OCR) of shrimps exposed with different nitrate concentrations was performed at day 75 with 9 shrimps from each pond. Detail of OCR study is shown in section 3.4.

3.3 Haemolymph analysis

Preparation of shrimp's haemolymph for physiological assays

Approximately 300 µL of haemolymph sample from each survived shrimp was collected from the last pair of walking legs (Supamattaya *et al*., 2000a) using small syringe (1 mL #26) as illustrated in Figure 4. Haemolymph sample was quickly separated into 3 Eppendrof tubes in an ice box for further analysis. Diagram in Figure 5 shows an overview of haemolymph preparation for physiological assays. Detail of each parameter is described in sections 3.3.1 to 3.3.4.

Figure 4. Haemolymph sampling: approximately 300 µL collected from the last pair of walking leg of survived shrimp for physiological assay.-

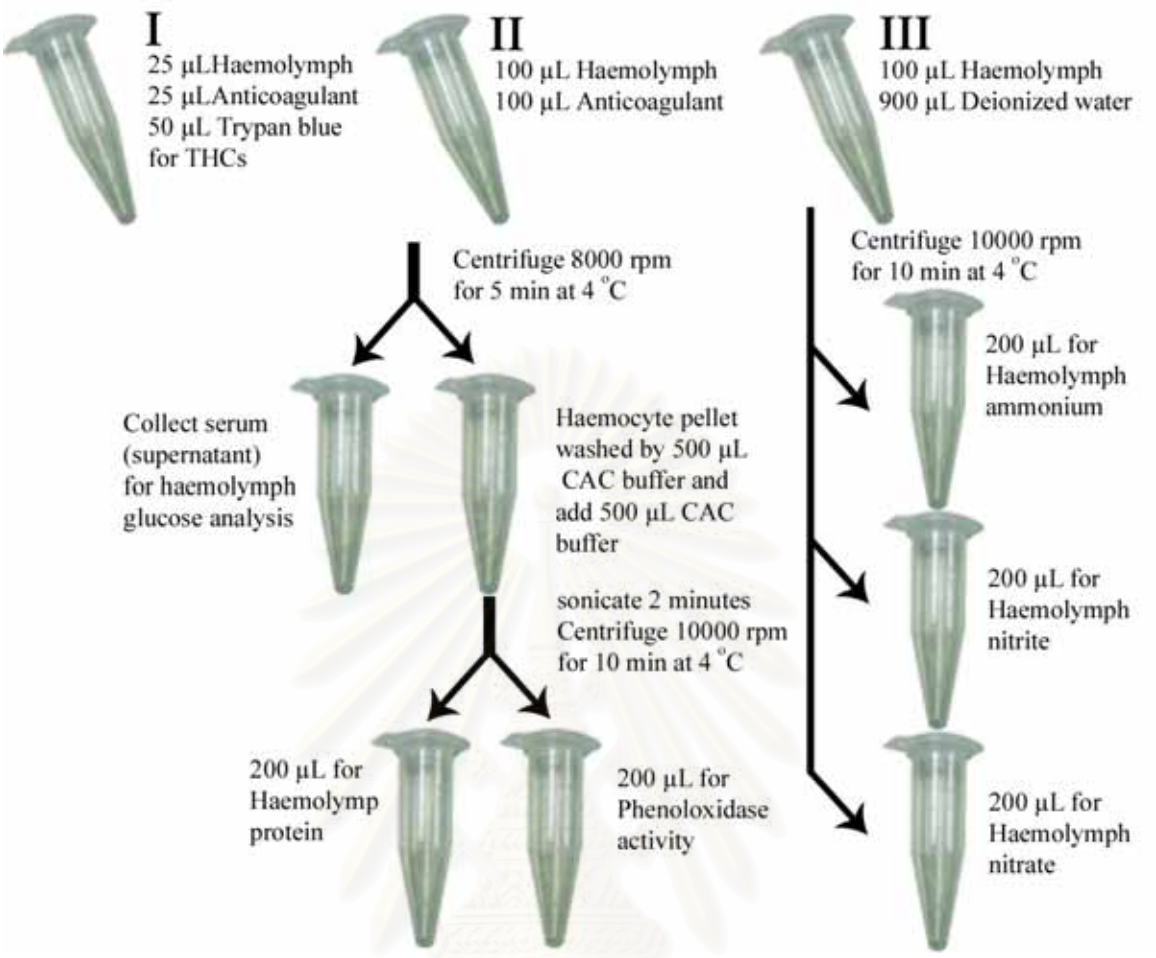

Figure 5. Haemolymph preparation: Tube I was prepared for total haemocyte counts, Tube II was prepared for haemolymph glucose, protein and phenoloxidase activity analysis and Tube III was prepared for haemolymph ammonium, nitrite, and nitrate analysis.

3.3.1 Total haemocyte counts (THCs)

Method for THCs was modified from Supamattaya *et al*. (2000a) with Trypan blue stained method. Twenty five microliter of haemolymph was mixed with anticoagulant (10% sodium citrate, pH 7.2) at 1:1 (v/v) and stained with 50 µL trypan blue then stored in an ice box. THCs were performed using haemacytometer under compound microscope. Living haemocytes (unstained) and death haemocytes (blue stained) as shown in Figure 6 were counted and calculated as following:

Total haemocyte counts (cell/ml) = total of cell count x 5 x 10^4 x dilution factor

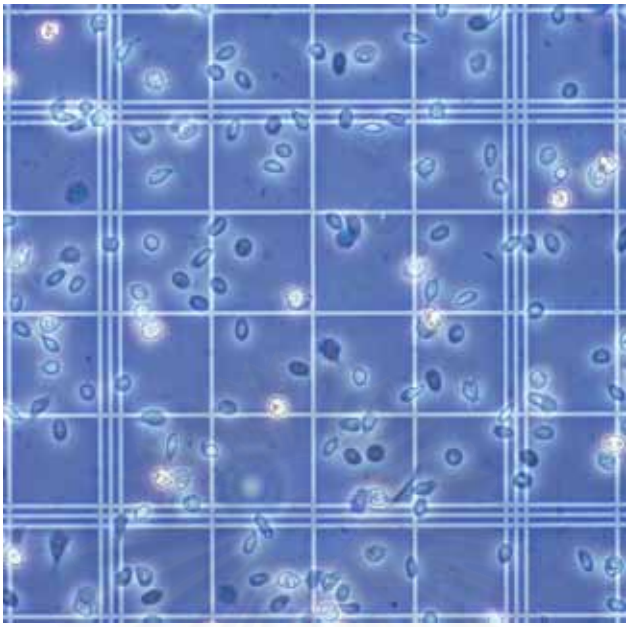

Figure 6. Haemocyte stained with Trypan blue for Total Haemocyte Counts (THCs) under compound microscope

3.3.2 Haemolymph glucose analysis

Hundred microliter of haemolymph sample (Tube II in figure 5) was mixed with anticoagulant (10% sodium citrate, pH 7.2) at 1:1 (v/v) and centrifuged at 8000 rpm for 5 minutes (at 4 ^{o}C). Serum (supernatant) was collected for haemolymph glucose analysis using Haltman method (Hyvarinen and Nikkila, 1962). Hundred microliter of serum was mixed with 100 µL TCA solution (30% Trichloroacetic acid in deionised water), centrifuged at 8000 rpm for 5 minutes. Eighty microliter of supernatant was added into 1.5 ml Eppendorf tube, mixed with 1000 µL colour reagent (0.15% Thiourea in 94% glacial acetic acid with 6% O-toluidine solution), and boiled for 10 minutes. Absorbance of coloured reagent was measured with spectrophotometer (GENESYS®; model 10 UV scanning) at 630 nm against glucose standard solution (100 mg glucose in 1000 ml of deionised water).

3.3.3 Haemocyte lysate supernatant (HLS) for phenoloxidase activity and haemolymph protein analysis

Haemocyte pellet after centrifugation was washed with 500 µL CAC buffer (10 mM Ca-codylic acid with 100 mM $CaCl_2$ in deionised water, was adjusted pH

to 6.8 with sodium hydroxide) and then resuspended in 500 µL CAC buffer. After 2 minute sonication, haemocyte sample was centrifuged at 10000 rpm for 10 minutes (at 4^{o}C). Haemocyte lysate supernatant (HLS) was finally collected for phenoloxidase activity and haemolymph protein analysis.

3.3.3.1 Phenoloxidase activity (modified from Supamattaya *et al*. (2000a))

Hundred microliter of HLS mixed with 100 µL trypsin solution (0.1% Trypsin in CAC buffer) incubated for 10 minutes at room temperature and 100 µL L-dihydroxyphenylalanine (L-DOPA 4 mg/ml in CAC buffer) was added into the 96 wells plastic plate. Kinetic activity of phenoloxidase enzyme was then measured with microplate reader (BIO-RAD®model 550) at 490 nm for 10 minutes. Phenoloxidase activity was calculated as:

Phenoloxidase activity = unit at $0.001(\Delta OD_{490})$/minute/mg.protein in solution

3.3.3.2 Haemolymph protein (modified from Lowry *et al*. (1951))

Freshly solution C was prepared by mixing 0.5 ml of solution A (2% sodium carbonate, 0.4% sodium hydroxide and 0.16% sodium potassium tartrate in deionized water) with 50 ml of solution B (0.04% $CuSO_4.3H_2O$). For protein analysis, 200 µL of HLS was mixed with 1000 µL of solution C, standed for 10 minutes, then mixed with 100 µL of solution D (Folin-Ciocalteau reagent 1:1 (v/v) deionized water). Measure absorbance of the solution using spectrophotometer (GENESYS®; model 10 UV scanning) at 660 nm against protein standard (200 µg/ml bovine serum albumin).

3.3.4 Preparation of serum for the analysis of ammonium, nitrite, and nitrate in haemolymph.

Hundred microliter of haemolymph (tube III in Figure 6) was mixed with 900 µL of deionised water and kept in the ice box. Prior to analysis, haemolymph was centrifuged at 10000 rpm for 5 minutes (at 4^{o}C) then supernatant (diluted serum) was collected for further analysis.

3.3.4.1 Ammonium Analysis

Ammonium in haemolymph was analyzed by Flow Injection Analysis (FIA) technique modified from Hunter and Uglow (1993) with the manifold showed in Figure 7. The flow rate of FIA system was fixed at 1 ml/min. Twenty microliter of diluted serum sample was injected through an injector port to be mixed with an alkali carrier solution (0.1 M NaOH). Ammonium (NH_4^+) from haemolymph sample was therefore converted into ammonia (NH_3) under strong alkali condition. At the diffusion block, ammonia gas was diffuse from carrier solution across the polytetrafluroethylene (PTFE) membrane into an indicator reagent (Bromothymol blue 0.4 g/L, pH 6.5-7.0). The result was a change in bromothymol blue colour which could be detected using an inline spectrophotometer (using red LED as the light source) with chart recorder or digital data logging system. Concentration of ammonium was analysed against the ammonium standard prepared with $(NH_4)_2SO_4$ (200 mg/L).

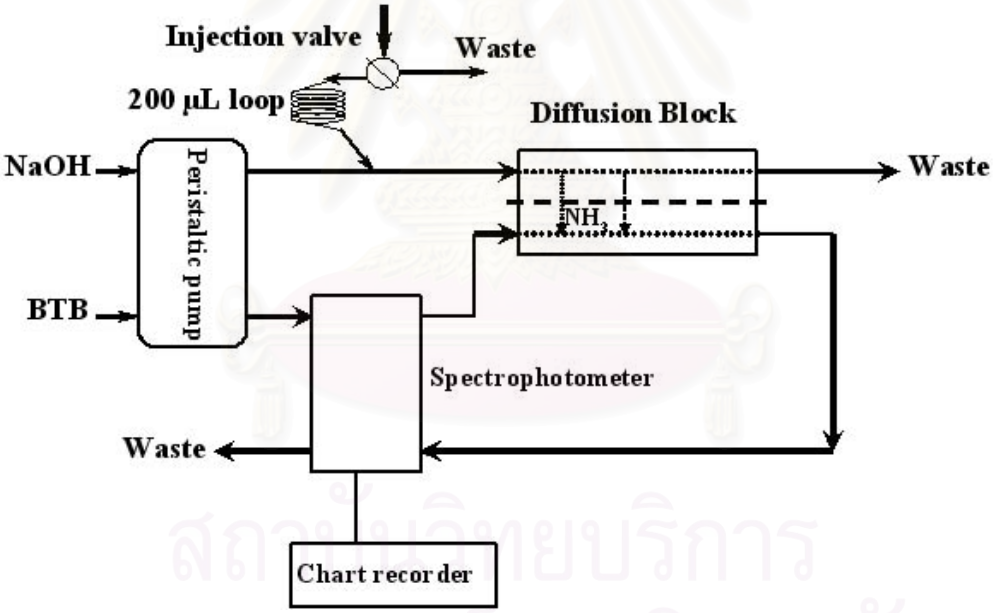

Figure 7. Diagram configuration of Flow Injection Analysis (FIA) for small volume ammonium analysis (modified from Hunter and Uglow, 1993).

3.3.4.2 Nitrite Analysis

Nitrite in diluted serum was analysed using a method modified from Strickland and Parsons (1972). One milliliter of diluted serum was mixed with 20 µL of sulphanilamine solution (1% sulphanilamide in 10% HCl) and 20 µL NNED solution

(0.1% N-(1-Naphthyl)-Ethylenediamine dihydrochloride solution in distilled water) and stand for 30 minutes. Thereafter, absorbance of the solution was measured using spectrophotometer (GENESYS®; model 10 UV scanning) at 543 nm against the nitrite standard (140 mg NO_2-N/L made from $NaNO_2$).

3.3.4.3 Nitrate Analysis

Nitrate was analysed by cadmium reduction method modified from Strickland and Parsons (1972). Diluted serum was reacted with 100 g of cadmium granule packed in 100 ml glass column. Cadmium then converted serum nitrate to nitrite. Nitrite was therefore analysed by the method already described in section 3.3.4.2. With this analysis, concentration of nitrite was the combination of nitrite converted from nitrate after cadmium reduction and nitrite originally found in the sample. Therefore, the correct nitrate-N concentration must be subtracted with nitrite-N concentration in the serum.

3.4 Oxygen consumption rate

Determination of oxygen consumption rate was conducted in a custom-made plastic chamber made of acrylic tube (7 cm in diameter and 39.5 cm length) containing 1.52 L of 30 psu seawater as shown in Figure 8. Dissolved oxygen probe (HANNA; HI 91410 Logging DO meter) was connected to the end of the chamber. Water in the chamber was stirred with magnetic bar to generate internal water movement. During experiment, one shrimp (approximately 25.46 g in weight) from experimental tank was transferred into the chamber then fresh oxygen-saturated seawater with desired nitrate concentration from the stock tank was pumped into the chamber. Reduction of oxygen concentration in the chamber, as a result of shrimp respiration, was recorded for approximately 10-12 minutes in each measurement.

The effect of nitrate on shrimp oxygen consumption rate was studied with nitrate concentrations between 50 to 500 mg $NaNO_3$-N/L. The experiment started with an evaluation of shrimp oxygen consumption rate in 50 mg NO_3-N/L. Thereafter, water in the chamber was exchanged with seawater containing 100, 250 and 500 mg NO_3-N/L respectively, following by seawater without nitrate (for recovery).

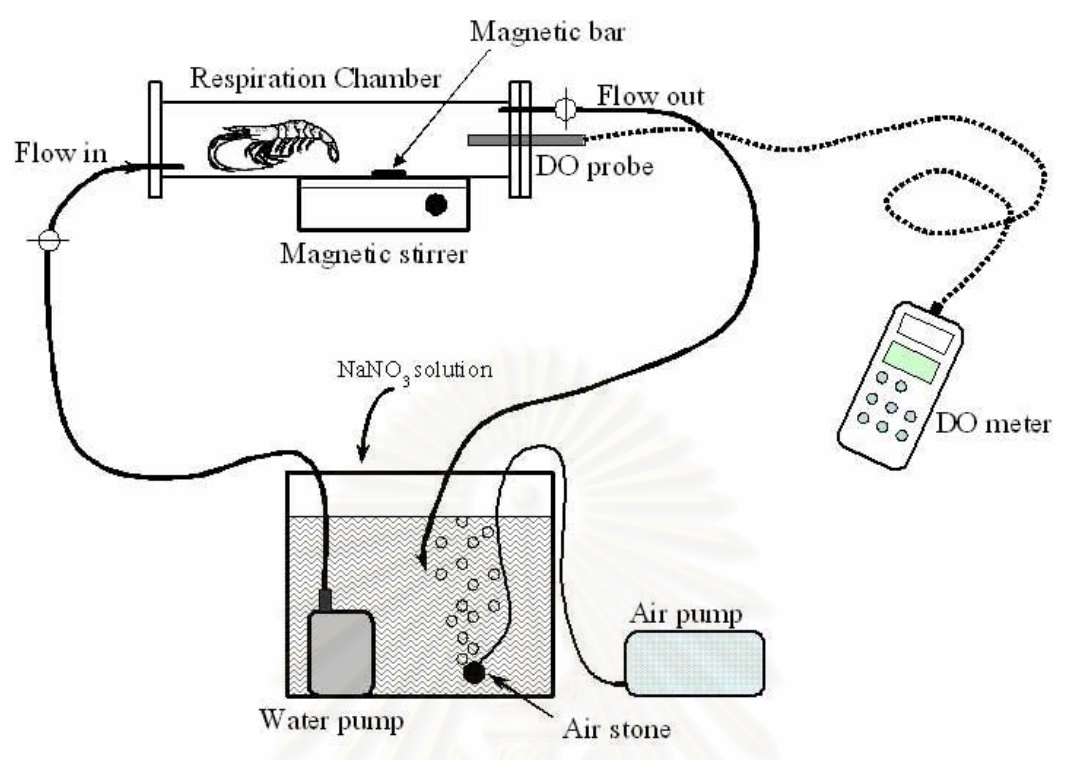

Figure 8. Diagram of a custom-made chamber for oxygen consumption rate measurement used in this study.

3.5 Water quality analysis

In this study, seawater was prepared by mixing high salinity seawater with fresh water to obtain 30 psu salinity, following by ozone treatment for 48 hours. During experiment, water salinity was measured using a hand refractometer (ATAGO® model 8608) and pH was measured using a pH meter (HI8418, Hanna Instrument). For chemical water analysis, water samples were filtered with Whatman GF/C filter and kept in the dark at -20ºC prior to analysis.

3.5.1 Ammonium (NH_4^+-N) analysis

Ammonium was analyzed by phenol-hypochlorite reaction method, modified from Strickland and Parsons (1972). Thousand microliter of filtered seawater in an Eppendrof tube was mixed with 40 µL of phenol solution (dissolve 20 g of crystalline analytical grade phenol in 200 ml of 95% (v/v) ethyl alcohol), 40 µL of sodium nitroprusside solution (0.5% $Na_2Fe(CN)_5NO.2H_2O$ in deionized water), and 100 µL of freshly prepared oxidizing reagent 20% sodium citrate and 1% sodium hydroxide in

deionized water 4:1 (v/v) sodium hypochlorite). After mixing and stand for 1 hour, the solution was transferred into a 1.4 ml cuvette with 1 cm light-path and measured the absorbance at 640 nm using spectrophotometer (GENESYS®; model 10 UV scanning).

3.5.2 Nitrite (NO_2^--N) and Nitrate (NO_3^--N) analysis

Nitrite was analyzed by sulfanilamide reaction, modified from the method of Strickland and Parsons (1972), which already described in section 3.3.4.2. Nitrate was converted to nitrite by cadmium reduction (section 3.3.4.3) and following with nitrite analysis.

3.5.3 Alkalinity

Alkalinity analysis was modified from the titration method described in Strickland and Parsons (1972), however, pH meter was used instead of observing colour changed of the methyl orange indicator. Hundred milliliter of filtered seawater sample in Erlenmeyer flasks, continuously stirred with magnetic bar, was titrated with 0.01 M H_2SO_4 (0.01 M H_2SO_4 in boiled distilled water) until the pH of the solution reached pH 4.40. Alkalinity was calculated as the following:

Alkalinity = (H_2SO_4 titrated x 1000)/ Volume of seawater sample.

CHAPTER IV

RESULTS

4.1 Acute toxicity (LC50) of nitrate to *Penaeus monodon*

4.1.1 Water Quality during acute toxicity study

Before the experiment, seawater was treated with ozone (O_3) for 30 minutes in order to reduced the number of bacteria and viruses. Daily monitoring of water quality during experiments showed that temperature, salinity, pH and alkalinity were within the normal range for *P. monodon* (Table 3).

Table 3. Physical water quality of 3 trials during LC_{50} study.

Parameters	**Trial I**	**Trial II**	**Trial III**
Temperature (^oC)	27.0-28.0	28.5-29.0	27.5-28.5
Salinity (psu)	30	30	30
pH	8.05-8.42	8.08-8.63	8.07-8.57
Alkalinity ($mgCO_3/L$)	92	86	94

In LC_{50} experiment, shrimps (*P. monodon*) were exposed to various-preset nitrate concentrations prepared by mixing $NaNO_3$ with the seawater. From nitrate analysis, the exact nitrate concentrations in experimental tanks during each trial are shown in Table 4.

Table 4. Nitrate concentrations in LC_{50} experiment after nitrate analysis.

Preset nitrate concentrations (mg NO_3-N/L)	**Correct nitrate concentrations (mg NO_3-N/L)**	**Remark**
0	1.81	Trial 1-3
500	463.61	Trial 1
1000	1079.50	Trial 1
1500	1738.89	Trial 1
2000	1901.93	Trial 1-3
2250	2133.03	Trial 3
2500	2525.43	Trial 1-3
2750	2726.24	Trial 3
3000	2914.27	Trial 1-3
3250	3256.34	Trial 3
3500	3487.56	Trial 1-3
4000	4000.28	Trial 2
4500	4449.87	Trial 2

4.1.2 Acute toxicity of nitrate to Penaeus monodon

The study on acute toxicity of nitrate to shrimp (*P. monodon*) consisted of 3 trials covering nitrate concentration from 1.81 (control) to 4449.87 mgNO_3-N/L. The results, as illustrated in Figure 9, showed that there was no mortality of shrimp in nitrate concentration lower than 1901.93 mgNO_3-N/L. On the other hand, 100% mortality of shrimp was found in nitrate concentration higher than 3256.34 mgNO_3-N/L. Median lethal concentration (LC_{50} at 96^{th} hours) evaluated by Probit analysis indicated that nitrate toxicity to *P. monodon* with average weight of 17.4±2.7 g in 30 psu seawater was 2636 mgNO_3-N/L. (Appendix 1)

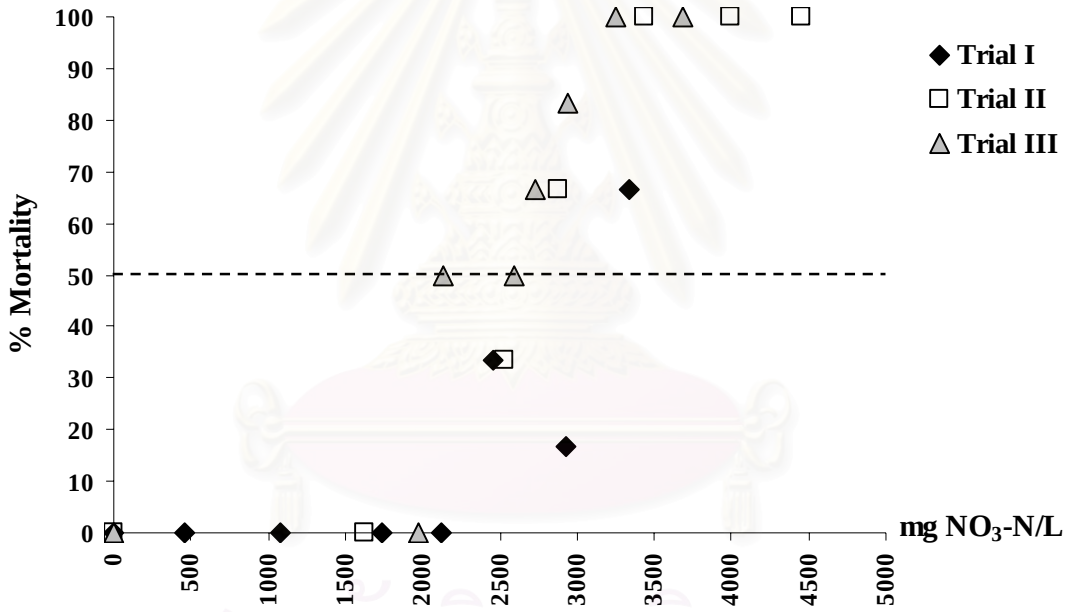

Figure 9. Mortality of *P. monodon* after 96 hours exposing with various nitrate concentrations in 30 psu seawater, illustrated by median lethal concentration (LC_{50} at 96 hours).

4.1.3 Physiological response of shrimp exposed with nitrate

Physiological response of survived shrimp after exposure to various nitrate concentrations was illustrated in Figures 10-13. The results illustrated large variation in all measuring parameters (total haemocyte counts (THCs), haemolymph protein, phenoloxidase activity and haemolymph glucose). High THCs was found in shrimp exposed to nitrate concentrations between 1079.50 and 1738.89 mgNO_3-N/L while shrimp exposed to nitrate concentrations higher than 1901.93 mgNO_3-N/L had significantly lower THCs (P<0.01 by Duncan multiple range test). Maximum THCs ($2.64x10^4$ cell/mm^3) was found in 1079.50 mgNO_3-N/L and minimum THCs ($0.42x10^4$ cell/mm^3) were found in 3256.34 mgNO_3-N/L (Figure 10).

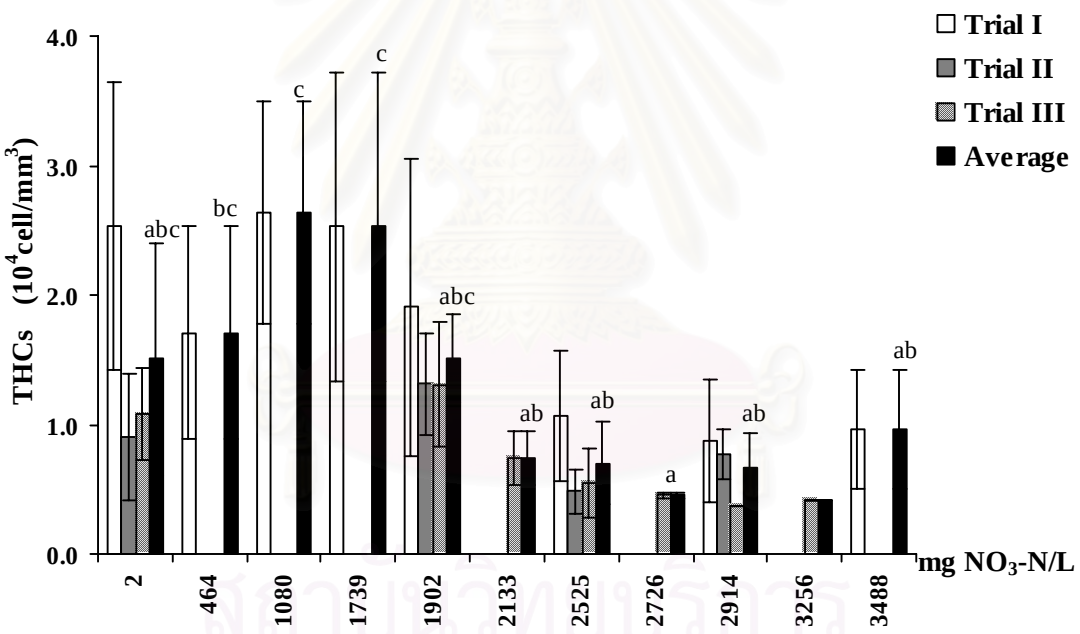

Figure 10. Total Haemocyte counts of *P. monodon* exposed to various nitrate concentrations after 96 hours experiment, while a, b or c indicate significant differences (P<0.01 by ANOVA and Duncan New Multiple Range Test).

Haemolymph protein (Figure 11) elicited greatly fluctuation as indicated by the large number of standard deviation. Maximum haemolymph protein was 4.17 mg/ml at 1901.93 mgNO_3-N/L and minimum haemolymph protein was 0.99 mg/ml at 2133.03 mgNO_3-N/L. However, statistic analysis showed that there was no significant difference in haemolymph protein among all nitrate concentrations (P>0.05).

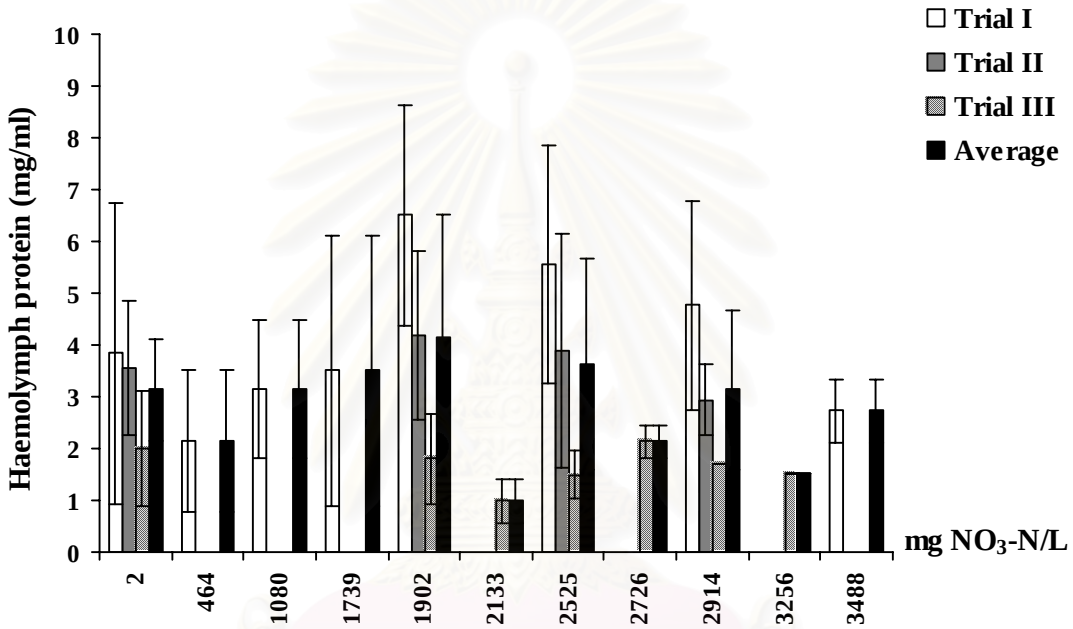

Figure 11. Haemolymph protein of survived *P. monodon* exposed to various nitrate concentrations after 96 hours experiment.

Immune response of *P. monodon*, indicated by phenoloxidase activity, had high fluctuation. Statistical analysis showed that shrimp exposed to 2133.03 mg NO_3-N/L had significantly higher phenoloxidase activity than control (Figure 12). However, in other concentrations of nitrate such as 1738.89, 1901.93, 2726.24, 2914.27 and 3487.56 mg NO_3-N/L, phenoloxidase activity was also higher than control but with lower significant level (indicated as "b" in Figure 12).

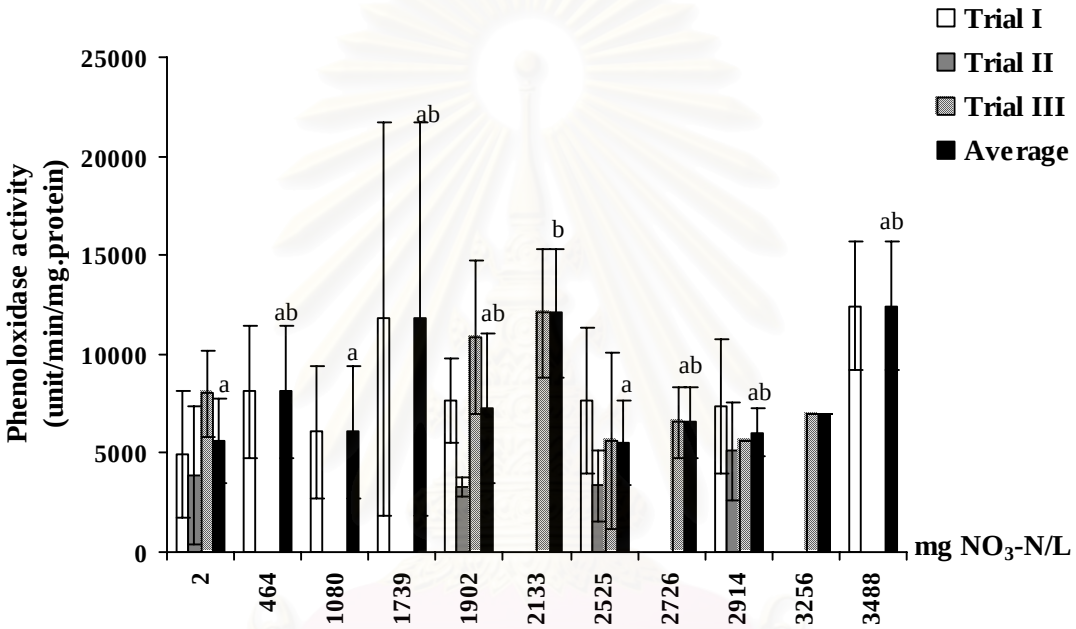

Figure 12. Phenoloxidase activity of *P. monodon* exposed to various nitrate concentrations after 96 hours experiment, while a or b indicate significant differences (*P*=0.045 by ANOVA and Duncan New Multiple Range Test).

Glucose in haemolymph of *P. monodon* exposed to various nitrate concentrations for 96 hours was between 32.18 to 148.15 mg% (Figure 13). However, statistical analysis using ANOVA did not show significant difference (P>0.05) in glucose concentrations.

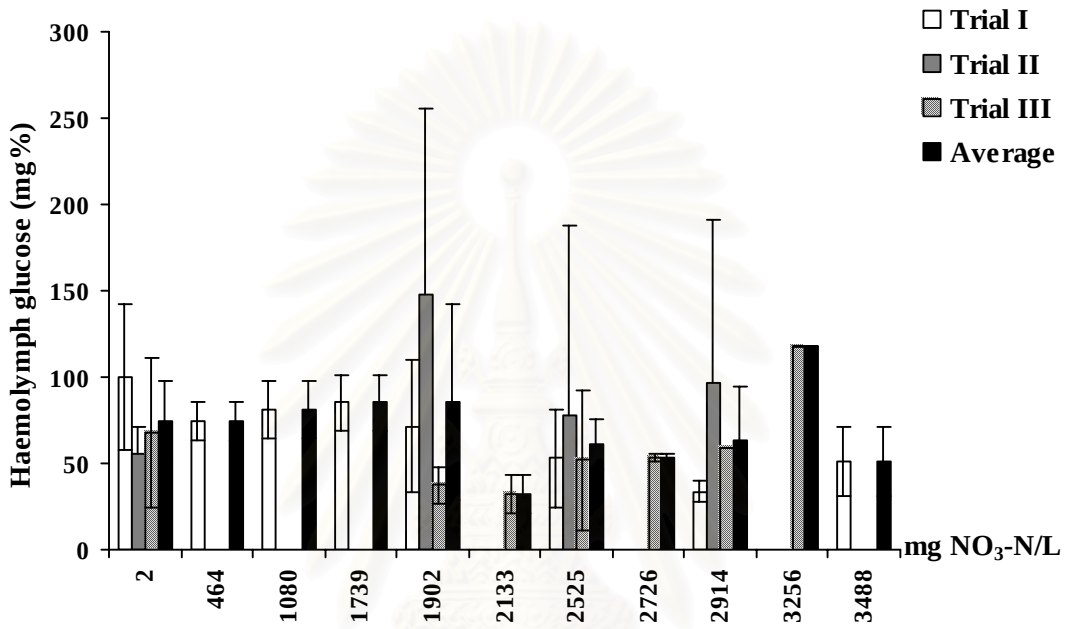

Figure 13. Haemolymph glucose of *P. monodon* exposed to various nitrate concentration for 96 hours.

4.1.4 Inorganic nitrogenous compounds

Inorganic nitrogenous compounds (ammonium, nitrite and nitrate) in water and in haemolymph of *P. monodon* after 96 hours exposed with various nitrate concentrations are shown in Figures 14 to 19. It was found that, after 96 hours experiment, shrimp in all tanks excreted high concentration of ammonia. As ammonium concentration in the water of trial 1 was between 2.5-6.5 mg NH_4^+-N/L. Therefore, in trials 2 and 3, water in all tanks was changed everyday in order to reduce the effect of excreted ammonium. Figure 14 shows that ammonium concentration in the haemolymph was ranged between 12.03-22.86 mg NH_4^+-N/L in shrimp exposed to nitrate concentration from 1.81-3487.56 mg NO_3-N/L except at 3256.34 mg NO_3-N/L that there was only one survival shrimp. Statistical analysis showed no significant difference of haemolymph ammonium concentration among treatments. The plot between ammonium in water and in haemolymph (Figure 15) shows that ammonium in haemolymph was between 12.03-22.86 mg NH_4^+-N/L which was higher than ammonium in the water. This also suggested that high ammonium concentration in the water of all trials did not affect ammonium concentration in haemolymph.

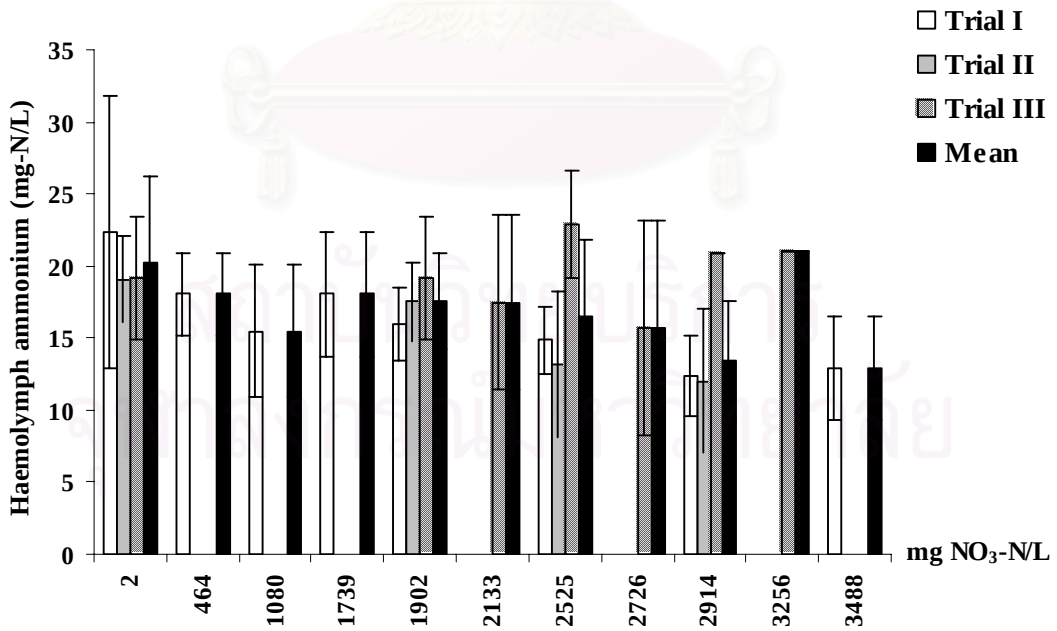

Figure 14. Concentration of ammonium in the haemolymph of *P. monodon* exposed to various nitrate concentrations for 96 hours.

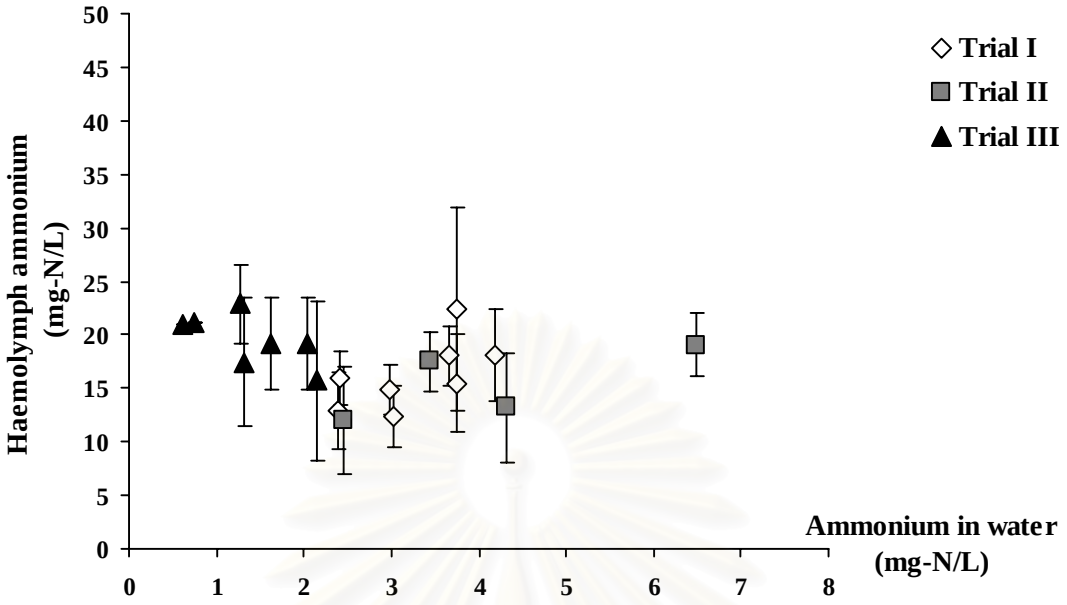

Figure 15. Concentration of ammonium in the water and in the haemolymph of *P. monodon* after exposed to various nitrate concentrations for 96 hours.

Concentration of nitrite in haemolymph increases as nitrate concentration in the water. The highest concentration of haemolymph nitrite was up to 10.29 mgNO_2-N/L in shrimp exposed to 3256.34 mgNO_3-N/L and significant difference $P<0.01$ in each nitrate concentrations (Figure 16). Haemolymph nitrite showed parallel increasing with nitrite in water. Regression analysis between nitrite in haemolymph and nitrite in water, as illustrated in Figure 17, showed the significant correlation ($P=0.034$).

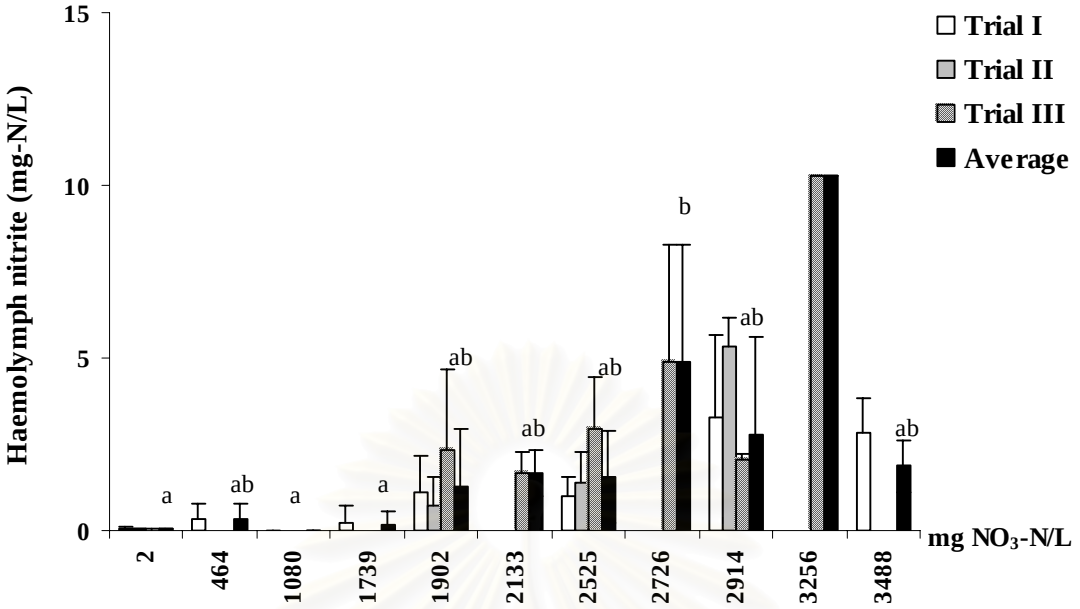

Figure 16. Concentration of nitrite in the haemolymph of *P. monodon* exposed to various nitrate concentrations for 96 hours, while a, b, and ab indicate significant differences (P<0.01 by ANOVA and Duncan New Multiple Range Test).

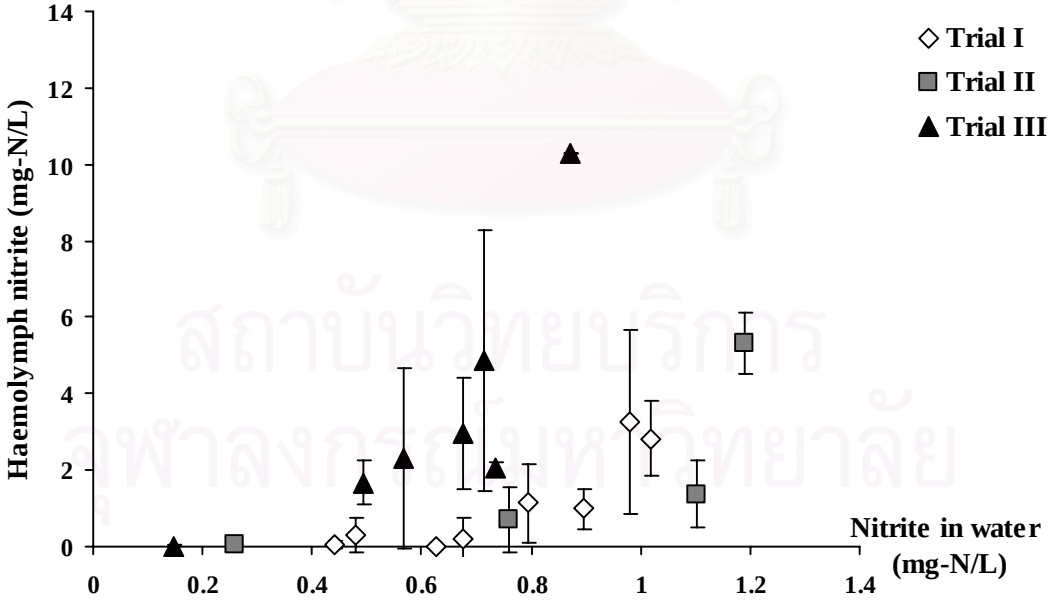

Figure 17. Concentration of nitrite in the water and in the haemolymph of *P. monodon* after exposed to various nitrate concentrations for 96 hours. Regression analysis showed significant correlation (P=0.034).

Concentration of nitrate in haemolymph was visibly found to increase with nitrate concentration in the water (Figure 18). It must be noted that, with a combination of data from 3 trials, concentration of nitrate in haemolymph and in water was amount equal. Regression analysis in Figure 19 showed highly significant correlation (P<0.01) between nitrate concentration in haemolymph and in water with the correlation coefficient close to 1 (y=1.0472x with R^2=0.9677).

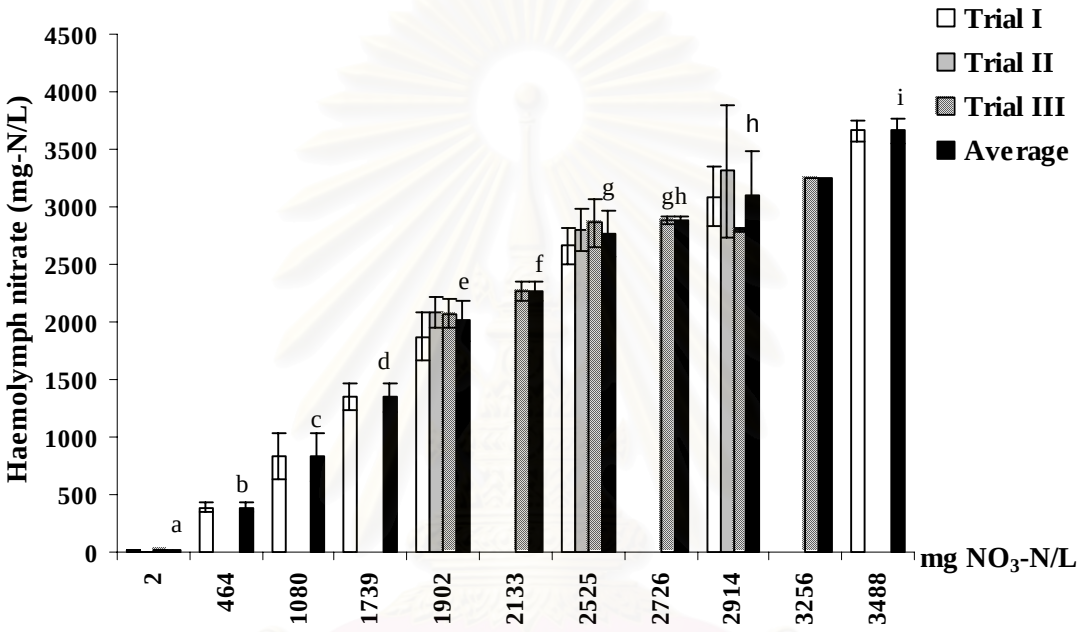

Figure 18. Concentration of nitrate in the haemolymph of *P. monodon* exposed to various nitrate concentrations for 96 hours, while a, b, c, d, e, f, g, gh, h and i indicate significant differences (P<0.01 by ANOVA and Duncan New Multiple Range Test).

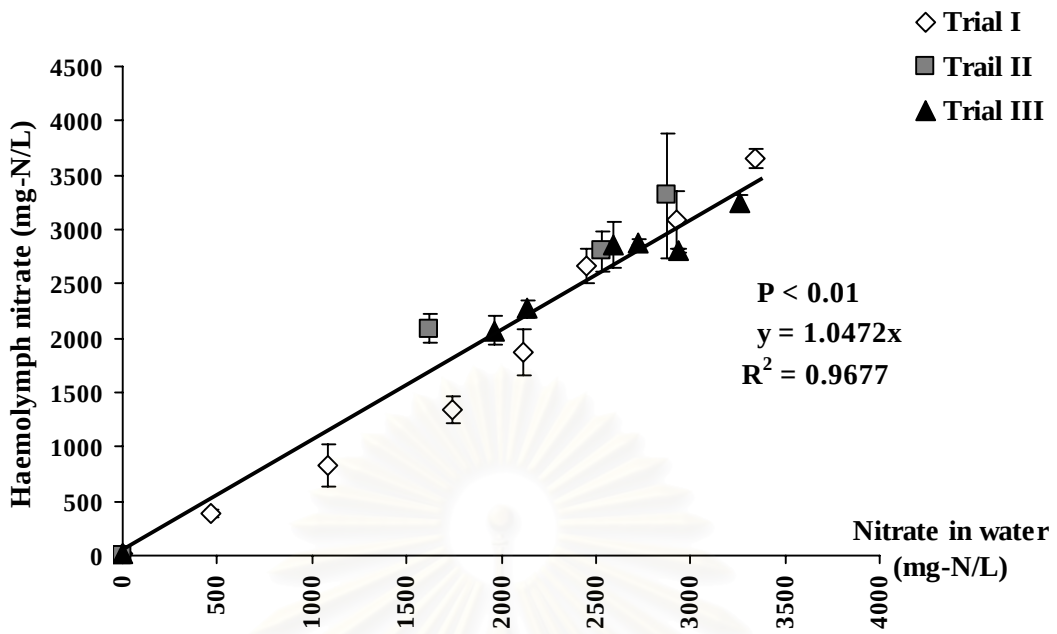

Figure 19. Concentration of nitrate in the water and in the haemolymph of *P. monodon* after exposed to various nitrate concentrations for 96 hours. Regression analysis showed highly significant correlation (P<0.01).

4.2 Long term nitrate toxicity of Penaeus monodon

Shrimps, 24.35±6.61 g in weight, collected from shrimp pond in Pathum Thani Province were reared in the closed recirculating seawater system (CRSS) for 216 days. One of the CRSS with the nitrate removal system using tubular denitrification reactor was assigned as treatment pond while the CRSS without nitrate removal system was assigned as control pond. Figure 20 shows that the accumulation of nitrate was found only in the control pond. Nitrate concentration in the control pond increased from 18.99 to 89.35 mg NO_3-N/L within 216 days. On the other hand, nitrate in treatment pond was constant at approximately 18.86 mg NO_3-N/L.

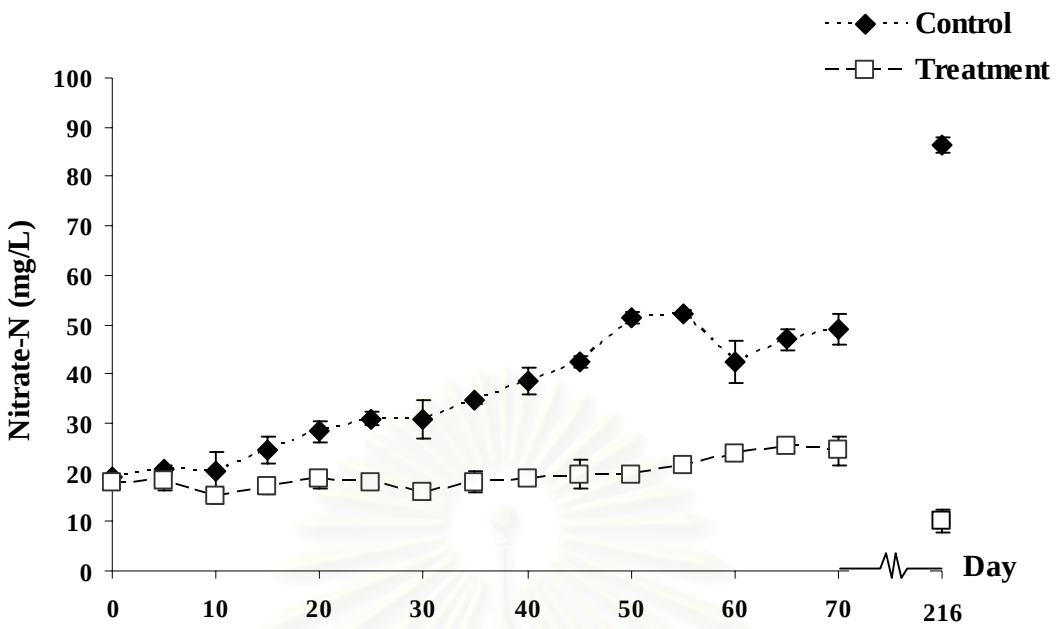

Figure 20. Nitrate concentration in control pond (without nitrate removal) and in treatment pond (with nitrate removal) along 216 day of the experiment.

During experiment, salinity in both ponds increased from 25 to 30 psu as a result of evaporation. Temperature of both ponds ranged between 26.5-28ºC. However, pH and alkalinity in control pond were found to decrease with time. After 70 days experiment, alkalinity in control pond clearly dropped from 108.5 to 50.5 mgCO_3/L while alkalinity in treatment pond slightly increased from 109.0 to 149.3 mg CO_3/L (Figure 21). To reduce the effect of low alkalinity to shrimps, Na_2HCO_3 was added in day 80 and alkalinity was increase to 196.0 mgCO_3/L. Trend of pH decline during the first 70 days in control pond was similar to alkalinity in which pH was found decrease from 8.11 to 7.40 while pH in treatment pond remained at approximately 8.04±0.08.

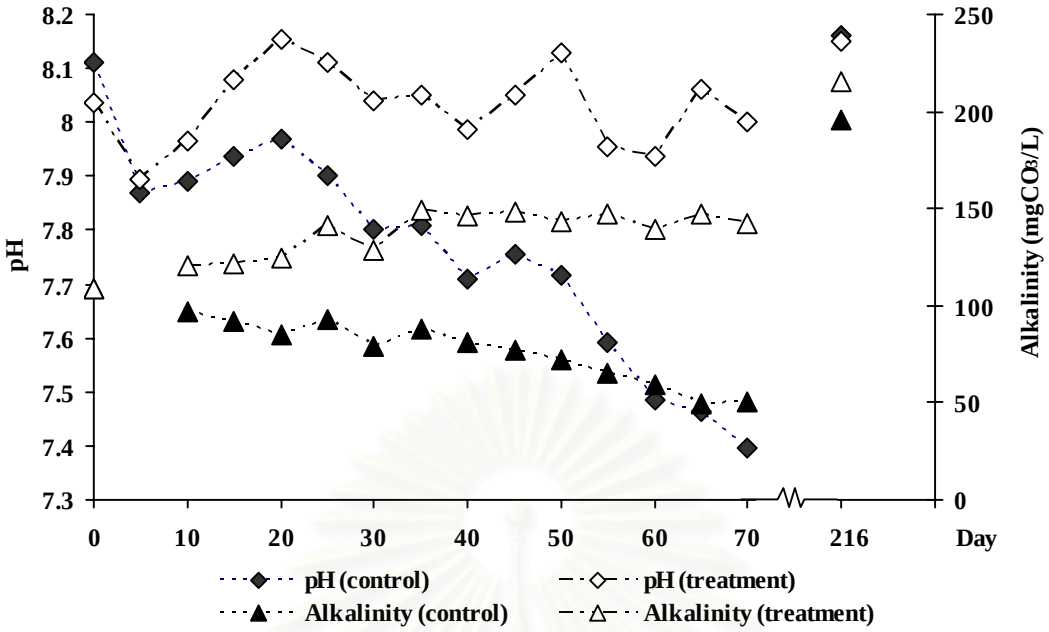

Figure 21. Alkalinity and pH in control pond (CRSS without nitrate removal) and treatment pond (CRSS with denitrification nitrate removal system) during experiment.

4.2.1 Growth and survival of shrimp

In this experiment, all shrimps in each pond were weighted and measured for theirs length every 30 days. The results in Table 5 showed that there are no difference in weight, length and survival rate of shrimp in control and treatment ponds. Average daily growth rate of shrimp in both ponds were 0.18 and 0.16 g/day with survival rate of 38 % and 54 % respectively.

Table 5. Growth and survival rate of *P. monodon* in control (without nitrate removal) and treatment (with nitrate removal) ponds during 216 days of the long term toxicity experiment.

Day	CRSS (control)			CRSS with DN (treatment)		
	Weight (g)	Length (cm)	Survival rate (%)	Weight (g)	Length (cm)	Survival rate (%)
initial	24.04±5.30	11.65±0.80	100	24.65±7.74	11.46±1.83	100
30	24.78±5.26	11.56±1.60	100	24.77±6.71	11.79±1.02	98
61	28.56±5.50	12.30±0.72	94	29.05±6.71	12.45±0.97	94
78	31.06±5.41	12.68±0.70	92	31.71±6.95	12.66±1.84	90
216	63.51±8.76	15.65±0.68	38	58.32±10.87	14.99±1.02	54

4.2.2 Physiological response bioassays

During the experiment in CRSS, haemolymph samples were collected from 10 shrimps in each pond every 10 days. For total haemocyte counts (THCs), the concentration of haemocyte from shrimps in control and in treatment ponds were similar (Figure 22). Average THCs found in this experiment ranged between $2.14\pm1.10 - 5.51\pm0.80$ x10^4cells/mm^3.

In this experiment, the data for initial day was from haemolymph samples of shrimps from shrimp farm before being released into the experimental ponds. As shown in Figure 23-25, initial haemolymph protein was 1.27 ± 0.36 mg/ml, phenoloxidase activity was 1.42 ± 1.28 unit/min/mg.protein, and haemolymph glucose was 20.43 ± 6.27 mg/L. Ammonium, nitrite and nitrate in haemolymph of shrimps from shrimp pond were 14.40 ± 4.65, 0.02 ± 0.02 and 10.64 ± 3.63 mg-N/L, respectively (Figure 26, 28, and 30).

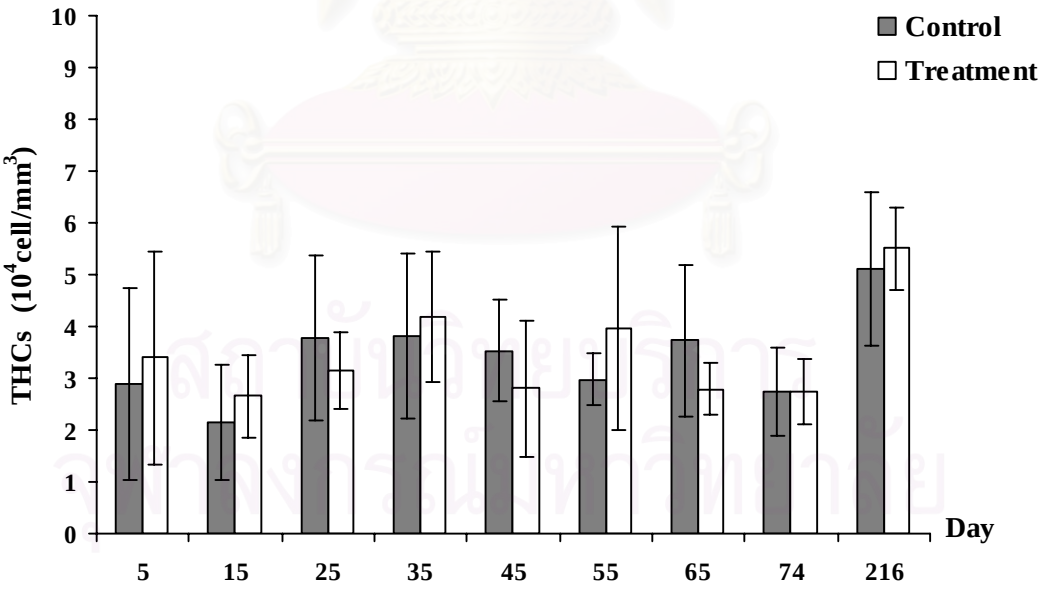

Figure 22. Illustrate of Total haemocyte counts of *P. monodon* reared in control and treatment (with nitrate removal) ponds along 216 days of the experiment. (Error bar represent standard deviation, $n = 10$)

There was significant difference of haemolymph protein at day 15 (P=0.007 by t-test) in which haemolymph protein of shrimps in treatment pond was higher than those in control pond (Figure 23). The range of haemolymph protein found in this experiment was between 0.98-4.15 mg/ml. However, significant difference of phenoloxidase activity of shrimp in control and treatment ponds was found at day 5 (P=0.023) day 15 (P=0.003) and day 45 (P=0.038) by t-test. The highest phenoloxidase activity found in this experiment was 10895.97±4785.06 unit/min/mg.protein in treatment pond at day 45 (Figure 24). For haemolymph glucose, most of the data obtained during experiment was higher than initial glucose concentration. As shown in Figure 25, significant difference of glucose in haemolymph of shrimp in control and treatment ponds was found at day 5 (P=0.010), day 25 (P=0.014) day 45 (P=0.013) and day 216 (P=0.029). It was found that haemolymph glucose elicited wide range from the lowest of 6.06±2.9 mg% at day 15 to the highest of 175.5±70.2 mg% at day 45.

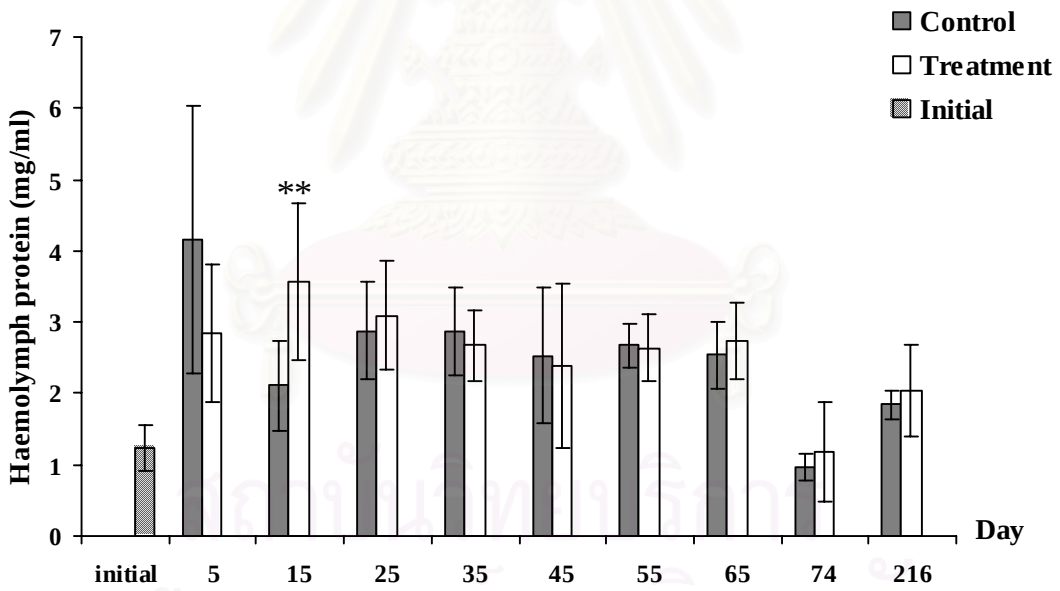

Figure 23. Haemolymph protein of *P. monodon* reared in control (without nitrate removal) and treatment (with nitrate removal) ponds. Significant difference at day 15 (P=0.007 by t-test) was indicated by the symbol (*). (Error bar represent standard deviation, n = 10)

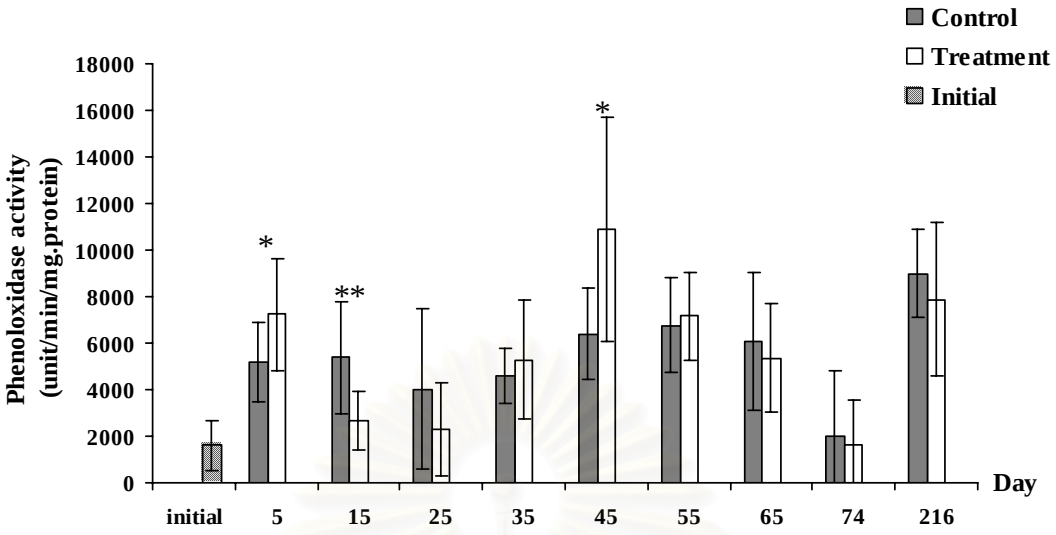

Figure 24. Phenoloxidase activity of *P. monodon* reared in control (without nitrate removal) and treatment (with nitrate removal) ponds. Significant difference at day 5 (P=0.023 by t-test) day 15 (P=0.003 by t-test) and day 45 (P=0.038 by t-test) was indicated by the symbol (*). (Error bar represent standard deviation, n = 10)

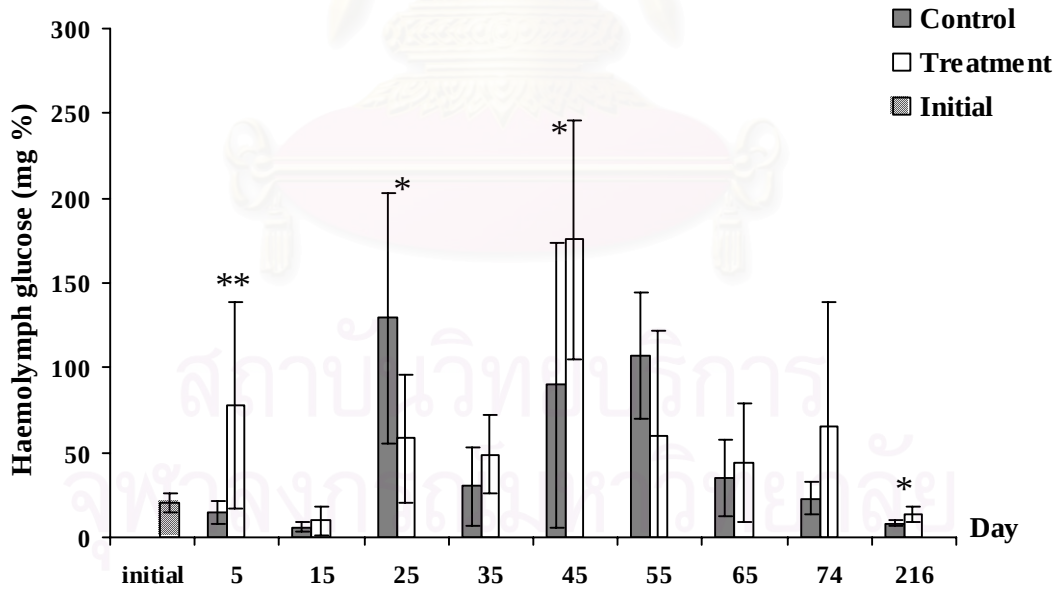

Figure 25. Haemolymph glucose of *P. monodon* reared in control (without nitrate removal) and treatment (with nitrate removal) ponds. Significant difference at day 5 (P=0.010 by t-test) day 25 (P=0.014 by t-test) day 45 (P=0.013 by t-test) and day 216 (P=0.029 by t-test) was indicated by the symbol (*). (Error bar represent standard deviation, n = 10)

4.2.3 *Haemolymph nitrogenous compound*

Concentrations of nitrogenous compounds (ammonium, nitrite and nitrate) in haemolymph are shown in Figures 26-31. It was found that haemolymph ammonium from initial time (shrimp captured from shrimp pond) to the end of the experiment was almost stable at approximately 13.2±4.8 to 21.9±5.1 mg NH_4-N/L in both control and treatment ponds (Figure 26) while ammonium concentration in the water never exceeded 0.148 mgNH_4-N/L (Figure 27). It has to be noted that haemolymph ammonium found in this experiment was close to the data found in acute nitrate toxicity experiment (section 4.1).

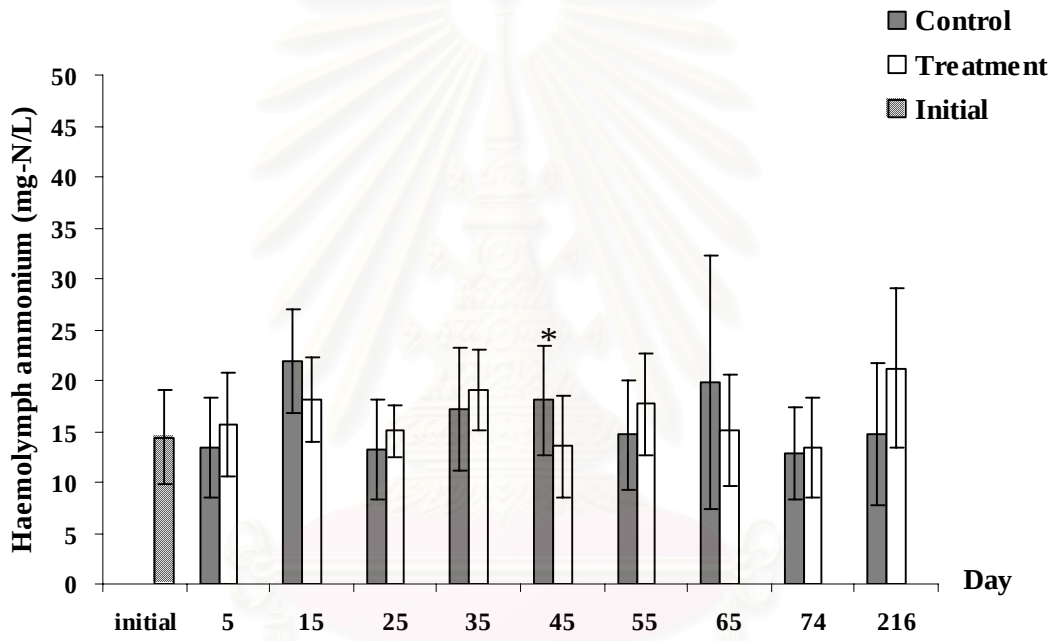

Figure 26. Haemolymph ammonium of *P. monodon* reared in control (without nitrate removal) and treatment (with nitrate removal) ponds, significant difference at day 45 (P=0.019 by t-test) was indicated by the symbol (*). (Error bar represent standard deviation, n = 10)

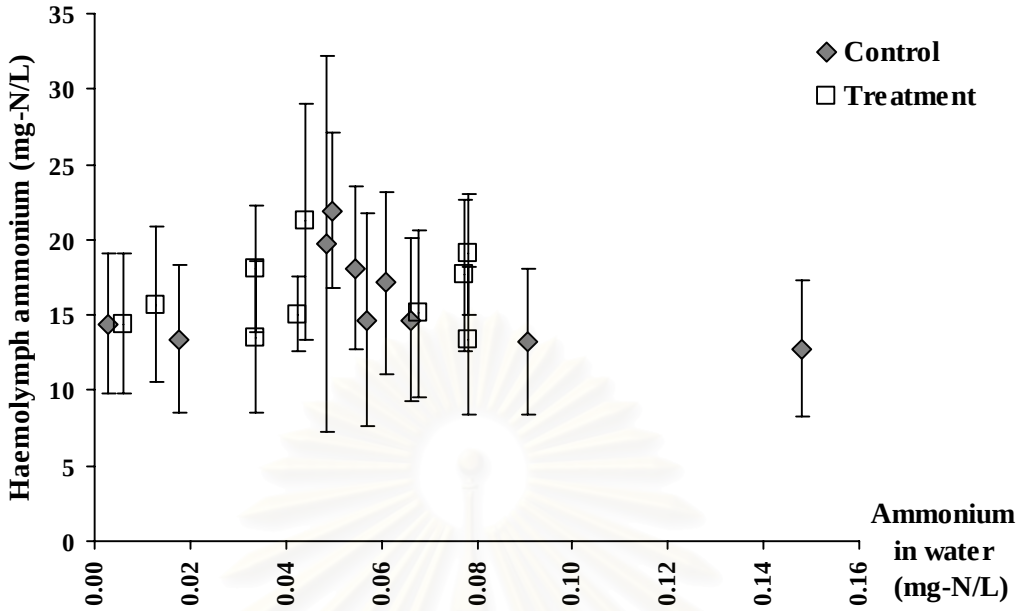

Figure 27. Concentration of ammonium in the water and in the haemolymph of shrimp in control (without nitrate removal) and treatment (with nitrate removal) ponds. (Error bar represent standard deviation, n = 10)

Nitrite concentration in haemolymph of shrimps from both ponds during the experiment illustrated wide range of standard deviation with the concentration ranged from 0 to 0.51$\pm$0.48 mg NO_2-N/L and the highest nitrite concentration of 0.51$\pm$0.48 mg NO_2-N/L was found at day 216. Nitrite concentration in the water of both ponds, as shown in Figure 29, was very low (0.011$\pm$0.002 to 0.055$\pm$0.008 mg NO_2-N/L). After statistical analysis, there was no significant difference of haemolymph nitrite between shrimps from control and treatment ponds at any day. However, most haemolymph nitrite data were higher than that found in shrimp at initial day.

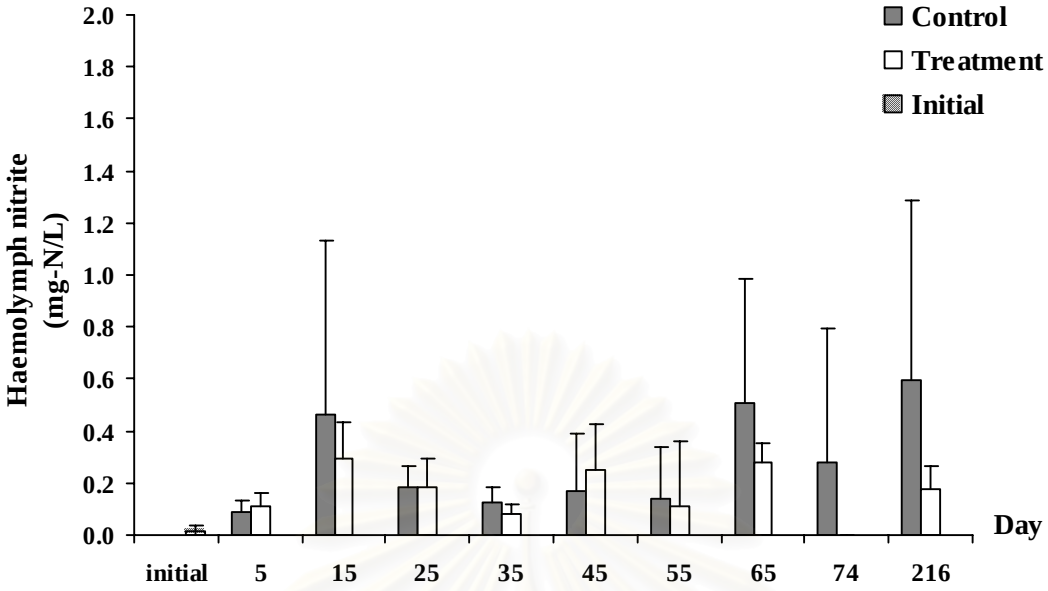

Figure 28. Haemolymph nitrite of *P. monodon* reared in control (without nitrate removal) and treatment (with nitrate removal) ponds. (Error bar represent standard deviation, *n* = 10)

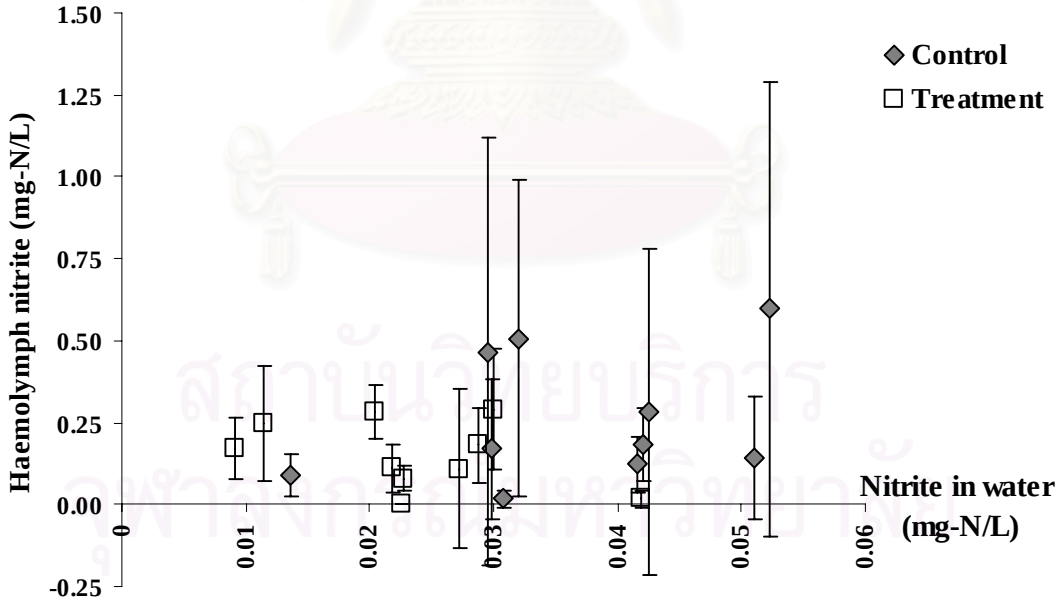

Figure 29. Concentration of nitrite in the water and in the haemolymph of shrimp in control (without nitrate removal) and treatment (with nitrate removal) ponds. (Error bar represent standard deviation, *n* = 10)

. It was found that haemolymph nitrate of shrimps in control pond was significantly higher than that found in treatment pond (Figure 30). During 216 days experiment, haemolymph nitrate of shrimps in control pond increase from 24.41±4.7 to 55.69±7.2 mg NO_3-N/L. On the other hand, haemolymph nitrate of shrimps in treatment pond remained constant between 16.96±5.77 and 19.36±4.82 mg NO_3-N/L except in day 5 that haemolymph nitrate was as high as 44.25±5.09 mg NO_3-N/L. With the combination of data from both control and treatment ponds, regression analysis between nitrate in the water and nitrate in the haemolymph showed significant correlation (P<0.01) with the x variable coefficient of 0.84 and R^2=0.75.

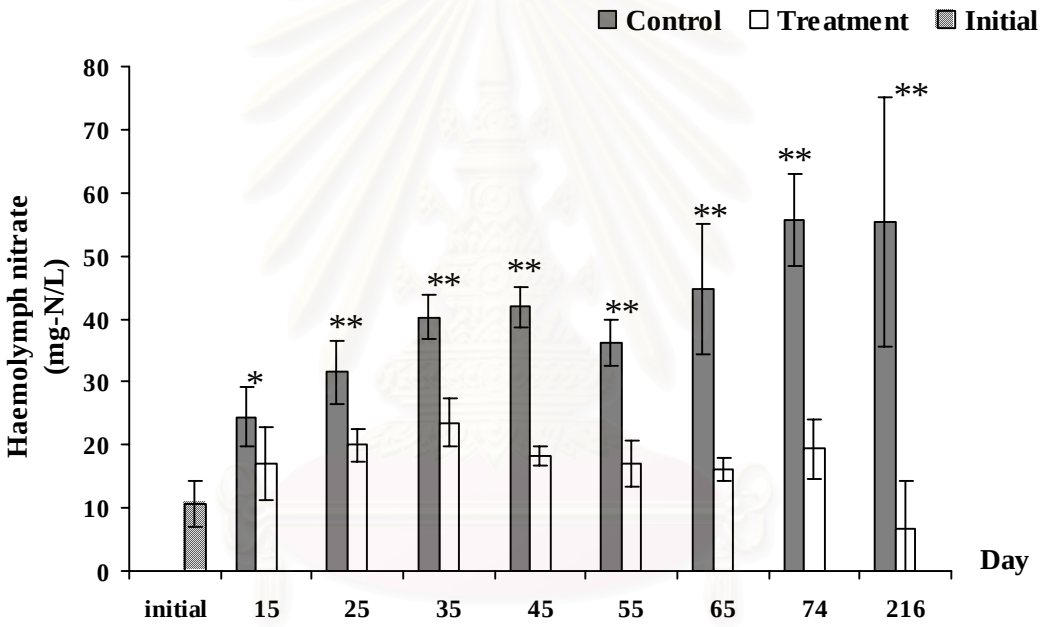

Figure 30. Haemolymph nitrate of *P. monodon* reared in control (without nitrate removal) and treatment (with nitrate removal) ponds. Significant difference (P<0.01 by t-test) was indicated by the symbol (*), (Error bar represent standard deviation, n = 10).

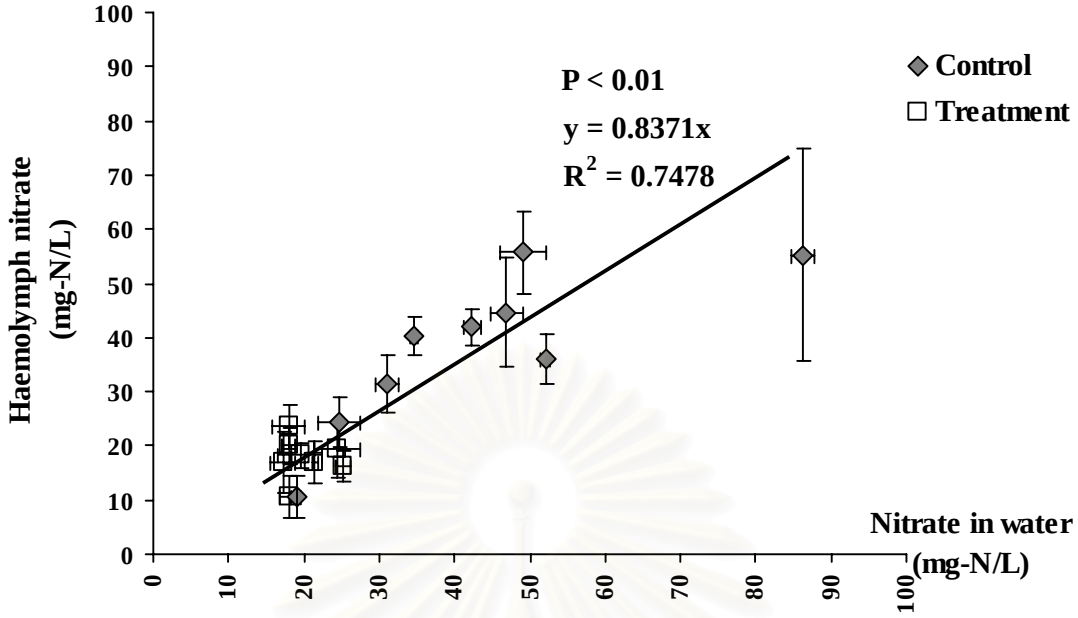

Figure 31. Relation between the concentration of nitrate in the water and in the haemolymph during 216 days of experiment analysed by regression analysis.

4.3 Effect of nitrate on oxygen consumption rate of P. monodon

The study on the effect of nitrate concentrations on oxygen consumption rate (respiration rate) of shrimps was separated into 3 trials. In the first trial, shrimp was placed in the respiration chamber and started with nitrate-free seawater. After detecting oxygen consumption for approximately 12 minutes, water in the chamber was changed consequently with seawater containing 50, 100, 250 or 500 mg NO_3-N/L and repeated the oxygen consumption measurement at each nitrate concentration. After exposed with 500 mg NO_3-N/L, shrimp was then taken out of the chamber and left in nitrate-free seawater for 30 minutes before taken back into the chamber in order to measured oxygen consumption rate after recovering period. Increase in nitrate concentrations in the chamber tended to decrease the oxygen consumption rate of shrimps (Figure 32). The average oxygen consumption rate decreased from 3.60, 3.12, 2.57, 2.21 to 2.12 µgO_2/g shrimp/minute when nitrate concentration increased from 0, 50, 100, 250 and 500 mg NO_3-N/L, respectively. After 30 minutes recovery in nitrate-free water, average oxygen consumption rate was found increase to 2.50

µgO_2/g shrimp/minute. However, with statistical analysis, the different of all oxygen consumption rates were not significant (P>0.05 by t-test).

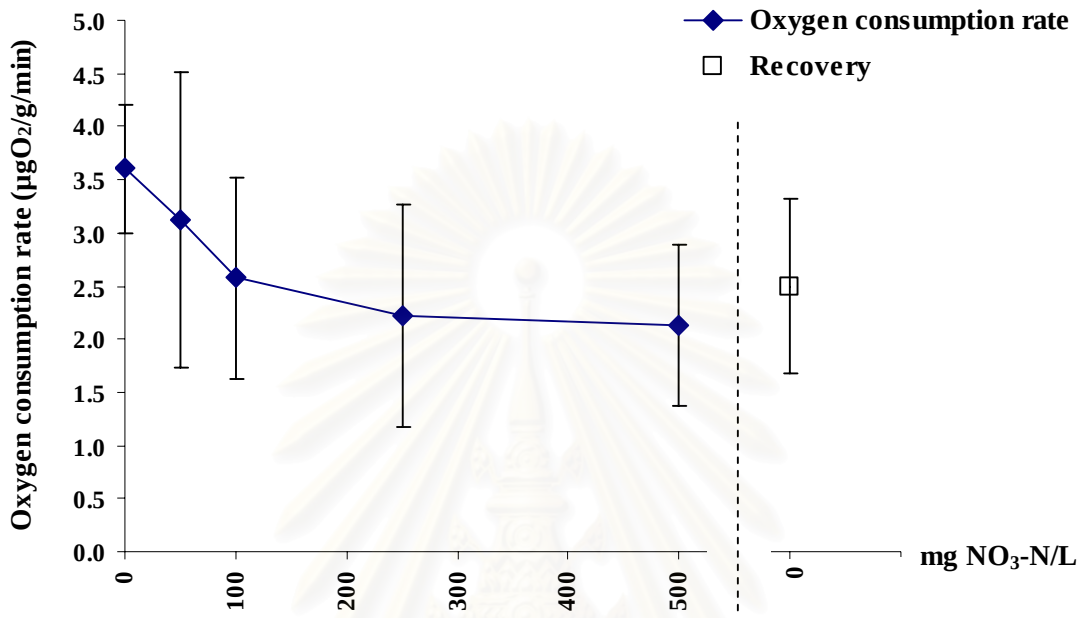

Figure 32. Oxygen consumption rate of *P. monodon* after increase nitrate concentration in seawater of the first trial (shrimps were removed from the chamber during recovering period, n = 7).

In the second trial, the procedure of oxygen consumption rate measurement was similar to the first trial except shrimp was left in the chamber during recovering period. The results, as illustrated in Figure 33, showed that nitrate did not clearly affect oxygen consumption rate with this short exposure time. Average oxygen consumption rate decreased from 2.70 to 1.88, 2.27, 1.78, and 1.96 µgO_2/g shrimp/minute when nitrate concentration increased from 0, 50, 100, 250 and 500 mg NO_3-N/L, respectively. Moreover, shrimp did not show the recovery of oxygen consumption after 30 minute exposed with nitrate-free seawater.

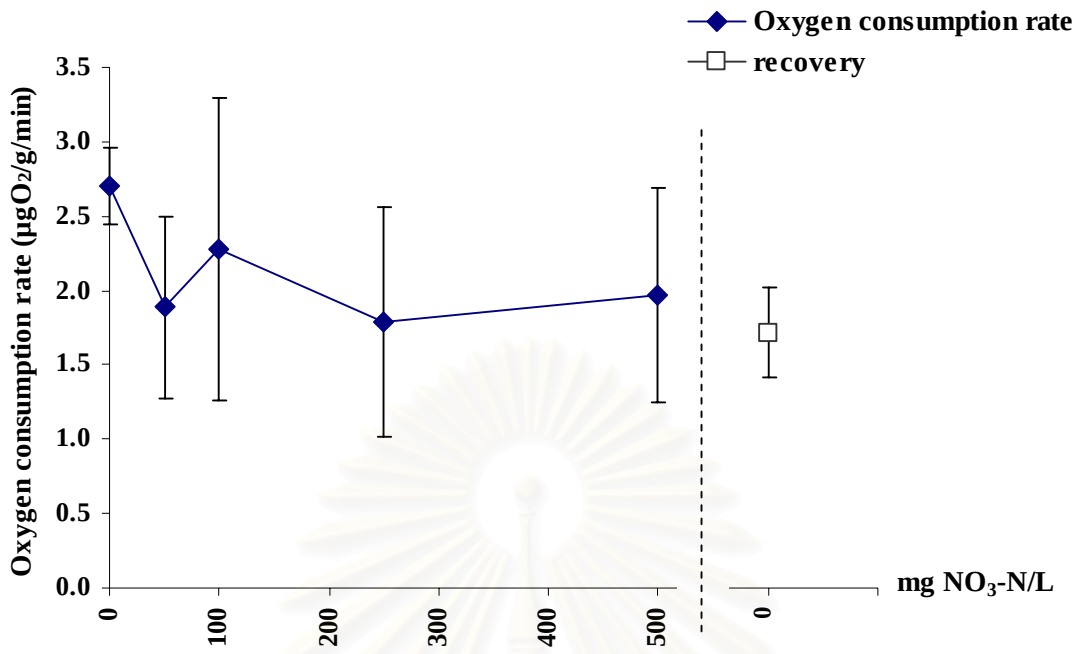

Figure 33. Oxygen consumption rate of *P. monodon* after increase nitrate concentration in seawater of the second trial (Shrimps were not removed from the chamber during recovering period).

The study of oxygen consumption of shrimp in the third trial was differed from the former trials. With this trial, oxygen consumption rates were measured at the 70th day of long term nitrate toxicity study (section 4.2) using 9 shrimps from control pond (exposed to 49.05 mg NO_3-N/L in CRSS) and 9 shrimps from treatment pond (exposed to 24.33 mg NO_3-N/L in CRSS with nitrate removal system) ponds. The results are shown in Figure 34. It was found that oxygen consumption of shrimp from control pond was ranged from 1.83-4.17 µgO_2/g shrimp/minute (average 3.13±0.80 µgO_2/g shrimp/minute) while oxygen consumption of shrimp from treatment pond was ranged from 3.12-3.90 µgO_2/g shrimp/minute (average 3.48±0.30 µgO_2/g shrimp/minute). Statistical analysis showed that oxygen consumption of shrimps from both ponds were not significant different (P>0.05 by t-test).

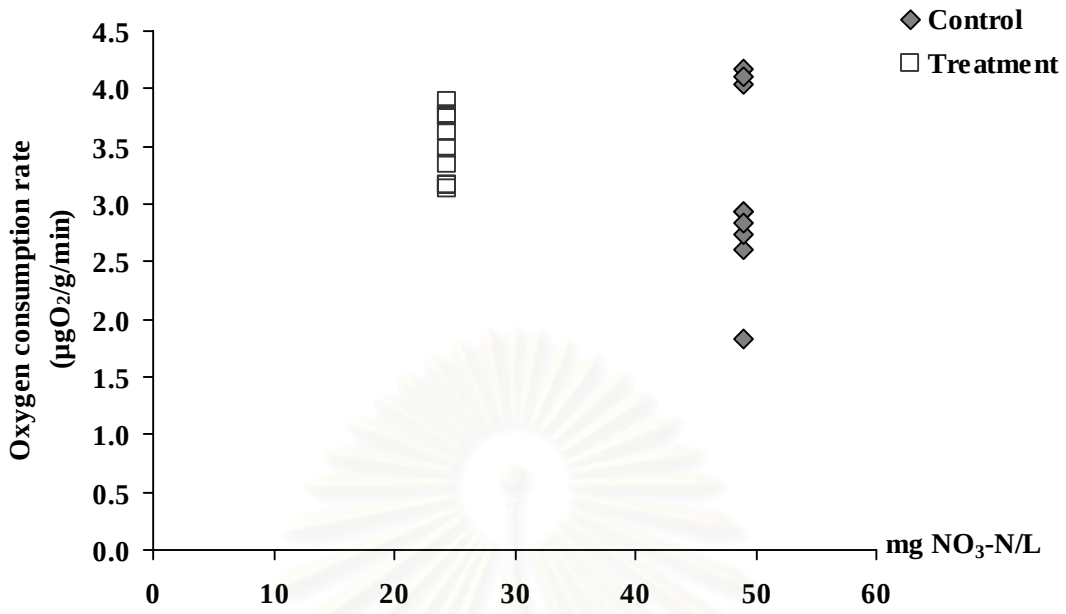

Figure 34. Oxygen consumption rate of *P. monodon* from control pond (CRSS) and treatment pond (CRSS with denitrification) at day 75. Each point represents value of one shrimp, $n = 9$.

CHAPTER V

DISCUSSION

5.1 Acute nitrate toxicity (LC_{50} at 96 hours)

The results from this study illustrated that the acute toxicity (median lethal concentration at LC_{50} 96 hours) of nitrate to *P. monodon* (17.4±2.7 g in weight) was 2636 mgNO_3-N/L. This result was among the range of acute nitrate toxicity of various aquatic animals as shown in Table 6.-

Table 6. The LC_{50} (median lethal concentration) of nitrate-N on several species of vertebrates and invertebrate compared with this study.

Species	Salinity	LC_{50} (mg/L)	References
Vetebrates			
Frog, *Pseudacris regilla* (embryo)	Freshwater	10 day 578	Schuytema and Nebeker (1999)
Frog, *Xenopus laevis* (embryo)	Freshwater	10 day 438-871	Schuytema and Nebeker (1999)
Channel catfish, *Ictaturas punctatus*	freshwater	96-h 6200	Colt and Tchobanoglous (1976)
Heteromycteris capensis	34.4-35.7psu	24-h 5050	Brownell (1980)
Gaidropsarus capensis	34.4-35.7psu	24-h >4000	Brownell (1980)
Diplodus sargus	34.4-35.7psu	24-h 3560	Brownell (1980)
Lithognathus mormyrus	34.4-35.7psu	24-h 3450	Brownell (1980)
Guadalupe bass, *Micropterus treculi*	Freshwater	96-h 1261	Tomasso and Carmichael (1986)
Carp, *Catla catla*	Freshwater	24-h 1565	Tilak *et al*. (2002)
Invertebrate			
Macrobrachium rosenbergii	1 psu	3-4 week 160	Wickins (1976)
Penaeid shrimps	28 psu	48-h 4300	Wickins (1976)
Cheumatopsyche pettiti (early instar)	Freshwater	96-h 113.5	Camargo and Ward (1992)
Cheumatopsyche pettiti (last instar)	Freshwater	96-h 165.5	Camargo and Ward (1992)
Hydropsyche occidentalis (early instar)	Freshwater	96-h 97.3	Camargo and Ward (1992)
Hydropsyche occidentalis (last instar)	Freshwater	96-h 109.0	Camargo and Ward (1992)
Penaeus paulensis (broodstock)	32 psu	96-h 2171	Cavalli *et al*. (1996)
Abalone, *Haliotis tuberculata*	Seawater	Safe level 100-250	Basuyaux and Mathieu (1999)
Urchin, *Paracentrotus lividus*	Seawater	Safe level 100	Basuyaux and Mathieu (1999)
Penaeus monodon (juveniles)	15 psu	96-h 1449	Tsai and Chen (2002)
P. monodon (juveniles)	25 psu	96-h 1575	Tsai and Chen (2002)
P. monodon (juveniles)	35 psu	96-h 2316	Tsai and Chen (2002)
P. monodon	30 psu	96-h 2649	This study

Table 6 shows that the lethal concentration of nitrate varied with high range, from 97 to 6200 mg/L. Teleost fishes seem to have higher nitrate tolerant than invertebrates. For *P. monodon*, the results from Tsai and Chen (2002) indicate less toxicity of nitrate when shrimps were grown in high salinity. It has to be noted that many shrimps exposed

with nitrate between 1901.93 - 3487.56 mg NO_3-N/L in this study were still alive but they were almost inactive with slightly movement of swimming legs. These shrimps were not counted as dead shrimp. Therefore, this must be concerned if there is an attempt to specify the safety concentration of nitrate for shrimp.

5.2 Long term toxicity test in the Closed Recirculating Seawater System (CRSS)

Most of the water quality parameters in the CRSS during long term toxicity test in both control and treatment ponds were within the acceptable range of water quality for marine shrimp culture suggested by Spotte (1979) and Howerton (2001). However, it was clearly found that nitrate concentration in the treatment pond was significantly lower than in the control pond. This was because the nitrate treatment reactor worked properly. Hence, nitrate concentration in the treatment pond was never exceed 25.27 mg-N/L throughout 216 days of the experiment while nitrate in the control pond reach 86.35 mg-N/L at the end of the experiment. In addition, alkalinity in control pond decreased from 108.5 mgCO_3/L at the starting day to 49 mgCO_3/L at day 65 (Fig. 21). This was probably a result from nitrification process in the nitrification tank which can be described with the equation below (Pansawasdi, 2001):

$$134.5\ NO_2^- + NH_4^+ + 62.25\ O_2 + HCO_3^- + 4\ H_2CO_3 \rightarrow C_5H_7O_2N + 134.5\ NO_3^- + 3\ H_2O$$

Because the lower limit of alkalinity for shrimp culture is 75 mgCO_3/L (Howerton, 2001 see Table 7), sodium bicarbonate was then added into the control pond in day 70 in order to increase alkalinity. As the result, alkalinity in control pond immediately rose to 196 mgCO_3/L and maintained at this concentration until the end of the experiment. On the other hand, alkalinity in the treatment tank was mostly constant because alkalinity consumed by nitrification process was compensated with alkalinity produced by denitrification process.

Table 7. The water quality in the closed recirculating seawater system (CRSS) during long term toxicity test

Water quality	CRSS (Control)	CRSS+DN (Treatment)	Water quality criteria	References
Ammonium-N (mg/L)	0.003 – 0.148	0.006 – 0.115	< 0.1	Spotte, 1979
Nitrite-N (mg/L)	0.014 – 0.055	0.009 – 0.042	< 0.1	Spotte, 1979
Nitrate-N (mg/L)	18.99 – 88.35	10.28 – 25.27	< 20	Spotte, 1979
Salinity (psu)	25 – 30	25 – 30	<2 – 40	Howerton, 2001
pH	7.40 – 8.16	7.90 – 8.16	8.0 – 9.0	Howerton, 2001
Alkalinity ($mgCO_3/L$)	49.0 – 196.0	109 – 215	75 – 150	Howerton, 2001

Remark: DN = Denitrification reactor (nitrate treatment system)

5.3 Physiological response of shrimp to nitrate

5.3.1 Haemocyte number

In general, total haemocyte count (THCs) has been reported as a parameter related with immune defense system in shrimp (Cheng *et al*., 2004). This was due to more than 90% of phenoloxidase activity is found in shrimp haemocyte (Galván *et al*., 1999). The results from acute toxicity study showed that high THCs was found in shrimp exposed with nitrate concentration below the LC_{50} (2636 mg-N/L). The highest haemocyte number of survived *P. monodon* during acute toxicity test was $2.64\pm0.87 \times 10^4$ cell/mm^3 at 1738 mg NO_3-N/L, while the lowest haemocyte number was 0.37×10^4 cell/mm^3 at 2914 mgNO_3-N/L. Statistical analysis using Duncan's New Multiple Range Test (Figure 10) indicated that THCs data had significantly different among treatments. The results from long term nitrate toxicity (Figure 22) was found related with the acute toxicity experiment since nitrate concentration in the ponds was much lower than that found during the acute toxicity study. In the CRSS, the THCs in was found within the range between 2.14 ± 1.10 - $5.12\pm1.49 \times 10^4$ cell/mm^3 and 2.66 ± 0.80 - $5.51\pm0.80 \times 10^4$ cell/mm^3, for the control pond and the treatment pond respectively. This range was close to the THCs data reported by Supamattaya *et al*. (2000a) in which the THCs of shrimp from the outdoor pond and from the laboratory were $6.49\pm2.33 \times 10^4$ cell/mm^3 and $3.07\pm1.91 \times 10^4$ cell/mm^3, respectively. Smith (1995) and Supamattaya *et al*. (2000a) suggested that low THCs found in shrimp under stress condition. However, nitrate concentration of control tank of the long term toxicity experiment was still in low concentration comparing with the acute toxicity experiment. This cause the THCs results

of control and treatment ponds during the long term toxicity experiment was in the same way.

5.3.2 Haemolymph protein

Concentration of haemolymph protein is one of the physiological parameter that related with stress response in shrimp (Rodriguez, 1981; Chen and Cheng, 1995). Cheng and Chan (2002b) reported that the serum protein of *Marsupenaeus japonicus* exposed with 104.48 mgNO_3-N/L was lower than the control with low nitrate. Similar result was found in *P. japonicus* as the haemolymph protein increased with the increase of nitrite concentration in the water (Chen and Cheng, 1995). Supamattaya *et al.* (2000a) found that haemolymph protein in the shrimp from the earthen pond (118.5$\pm$51.7 mg/ml) was higher than those from the captured tank in the laboratory (114.3$\pm$38.1 mg/ml).

With the acute toxicity test, the highest haemolymph protein was 6.51$\pm$2.14 mg/ml in shrimp exposed with 1901 mgNO_3-N/L and the lowest haemolymph protein was 0.99$\pm$0.41 mg/ml in shrimp exposed with 2133 mgNO_3-N/L (Figure 11). However, no significant difference of haemolymph protein (P>0.05 by ANOVA) was found. This probably due to high variation of the protein concentration detected from each shrimp. For the long term toxicity experiment in CRSS, the range of haemolymph protein found in shrimp from control and treatment ponds were 0.98$\pm$0.19 - 4.15$\pm$1.88 mg/ml and 1.19$\pm$0.70 - 3.57$\pm$1.11 mg/ml, respectively (Figure 23). Significant difference of haemolymph protein was found only on day 15 (P=0.007 by t-test).

With these results, it could be considered that the effect of nitrate on haemolymph protein of *P. monodon* was still unclear. One of the possible reason might because haemolymph protein in this study was measured using the haemocyte lysate supernatant which was generally lower than total serum protein. Moreover, haemolymph protein of crustaceans could also be related with molting stages and starvation (Cheng *et al*., 2001).

5.3.3 Haemolymph glucose

Concentration of haemolymph glucose in an acute toxicity experiment was found with greatly fluctuation (Figure 13). The highest glucose concentration was

148.15±107 mg% at 1901 mgNO_3-N/L and the lowest was 32.18±11.08 mg% at 2133 mgNO_3-N/L, but all data were not significant difference (P>0.05 by ANOVA). Supamattaya *et al*. (2000a) suggested that when shrimp was exposed to stress condition or being captured in the limited area, the glucose concentration had shown highly fluctuation. The results from their study showed high variation of glucose concentration in shrimp from the outdoor pond (32.91+27.95 mg%) and from the laboratory (53.87+54.84 mg%). Furthermore, under stress condition, haemolymph glucose of the estuarine crab, *Chasmagnathus granulate*, exposed to freshwater quickly decreased to 0 mg% at 72 hours after salinity changed. When it was recovered in high salinity for 168 hour, haemolymph glucose could be restored back to 7.47±1.53 mg% (Santos and Nery, 1987). Another example is the glucose concentrations in haemolymph of the river crab, *Potamonautes warreni*, exposed with anoxia condition for 6 hours rose from 8.52±7.53 mg% to 173.80±77.29 mg% (Van Aardt, 1988). The same result was found in chinese freshwater crab, *Eriocheir sinensis* exposed with hypoxia condition. Haemolymph glucose of the crab increased and reached the peak and then declined slowly to the original level (Zou *et al*., 1996).

For the long term toxicity test of shrimp in the CRSS, significant difference of haemolymph glucose between control and treatment ponds was found on day 5 (P=0.010), day 25 (P=0.014), day 45 (P=0.013), and day 216 (P=0.029) in Figure 25. Although some significant difference was found, however, the correlation between nitrate toxicity in both acute and long term period and haemolymph glucose could not be acceptably described so further studies is therefore needed.

5.3.4 Phenoloxidase activity

Phenoloxidase (PO) activity which stimulated by trypsin and L-DOPA as substrate is one of the physiological parameter related with the immune defense system in shrimp (Perazzolo and Barracco, 1997; Galván *et al*., 1999; Supamattaya *et al*., 2000a; Van de Braak, 2002). Generally, variation of PO activity usually has the similar trend with total haemocyte number because approximately 90% of PO activity is found in haemocyte (Galván *et al*., 1999) and also with highly fluctuation Supamattaya *et al*. (2000a). The results from this study indicated that PO activity in shrimps from the acute toxicity experiment had highly fluctuation as indicate by the error bars in Figure 12. The PO activity of shrimp in the CRSS also showed high standard deviation with the

significant difference between shrimp from control and treatment ponds on day 5 (P=0.0230), day 15 (P=0.003), and day 45 (P=0.038) (Figure 24).

5.3.5 Inorganic nitrogenous compound in haemolymph

It was found that ammonia concentration in haemolymph of *P. monodon* was within the range between 12-22 mgNH_4-N/L. Figures 14 and 26 suggested that haemolymph ammonium concentration did not relate with neither ammonium nor nitrate concentration in the water. In general, ammonium is the nitrogenous waste excreted by most of aquatic animals (Schmitt and Santos, 1999). Concentration of ammonium in haemolymph is therefore depended mostly on cellular metabolism. Previous studies showed that shrimp usually response to ammonium in the water within 6-24 hours in which a peak of ammonium could be detected in haemolymph (Cheng and Chen, 2001; Schmitt and Uglow, 1997; Schmitt and Santos, 1999). The similar result was also found in giant clam, *Tridacna gigas* (Shepherd *et al*., 1999). Although the results of haemolymph ammonium analysis in this study was done using shrimps that exposed to various ambient ammonium concentrations, most of those shrimps were never exposed with a sudden change of ammonium.

It was found that the concentration of nitrite in haemolymph of *P. monodon* had significant correlation (P<0.05 by regression analysis) with nitrite concentration in the water (Figure 17). The accumulation of nitrite in haemolymph of shrimp was previously reported by Cheng and Chen (2001), Chen and Cheng (1995), Cheng and Chen (2002a) and Chen and Lee (1997) in which the haemolymph nitrite of *Penaeus japonicus* and *Macrobrachium rosenbergii* had a positive linear relationship with exposure time and nitrite concentration in the water.

One of the most notable results from this study is that nitrate concentration in haemolymph of *P. monodon* from both acute and long term toxicity experiments was strongly related with nitrate concentrations in the water (Figures 18 and 30). Regression analysis showed highly significant correlation (P<0.01) with high R^2 (0.75-0.97). The slope of the regression line was 1.0472 and 0.8371 for acute and long term toxicity experiments respectively. This indicated that concentration of nitrate in haemolymph is almost equal to the concentration of nitrate in the surrounding water. Hence, it could be suggested that there was a passive transport of nitrate into shrimp body. However, this

passive process might not a rapid response since Cheng *et al* (2002) found that haemolymph nitrate of shrimp was gradually increased after 24 hours of nitrate exposure. The similar results also reported with freshwater crayfish *Astacus astacus* (Jensen, 1996) and giant clam *Tridacna gigas* (Shepherd *et al*., 1999)

5.3.6 Oxygen consumption rate

Oxygen consumption or respiration rate is one of the physiological parameter that could be used to indicate the response of aquatic animals to stress conditions (Alikhan, 1983; Van Holde *et al*., 2001; Valarmathi and Azariah, 2002). For example, Chen and Nan (1993) found that ammonia concentration could affect oxygen consumption rate of *P. chinensis*. With the acute toxicity test in the present study, effect of nitrate concentrations at sub-lethal dose was still unclear whereas a recovery of oxygen consumption in Figure 32 was found. It must be noted that water used in the chamber was freshly prepared before each measurement. The results from those experiments might not project the adaptation of shrimp to nitrate in the real environment. The long term toxicity test, on the other hand, used water that directly pumped from shrimp pond into the chamber following with the oxygen consumption measurement. The results in Figure 34 showed that oxygen consumption rates of shrimps from both pond were not significant difference (P=0.236 by ANOVA). For more detail, mean and standard deviation of oxygen consumption rates of shrimp were 3.13±0.8 and 3.48±0.3 $\mu gO_2/g/min$ for shrimp from control pond and treatment pond respectively. Although the oxygen consumption rate was not different, it was found that shrimps from control pond (exposed with higher nitrate concentration) had significantly larger variation of oxygen consumption rate than shrimp in treatment pond (P=0.015 by homogeneity test of variance). This might be an evident proposing the effect of nitrate to shrimp thus further study is needed.

CHAPTER VI

CONCLUSION

1. Acute toxicity (Median lethal concentration at 96 hours) of Sodium nitrate ($NaNO_3$) to Black tiger shrimp *Penaeus monodon* (17.4±2.7 g in weight) at 30 psu salinity was 2636 mg NO_3-N/L.

2. Total haemocyte counts (THCs) and phenoloxidase activity of survived shrimp exposed to various nitrate concentrations during an acute nitrate toxicity test were significant difference ($P<0.05$ by ANOVA).

3. For long-term nitrate toxicity test (216 days) with shrimps cultured in the closed recirculating seawater systems, significant difference in various physiological parameters of shrimps in control pond and treatment pond (with nitrate removal system) was also found. These parameters included haemolymph protein at day 15 (P=0.007), haemolymph glucose at day 5 (P=0.01), day 25 (P=0.014), day 45 (P=0.013), and day 216 (P=0.029), respectively and phenoloxidase activity at day 5 (P=0.023), day 15 (P=0.003), and day 45 (P=0.038).

4. For inorganic nitrogenous compound in haemolymph of survived shrimps in both acute toxicity test and the long-term study with the closed recirculating seawater systems, it was found that haemolymph nitrate had significantly correlation with nitrate in seawater ($P<0.01$, $y=1.04x$, $R^2= 0.96$ with acute study and $P<0.01$, $y=0.83x$, $R^2= 0.75$ with long-term study).

5. Oxygen consumption rates of shrimps exposed to various nitrate concentrations were not significant different. However, greater variation of oxygen consumption rate was found in shrimps exposed with high nitrate during the long-term study.

RECOMMENDATION:

1. Physiological parameters should be more frequently monitored such as every 3-6 hours during acute toxicity study. This might indicate the short time response and the adaptation of shrimp to nitrate during 96 hours of exposure duration.

2. Further study on the effect of nitrate to body fluid, tissue and waste regulatory organ such as hepatopancreas is needed and the toxicity of nitrate with special emphasis on the oxygen transport efficiency of haemocyanin in haemolymph should be intensively studied.

3. Detoxification of nitrate in shrimp should be studied to understand the detoxified capability of shrimp after exposed to high nitrate concentrations

4. Safety concentration of nitrate should be studied and recommended as a guideline for the cultivation of shrimp in the closed recirculating seawater system.

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Appendices

Appendix A. Probit analysis in LC_{50} 96 hr of *P. monodon* to nitrate in 30 psu seawater.

Regression line: y = A + slope x (X - M)

A = 5.141495 +/- .585556 4.98293 < A < 5.30005

Slope = 9.297923 +/- 2.547971 6.749953 < Slope < 11.84589

M = 13.43627

Heterogeneity = 1

LC	Level of Confidence	Range
2 = %1585.33900	.95	833.53020 < LC < 1936.74000
50 = %2636.64700	.95	%2363.30100 < LC < 2858.88400
90 = %3621.61900	.95	%3220.16100 < LC < 5125.85900
95 = %3962.74400	.95	%3428.89600 < LC < 6201.56800
98 = %4385.12700	.95	%3673.26200 < LC < 7698.12600

Survival of shrimps in acute toxicity test at 96 hours of 3 trials

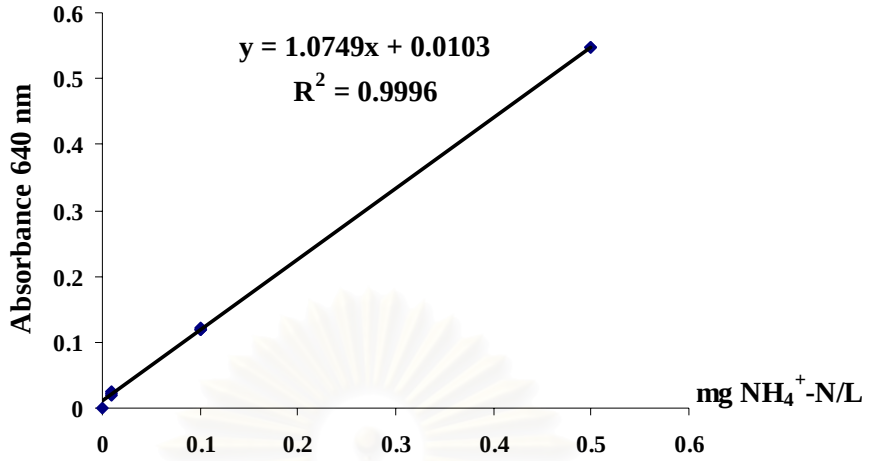
Trial	Nitrate concentration (mg-N/L)												
	2	**463**	**1080**	**1739**	**1902**	**2133**	**2525**	**2726**	**2914**	**3256**	**3487**	**4000**	**4449**
I	6	6	6	6	6	-	4	-	5	-	2	-	-
II	6	-	-	-	6	-	4	-	2	-	0	0	0
III	6	-	-	-	6	3	3	2	1	0	0	-	-

Appendix A. (cont.) Survival shrimp's behavior in trial III of acute toxicity study

Time (hours)	Nitrate concentrations (mg-N/L)							
	2	1902	2133	2525	2726	2914	3256	3487
initial	******	******	******	******	******	******	******	******
4	******	******	******	******	******	******	******	***oo
8	******	******	******	******	******	******	***ooo	***oo
12	******	******	******	******	******	******	***oo	**ooo
16	******	******	******	******	******	******	*ooo	*oo
20	******	******	******	****oo	***ooo	******	*ooo	*oo
24	******	******	******	****oo	****oo	*****o	*oo	*oo
28	******	******	******	****oo	***oo	*****o	*oo	*oo
32	******	******	******	****oo	***o	*****o	*oo	*oo
36	******	******	*****	****oo	***o	****o	*oo	*oo
40	******	******	****	****oo	***o	****o	*o	*oo
44	******	******	**oo	***o	***o	**oo	*o	*oo
48	******	******	**oo	***o	***o	**o	*o	*oo
52	******	******	**oo	***o	***o	**o	*o	*o
56	******	******	**oo	***o	***o	*oo	*o	oo
60	******	******	**oo	***o	**oo	*oo	*o	o
64	******	******	**o	***o	**oo	*oo	o	o
68	******	******	**o	**o	**oo	*oo	o	-
72	******	******	**o	**o	*ooo	*oo	o	-
76	******	******	**o	**o	*ooo	*oo	o	-
80	******	******	**o	**o	oo	o	o	-
84	******	******	**o	**o	oo	o	o	-
88	******	******	**o	**o	oo	o	o	-
92	******	******	**o	**o	oo	o	o	-
96	******	******	**o	**o	oo	o	-	-

Remark: * = No. of active shrimp
o = No. of inactive shrimp

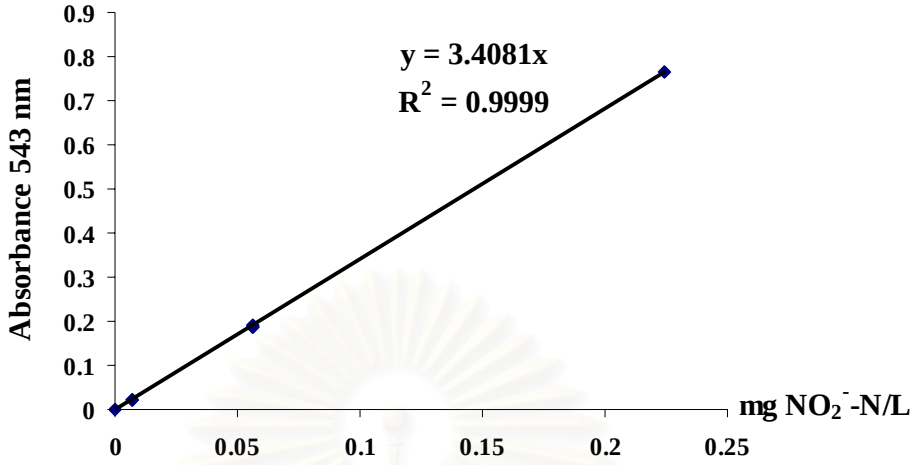
Appendix B. Ammonium-N in seawater of acute toxicity test and in CRSS.



Nitrate (mg-N/L)	Ammonium(mg-N/L)			
	Trial I	Trial II	Trial III	Mean 3 Trials
1.81	3.76±1.00	6.48±0.31	2.05±0.07	4.10±2.01
463.61	3.66±0.53			3.66±0.53
1079.5	3.75±0.46			3.75±0.46
1738.89	4.19±0.47			4.19±0.47
1901.93	2.41±0.37	3.44±0.28	1.62±0.43	2.49±0.85
2133.03			1.31±0.20	1.31±0.20
2525.43	2.98±0.74	4.31±0.29	1.27±0.02	2.86±1.38
2726.24			2.15±0.06	2.15±0.06
2914.27	3.03±0.17	2.45±0.18	0.61±0.15	2.03±1.10
3256.34			0.74±0.09	0.74±0.09
3487.56	2.38±0.23			2.38±0.23

Date	Day	Ammonium (mg-N/L)	
		Control	Treatment
21 Aug 03	0	0.003±0.005	0.006±0.008
26 Aug 03	5	0.018±0.020	0.013±0.014
31 Aug03	10	0.032±0.010	0.040±0.016
5 Sep 03	15	0.050±0.029	0.034±0.020
10 Sep 03	20	0.047±0.024	0.041±0.012
15 Sep 03	25	0.091±0.073	0.042±0.030
20 Sep 03	30	0.069±0.077	0.032±0.023
25 Sep 03	35	0.061±0.025	0.078±0.063
30 Sep 03	40	0.034±0.026	0.016±0.017
5 Oct 03	45	0.054±0.019	0.034±0.024
10 Oct 03	50	0.125±0.078	0.103±0.060
15 Oct 03	55	0.066±0.035	0.077±0.050
20 Oct 03	60	0.100±0.038	0.115±0.046
25 Oct 03	65	0.048±0.029	0.068±0.025
30 Oct 03	70	0.148±0.054	0.078±0.074
19 Mar 04	216	0.057±0.015	0.044±0.013

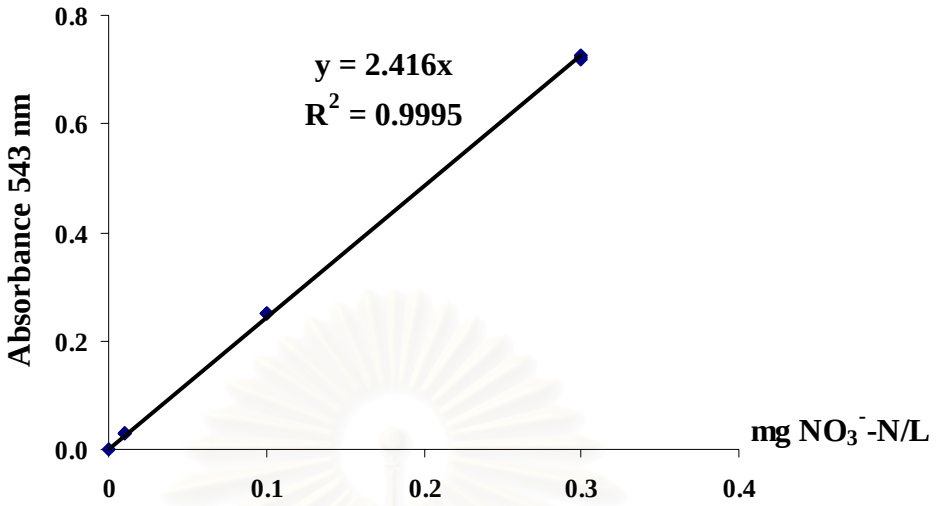
Appendix B. (cont.) Nitrite-N in seawater of acute toxicity test and in CRSS.



Nitrate (mg-N/L)	Nitrite(mg-N/L)			
	Trial I	Trial II	Trial III	Mean 3 Trials
1.81	0.44±0.03	0.26±0.01	0.15±0.02	0.28±0.13
463.61	0.48±0.01			0.48±0.01
1079.5	0.63±0.01			0.63±0.01
1738.89	0.68±0.03			0.68±0.03
1901.93	0.79±0.01	0.76±0.00	0.57±0.05	0.71±0.11
2133.03			0.49±0.03	0.49±0.03
2525.43	0.90±0.08	1.10±0.06	0.67±0.02	0.89±0.19
2726.24			0.71±0.05	0.71±0.05
2914.27	0.98±0.02	1.19±0.04	0.74±0.08	0.97±0.20
3256.34			0.87±0.02	0.87±0.02
3487.56	1.02±0.03			1.02±0.03

Date	Day	Nitrite (mg-N/L)	
		Control	Treatment
21 Aug 03	0	0.031±0.003	0.042±0.012
26 Aug 03	5	0.014±0.002	0.022±0.005
31 Aug03	10	0.016±0.003	0.024±0.001
5 Sep 03	15	0.030±0.001	0.030±0.012
10 Sep 03	20	0.022±0.002	0.019±0.002
15 Sep 03	25	0.042±0.011	0.029±0.005
20 Sep 03	30	0.030±0.004	0.016±0.003
25 Sep 03	35	0.042±0.012	0.023±0.005
30 Sep 03	40	0.024±0.008	0.017±0.002
5 Oct 03	45	0.030±0.011	0.011±0.002
10 Oct 03	50	0.026±0.006	0.020±0.006
15 Oct 03	55	0.051±0.016	0.027±0.005
20 Oct 03	60	0.055±0.008	0.028±0.004
25 Oct 03	65	0.032±0.010	0.020±0.003
30 Oct 03	70	0.043±0.016	0.023±0.001
19 Mar 04	216	0.052±0.016	0.009±0.001

Appendix B. (cont.) Nitrate-N in seawater of acute toxicity test and in CRSS.



Nitrate (mg-N/L)	Nitrate(mg-N/L)			
	Trial I	Trial II	Trial III	Mean 3 Trials
1.81	2.03±0.22	2.140.00	1.260.22	1.81±0.44
463.61	463.61±35.20			463.61±35.20
1079.5	1079.50±67.89			1079.50±67.89
1738.89	1738.89±17.96			1738.89±17.96
1901.93	2115.50±115.88	1621.35±6.13	1968.95±75.73	1901.93±230.46
2133.03			2133.03±21.86	2133.03±21.86
2525.43	2454.20±253.88	54.69±6.13	2587.40±21.86	2525.43±140.06
2726.24			2726.24	2726.24
2914.27	2923.93±253.98	2878.08±10.62	2940.81±57.84	2914.27±133.35
3256.34			3256.34	3256.34
3487.56	3339.79±43.09			3339.79±43.09

Date	Day	Nitrate (mg-N/L)	
		Control	Treatment
21 Aug 03	0	18.99±0.56	17.95±1.05
26 Aug 03	5	20.53±0.95	18.20±1.80
31 Aug03	10	20.17±3.81	15.25±1.55
5 Sep 03	15	24.64±2.74	17.19±1.64
10 Sep 03	20	28.24±1.98	18.57±1.66
15 Sep 03	25	30.93±1.54	17.95±1.00
20 Sep 03	30	30.77±4.01	15.92±1.67
25 Sep 03	35	34.55±0.52	18.02±2.18
30 Sep 03	40	38.50±2.75	18.82±0.64
5 Oct 03	45	42.36±1.19	19.48±2.92
10 Oct 03	50	51.33±1.18	19.49±0.62
15 Oct 03	55	52.07±0.73	21.47±0.42
20 Oct 03	60	42.28±4.27	23.61±0.62
25 Oct 03	65	46.92±2.18	25.27±1.06
30 Oct 03	70	49.05±2.99	24.33±3.08
19 Mar 03	216	86.35±1.56	10.28±2.34

Appendix B. (cont.) pH and Alkalinity in CRSS.

Date	Day	pH	
		Control	Treatment
21 Aug 03	0	8.11	8.04
26 Aug 03	5	7.87	7.90
31 Aug03	10	7.89	7.97
5 Sep 03	15	7.94	8.08
10 Sep 03	20	7.97	8.16
15 Sep 03	25	7.90	8.11
20 Sep 03	30	7.80	8.04
25 Sep 03	35	7.81	8.05
30 Sep 03	40	7.71	7.99
5 Oct 03	45	7.76	8.05
10 Oct 03	50	7.72	8.13
15 Oct 03	55	7.59	7.96
20 Oct 03	60	7.49	7.94
25 Oct 03	65	7.47	8.06
30 Oct 03	70	7.40	8.00
19 Mar 03	216	8.16	8.15

Date	Day	Alkalinity ($mgCO_3/L$)	
		Control	Treatment
21 Aug 03	0	108.50	109.00
26 Aug 03	5	-	-
31 Aug03	10	96.50	121.00
5 Sep 03	15	91.50	121.50
10 Sep 03	20	85.00	125.00
15 Sep 03	25	93.00	141.50
20 Sep 03	30	79.00	128.50
25 Sep 03	35	87.50	149.25
30 Sep 03	40	81.00	146.00
5 Oct 03	45	77.00	148.50
10 Oct 03	50	72.00	143.50
15 Oct 03	55	65.00	147.00
20 Oct 03	60	59.00	139.00
25 Oct 03	65	49.00	147.50
30 Oct 03	70	50.50	142.00
19 Mar 03	216	196.00	215.00

Appendix C. Weight gain, length and survival rate of shrimp reared in CRSS.

Date	Day	Weight gain (g. fresh weight)	
		Control	Treatment
14 Aug 03	1	24.04±5.30	24.65±7.74
13 Sep 03	30	24.78±5.26	24.77±6.71
14 Oct 03	61	28.56±5.50	29.05±6.71
31 Oct 03	78	31.06±5.41	31.71±6.95
19 Mar 03	216	63.51±8.76	58.32±10.87

Date	Day	Length (cm)	
		Control	Treatment
14 Aug 03	1	11.65±0.80	11.46±1.83
13 Sep 03	30	11.56±1.60	11.79±1.02
14 Oct 03	61	12.30±0.72	12.45±0.97
31 Oct 03	78	12.68±0.70	12.66±1.84
19 Mar 03	216	15.65±0.68	14.99±1.02

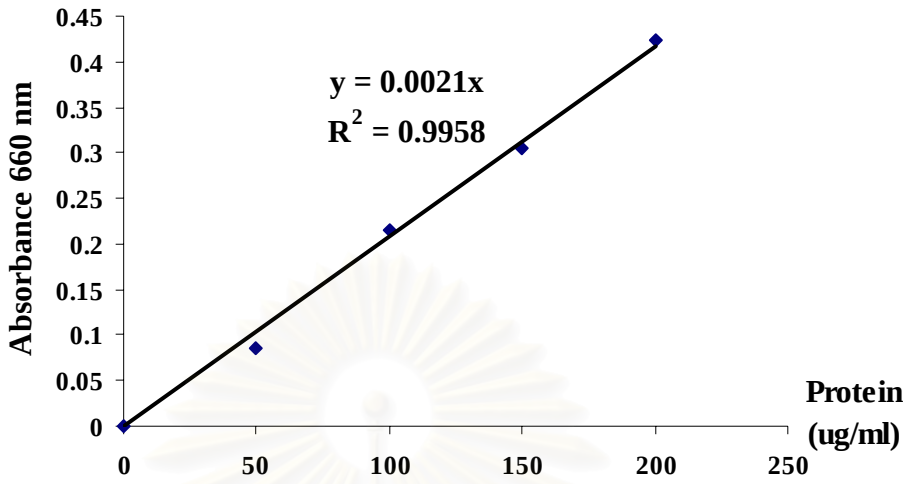
Date	Day	No. of shrimps (pcs)		Survival rate (%)	
		Control	Treatment	Control	Treatment
14 Aug 03	1	50	50	100	100
13 Sep 03	30	50	49	100	98
14 Oct 03	61	47	47	94	94
31 Oct 03	78	46	45	92	90
19 Mar 03	216	19	27	38	54

Appendix D. Total haemocyte counts of *P. monodon* in acute toxicity test and in CRSS.

Nitrate (mg-N/L)	THCs (10^4 cell/mm^3)			
	Trial I	**Trial II**	**Trial III**	**Mean 3 Trials**
1.81	2.54±1.11	0.90±0.49	1.09±0.35	1.51±0.90
463.61	1.71±0.82			1.71±0.82
1079.5	2.64±0.87			2.64±0.87
1738.89	2.53±1.19			2.53±1.19
1901.93	1.91±1.15	1.31±0.39	1.31±0.48	1.51±0.34
2133.03			0.74±0.21	0.74±0.21
2525.43	1.07±0.50	0.49±0.17	0.55±0.26	0.70±0.32
2726.24			0.46±0.02	0.46±0.02
2914.27	0.87±0.48	0.78±0.19	0.37±0.00	0.67±0.27
3256.34			0.42±0.00	0.42±0.00
3487.56	0.97±0.46			0.97±0.46

Date	Day	THCs (10^4 cell/mm^3)	
		Control	**Treatment**
21 Aug 03	5	2.88±1.86	3.39±2.02
31 Aug 03	15	2.14±1.10	2.66±0.80
10 Sep 03	25	3.78±1.58	3.15±0.74
20 Sep 03	35	3.81±1.59	4.19±1.24
30 Sep 03	45	3.53±0.99	2.80±1.32
10 Oct 03	55	2.98±0.50	3.97±1.97
20 Oct 03	65	3.72±1.46	2.79±0.50
29 Oct 03	74	2.73±0.84	2.74±0.64
19 Mar 04	216	5.12±1.49	5.51±0.80

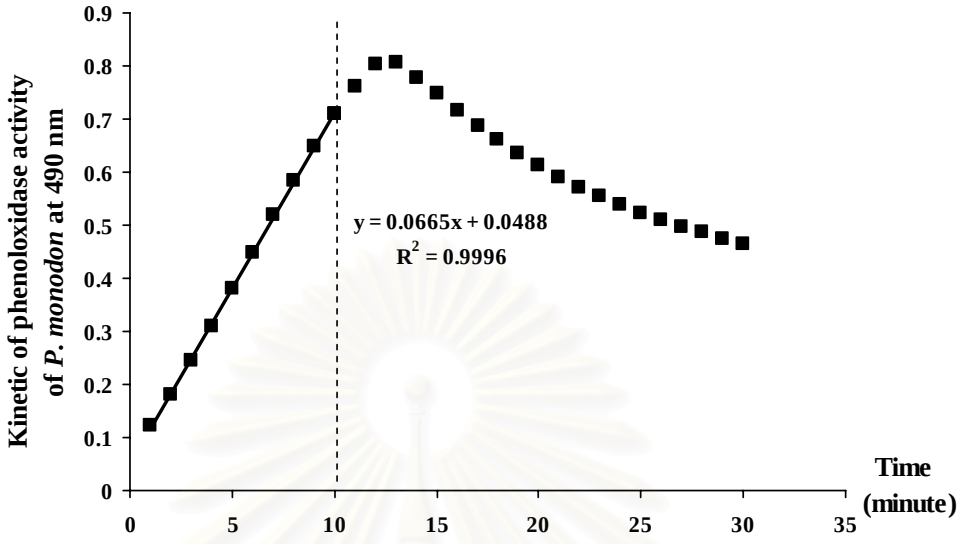
Appendix D. (cont.) Haemolymph protein of *P. monodon* in acute toxicity test and in CRSS.



Nitrate (mg-N/L)	Haemolymph protein(mg/ml)			
	Trial I	Trial II	Trial III	Mean 3 Trials
1.81	3.84± 2.90	3.56±1.29	2.01±1.11	3.14±0.99
463.61	2.16±1.37			2.16±1.37
1079.5	3.14±1.32			3.14±1.32
1738.89	3.52±2.61			3.52±2.61
1901.93	6.51±2.14	4.19±1.63	1.80±0.88	4.17±2.35
2133.03			0.99±0.41	0.99±0.41
2525.43	5.55±2.29	3.88±2.25	1.49±0.47	3.64±2.04
2726.24			2.14±0.32	2.14±0.32
2914.27	4.77±2.02	2.93±0.69	1.70±0.00	3.14±1.54
3256.34			1.51±0.00	1.51±0.00
3487.56	2.72±0.61			2.72±0.61

Date	Day	Haemolymph protein (mg/ml)	
		Control	Treatment
16 Aug 03	initial	1.24±0.32	1.24±0.32
21 Aug 03	5	4.15±1.88	2.84±0.96
31 Aug 03	15	2.11±0.63	3.57±1.11
10 Sep 03	25	2.87±0.68	3.09±0.76
20 Sep 03	35	2.87±0.63	2.67±0.49
30 Sep 03	45	2.53±0.95	2.38±1.15
10 Oct 03	55	2.67±0.32	2.64±0.47
20 Oct 03	65	2.54±0.46	2.73±0.53
29 Oct 03	74	0.98±0.19	1.19±0.70
19 Mar 04	216	1.85±0.20	2.04±0.65

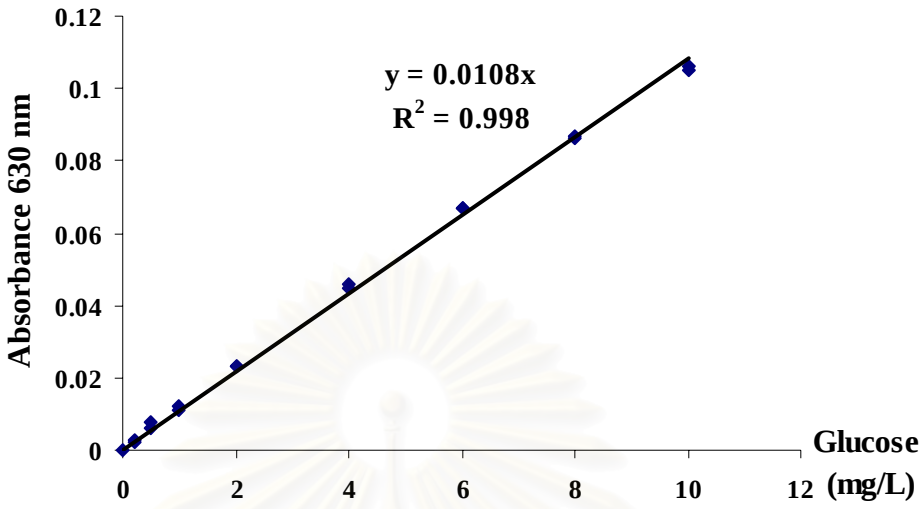
Appendix D. (cont.) Phenoloxidase activity of *P. monodon* in acute toxicity test and in CRSS.



Nitrate (mg-N/L)	Phenoloxidase activity (unit/min/mg.protein)			
	Trial I	Trial II	Trial III	Mean 3 Trials
1.81	4940.74±3187.78	3902.54±3505.01	7996.90±2181.67	5613.39±2128.45
463.61	8099.26±3306.44			8099.26±3306.44
1079.5	6061.10±3368.41			6061.10±3368.41
1738.89	11783.51±9923.09			11783.51±9923.09
1901.93	7639.07±2138.40	3271.84±463.57	10856.50±3869.49	7255.80±3806.83
2133.03			12067.91±3251.90	12067.91±3251.90
2525.43	7646.59±3705.03	3363.86±1815.81	5620.76±4416.30	5543.73±2142.40
2726.24			6562.92±1807.35	6562.92±1807.35
2914.27	7378.16±3364.73	5099.67±2469.71	5573.98±0.00	6017.27±1202.19
3256.34			6939.66±0.00	6939.66±0.00
3487.56	12446.43±3282.19			12446.43±3282.19

Date	Day	Phenoloxidase activity (unit/min/mg.protein)	
		Control	Treatment
16 Aug 03	initial	1596.00±1052.72	1596.00±1052.72
21 Aug 03	5	5181.90±1689.35	7233.56±2387.12
31 Aug 03	15	5393.07±2401.63	2654.32±1255.08
10 Sep 03	25	4016.12±3428.86	2277.20±2015.41
20 Sep 03	35	4594.89±1191.21	5296.27±2580.11
30 Sep 03	45	6407.08±1954.68	10895.97±4785.06
10 Oct 03	55	6774.74±2012.60	7159.40±1878.62
20 Oct 03	65	6073.84±2970.43	5342.25±2328.11
29 Oct 03	74	1997.01±2796.20	1656.57±1925.73
19 Mar 04	216	8984.93±1896.19	7887.07±3274.08

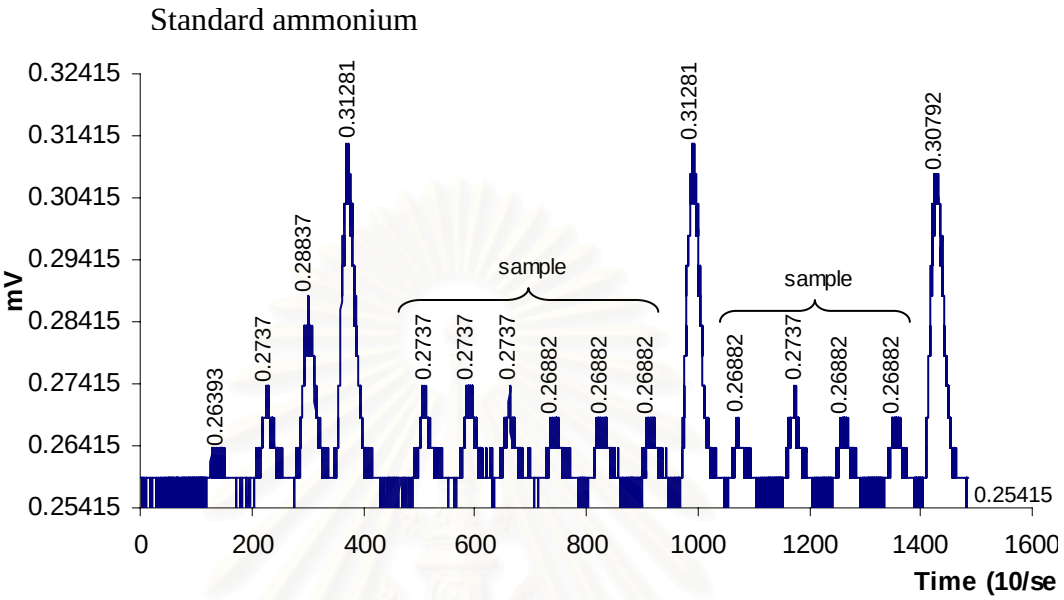
Appendix D. (cont) Haemolymph glucose of *P. monodon* in acute toxicity test and in CRSS.

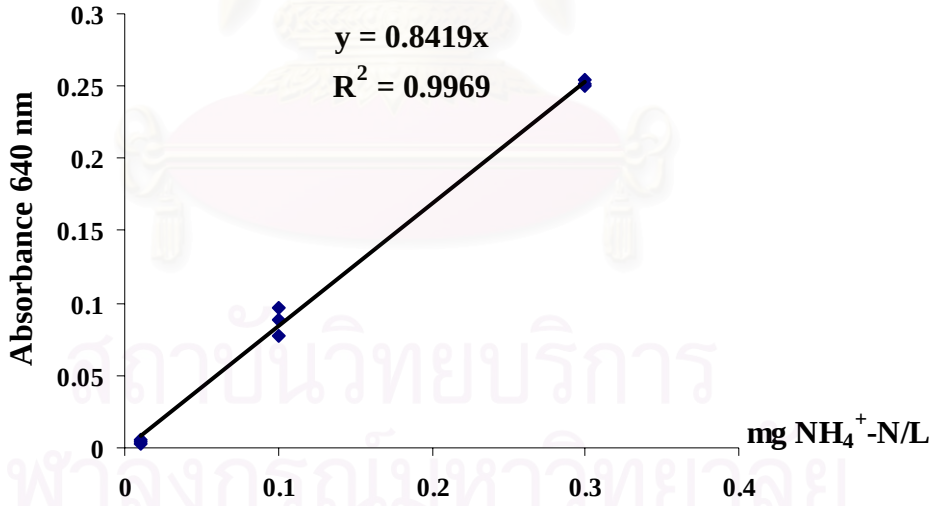


Nitrate (mg-N/L)	Haemolymph glucose (mg%)			
	Trial I	Trial II	Trial III	Mean 3 Trials
1.81	100.00±41.73	55.14±16.35	67.82±43.33	74.32±23.12
463.61	74.36±10.91			74.36±10.91
1079.5	81.03±16.70			81.03±16.70
1738.89	85.13±16.36			85.13±16.36
1901.93	71.28±38.35	148.15±107.83	37.36±10.33	85.60±56.77
2133.03			32.18±11.08	32.18±11.08
2525.43	53.08±28.24	77.78±109.79	51.72±40.65	60.86±14.67
2726.24			53.45±2.44	53.45±2.44
2914.27	33.85±6.15	96.30±94.28	58.62±0.00	62.92±31.45
3256.34			117.24±0.00	117.24±0.00
3487.56	51.23±20.23			51.23±20.23

Date	Day	Haemolymph glucose (mg%)	
		Control	Treatment
16 Aug 03	initial	20.43±5.23	20.43±5.23
21 Aug 03	5	14.74±7.23	78.08±60.94
31 Aug 03	15	6.06±2.90	9.72±8.08
10 Sep 03	25	129.47±73.65	58.16±37.31
20 Sep 03	35	30.13±22.79	48.70±23.29
30 Sep 03	45	89.74±84.17	175.51±70.19
10 Oct 03	55	107.16±36.95	59.28±62.84
20 Oct 03	65	35.09±22.54	43.45±34.95
29 Oct 03	74	23.02±9.56	64.91±74.31
19 Mar 04	216	8.40±1.66	13.86±4.67

Appendix D. (cont.) Haemolymph ammonium-N of *P. monodon* in acute toxicity test and CRSS.



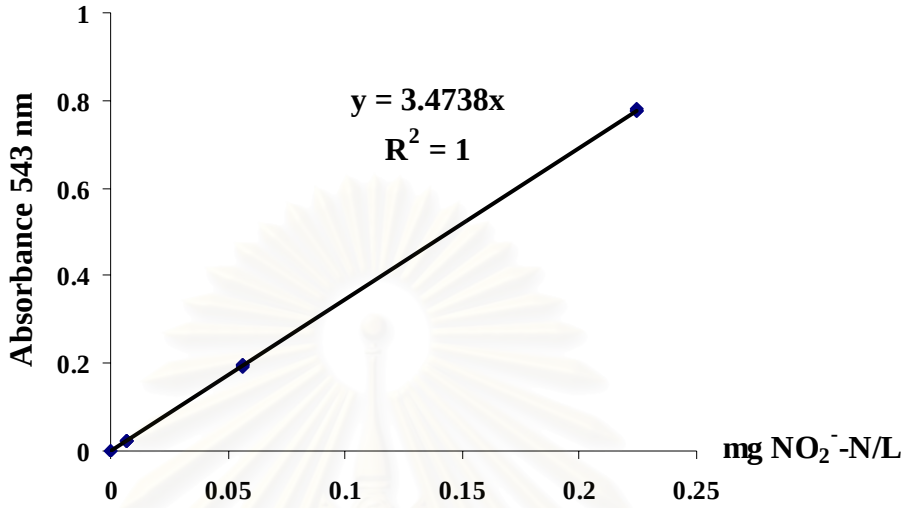


Appendix D. (cont.)

Nitrate (mg-N/L)	Haemolymph ammonium(mg-N/L)			
	Trial I	**Trial II**	**Trial III**	**Mean 3 Trials**
1.81	22.37±9.46	19.08±2.98	19.21±4.28	20.22±6.06
463.61	18.03±2.81			18.03±2.81
1079.5	15.48±4.62			15.48±+4.62
1738.89	18.07±4.32			18.07±4.32
1901.93	15.99±2.50	17.52±2.74	19.21±4.28	17.57±3.35
2133.03			17.46±6.05	17.46±6.05
2525.43	14.85±2.28	13.22±5.07	22.6±3.72	16.44±5.44
2726.24			15.72±7.41	15.72±7.41
2914.27	12.40±2.83	12.03±5.04	20.96±0.00	13.38±4.20
3256.34			21.06±0.00	21.06±0.00
3487.56	12.91±3.65			12.91±3.65

Date	Day	Haemolymph ammonium(mg-N/L)	
		Control	**Treatment**
16 Aug 03	initial	14.47±4.65	14.40±4.65
21 Aug 03	5	13.42±4.94	15.72±5.11
31 Aug 03	15	21.93±5.11	18.09±4.19
10 Sep 03	25	13.23±4.89	15.05±2.51
20 Sep 03	35	17.13±6.02	19.05±4.00
30 Sep 03	45	18.08±5.40	13.52±5.02
10 Oct 03	55	14.66±5.40	17.67±5.02
20 Oct 03	65	19.76±12.45	15.09±5.56
29 Oct 03	74	12.78±4.49	13.33±4.91
19 Mar 04	216	14.70±7.03	21.21±7.83

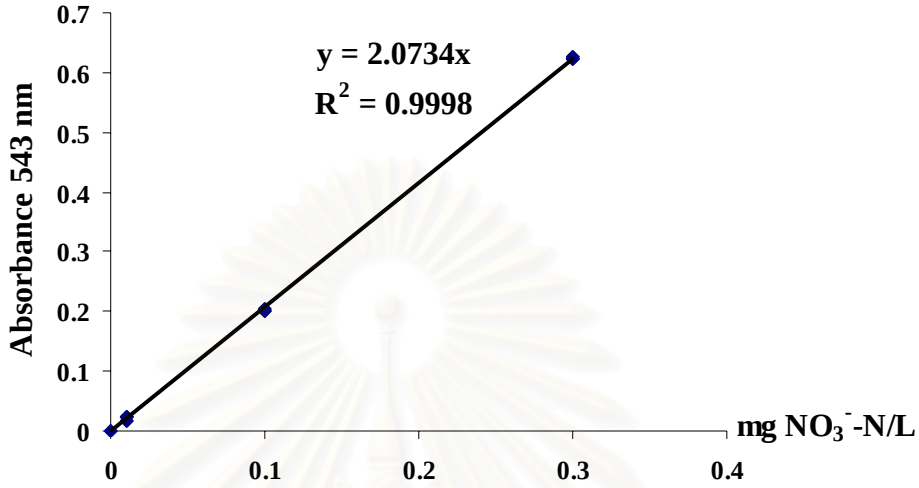
Appendix D. (cont.) Haemolymph nitrite-N of *P. monodon* in acute toxicity test and CRSS.



Nitrate (mg-N/L)	Haemolymph nitrite(mg-N/L)			
	Trial I	Trial II	Trial III	Mean 3 Trials
1.81	0.03±0.09	0.04±0.03	0.01±0.03	0.03±0.05
463.61	0.31±0.46			0.31±0.46
1079.5	0.00±0.00			0.00±0.00
1738.89	0.20±0.54			0.18±0.39
1901.93	1.13±1.04	0.71±0.86	2.32±2.35	1.26±1.66
2133.03			1.67±0.59	1.67±0.59
2525.43	1.00±0.53	1.37±0.89	2.97±1.45	1.55±1.32
2726.24			4.88±3.40	4.88±3.40
2914.27	3.26±2.41	5.32±0.82	2.04±0.18	2.76±2.85
3256.34			10.29±0.00	10.29±0.00
3487.56	2.82±0.99			1.88±0.75

Date	Day	Haemolymph nitrite(mg-N/L)	
		Control	Treatment
16 Aug 03	initial	0.02±0.03	0.02±0.03
21 Aug 03	5	0.09±0.06	0.11±0.07
31 Aug 03	15	0.47±0.65	0.29±0.18
10 Sep 03	25	0.18±0.11	0.18±0.12
20 Sep 03	35	0.12±0.08	0.08±0.04
30 Sep 03	45	0.17±0.21	0.25±0.17
10 Oct 03	55	0.14±0.19	0.11±0.24
20 Oct 03	65	0.51±0.48	0.28±0.08
29 Oct 03	74	0.28±0.50	0.00±0.00
19 Mar 04	216	0.60±0.70	0.17±0.09

Appendix D. (cont.) Haemolymph nitrate-N of *P. monodon* in acute toxicity test and CRSS.



Nitrate (mg-N/L)	Haemolymph nitrate (mg-N/L)			
	Trial I	Trial II	Trial III	Mean 3 Trials
1.81	13.00±3.35	1.00±1.86	12.13±3.36	8.73±6.25
463.61	390.80±35.91			390.80±37.19
1079.5	830.36±199.07			830.36±209.78
1738.89	1346.96±121.28			1346.96±126.24
1901.93	1870.52±206.15	2086.48±131.31	2071.21±129.08	2009.40±182.22
2133.03			2270.56±83.88	2270.56±87.21
2525.43	2659.88±157.03	2804.97±185.44	2858.43±206.97	2766.79±198.99
2726.24			2884.96±33.75	2884.96±17.05
2914.27	3088.00±259.19	3309.38±573.39	2802.16±17.39	3107.62±377.85
3256.34			3245.88±67.00	3245.88±0.00
3487.56	3661.46±88.38			3661.46±105.72

Date	Day	Haemolymph nitrate (mg-N/L)	
		Control	Treatment
16 Aug 03	initial	10.64±3.84	10.64±3.84
21 Aug 03	5	26.45±5.42	44.25±5.09
31 Aug 03	15	24.41±4.68	16.96±5.75
10 Sep 03	25	31.53±5.26	20.00±3.34
20 Sep 03	35	40.21±3.55	23.56±4.07
30 Sep 03	45	41.89±3.51	18.20±2.32
10 Oct 03	55	36.17±4.54	16.96±4.00
20 Oct 03	65	44.69±10.12	16.16±2.78
29 Oct 03	74	55.69±7.69	19.36±5.14
19 Mar 04	216	55.29±19.74	6.56±7.88

Appendix E. Oxygen consumption rate (OCR) of *P. monodon* in acute toxicity and in CRSS

Nitrate (mg-N/L)	Oxygen consumption rate (μgO_2/g/min)						
	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7
0	4.01	2.40	3.87	3.16	3.81	4.04	3.93
50	2.52	1.42	2.09	3.11	5.70	3.79	3.22
100	1.90	1.02	2.83	2.40	3.68	3.66	2.54
250	0.54	1.35	2.13	2.33	3.30	3.56	2.28
500	3.06	0.99	1.52	2.17	1.79	3.01	2.32
0	2.67	2.61	2.85	3.57	2.96	1.74	1.10

Nitrate (mg-N/L)	Oxygen consumption rate (μgO_2/g/min)				
	Trial 8	Trial 9	Trial 10	Trial 11	Trial 12
0	3.04	2.88	2.56	2.41	2.62
50	1.48	1.77	2.75	2.20	1.22
100	1.69	3.72	2.92	1.81	1.24
250	1.55	3.06	1.92	1.26	1.16
500	1.56	2.64	1.41	1.37	2.86
0	1.61	2.05	1.71	1.27	1.94

Control			Treatment		
Nitrate (mg-N/L)	Shrimp weight (g)	OCR μgO2/g/min	Nitrate (mg-N/L)	Shrimp weight (g)	OCR μgO2/g/min
49.05	36.5	2.61	24.33	33	3.78
49.05	40.3	2.74	24.33	29.7	3.16
49.05	28.3	4.03	24.33	40.9	3.12
49.05	39.5	1.83	24.33	34.4	3.35
49.05	33.1	4.17	24.33	33.7	3.76
49.05	32.4	2.94	24.33	40.3	3.62
49.05	35.8	2.94	24.33	32.5	3.48
49.05	33.4	4.11	24.33	36.7	3.90
49.05	36.7	2.83	24.33	34	3.16

Appendix F. Statistical test of physiological parameters to nitrate concentration by Duncan multiple range test (ANOVA).

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
THCs	Between Groups	29.389	9	3.265	5.088	**.000**
	Within Groups	44.925	70	.642		
	Total	74.313	79			
PROTEIN	Between Groups	47.451	9	5.272	1.232	.290
	Within Groups	299.556	70	4.279		
	Total	347.007	79			
PO	Between Groups	3386599.061	9	376288.785	2.062	**.045**
	Within Groups	12772071.867	70	182458.170		
	Total	16158670.928	79			
GLUCOSE	Between Groups	15466.878	9	1718.542	.624	.772
	Within Groups	192666.975	70	2752.385		
	Total	208133.853	79			
H_AMMO	Between Groups	356.027	9	39.559	1.713	.102
	Within Groups	1616.389	70	23.091		
	Total	1972.417	79			
H_NITRIT	Between Groups	92.029	9	10.225	5.052	**.000**
	Within Groups	141.674	70	2.024		
	Total	233.703	79			
H_NITRAT	Between Groups	107601667.323	9	11955740.814	364.857	**.000**
	Within Groups	2293783.349	70	32768.334		
	Total	109895450.672	79			

Post Hoc Tests

Homogeneous subsets

Total haemocyte counts (THCs)

Duncan [a,b]

	N	Subset for alpha = .05		
NITRATE		**1**	**2**	**3**
2726.24	2	4.5500		
2525.43	11	7.1364	7.1364	
2133.03	3	7.4000	7.4000	
2914.27	8	7.8625	7.8625	
3339.79	2	9.6500	9.6500	
1.81	18	15.0778	15.0778	15.0778
1901.93	18	15.0889	15.0889	15.0889
463.61	6		17.1000	17.1000
1738.89	6			25.2667
1079.50	6			26.3833
Sig.		.088	.107	.058

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 4.629.

b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Appendix F. (cont.)

Phenoloxidase activity (PO)

Duncan [a,b]

	N	Subset for alpha = .05	
NITRATE		**1**	**2**
2525.43	11	5.5191	
1.81	18	5.5739	
1079.50	6	5.9733	5.9733
2914.27	8	6.5425	6.5425
2726.24	2	6.5600	6.5600
1901.93	18	7.2422	7.2422
463.61	6	7.9450	7.9450
1738.89	6	11.6600	11.6600
2133.03	3	12.0667	12.0667
3339.79	2		12.3500
Sig.		.050	.054

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 4.629.

b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

H_NITRITE

Duncan [a,b]

	N	Subset for alpha = .05	
NITRATE		**1**	**2**
1079.50	6	.0000	
1.81	18	2.722E-02	
1738.89	6	.2650	
1901.93	18	1.3889	1.3889
2133.03	3	1.6700	1.6700
463.61	6	1.7533	1.7533
3339.79	2	2.8250	2.8250
2726.24	2	4.8750	4.8750
2525.43	11	11.2245	11.2245
2914.27	8		15.2800
Sig.		.138	.059

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 4.629.

b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Appendix F. (cont.)

H_NITRATE

Duncan [a,b]

N		Subset for alpha = .05								
NITRATE		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1.81	18	8.73								
463.61	6		390.80							
1079.50	6			830.36						
1738.89	6				1346.96					
1901.93	18					2009.40				
2133.03	3						2270.56			
2525.43	11							2766.79		
2726.24	2							2884.96	2884.96	
2914.27	8								3107.62	
3339.79	2									3661.46
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	.324	.065	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 4.629.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Appendix G. Statistical test of physiological parameters to nitrate concentration in CRSS compared with CRSS with denitrification by *t*- test at day 5 and day 15.

Paired Samples Test at day 5

		Paired Differences					*t*	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	N_THCS - DN_THCS	-.5060	1.2300	.3889	-1.3859	.3739	-1.301	9	.226
Pair 2	N_PROT - DN_PROT	1.2411	2.4070	.8023	-.6090	3.0913	1.547	8	.160
Pair 3	N_PO - DN_PO	-181.8133	194.5998	64.8666	-331.3960	-32.2307	-2.803	8	**.023**
Pair 4	N_GLUC - DN_GLUC	-69.0811	61.8574	20.6191	-116.6289	-21.5333	-3.350	8	**.010**
Pair 5	N_H_NH4 - DN_H_NH4	-2.3000	7.4148	2.3448	-7.6043	3.0043	-.981	9	.352
Pair 6	N_H_NO2 - DN_H_NO2	-2.5000E-02	7.590E-02	2.400E-02	-7.9297E-02	2.930E-02	-1.042	9	.325
Pair 7	N_H_NO3 - DN_H_NO3	-17.8050	7.6597	2.4222	-23.2844	-12.3256	-7.351	9	**.000**

Paired Samples Test at day 15

		Paired Differences					*t*	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	N_THCS - DN_THCS	-.5260	1.1363	.3593	-1.3389	.2869	-1.464	9	.177
Pair 2	N_PROT - DN_PROT	-1.4600	1.3261	.4194	-2.4086	-.5114	-3.482	9	**.007**
Pair 3	N_PO - DN_PO	273.8740	212.9805	67.3504	121.5169	426.2311	4.066	9	**.003**
Pair 4	N_GLUC - DN_GLUC	-3.6700	8.8562	2.8006	-10.0053	2.6653	-1.310	9	.222
Pair 5	N_H_NH4 - DN_H_NH4	3.8450	8.5061	2.6899	-2.2399	9.9299	1.429	9	.187
Pair 6	N_H_NO2 - DN_H_NO2	.1760	.6120	.1935	-.2618	.6138	.909	9	.387
Pair 7	N_H_NO3 - DN_H_NO3	7.4440	8.5069	2.6901	1.3585	13.5295	2.767	9	**.022**

Appendix G. (cont.) Statistical test of physiological parameters to nitrate concentration in CRSS compared with CRSS with denitrification by *t*-test at day 25 and day 35.

Paired Samples Test at day 25

		Paired Differences					*t*	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	N_THCS - DN_THCS	.6250	1.9274	.6095	-.7538	2.0038	1.025	9	.332
Pair 2	N_PROT - DN_PROT	-.2160	1.0561	.3340	-.9715	.5395	-.647	9	.534
Pair 3	N_PO - DN_PO	165.2713	291.5526	103.0794	-78.4729	409.0154	1.603	7	.153
Pair 4	N_GLUC - DN_GLUC	71.3090	74.3988	23.5270	18.0873	124.5307	3.031	9	**.014**
Pair 5	N_H_NH4 - DN_H_NH4	-1.8130	6.3368	2.0039	-6.3461	2.7201	-.905	9	.389
Pair 6	N_H_NO2 - DN_H_NO2	4.000E-03	8.758E-02	2.770E-02	-5.8654E-02	6.665E-02	.144	9	.888
Pair 7	N_H_NO3 - DN_H_NO3	11.5290	5.3039	1.6772	7.7348	15.3232	6.874	9	**.000**

Paired Samples Test at day 35

		Paired Differences					*t*	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	N_THCS - DN_THCS	-.3740	2.1053	.6657	-1.8800	1.1320	-.562	9	.588
Pair 2	N_PROT - DN_PROT	.2040	.6701	.2119	-.2753	.6833	.963	9	.361
Pair 3	N_PO - DN_PO	-70.1380	352.0709	111.3346	-321.9944	181.7184	-.630	9	.544
Pair 4	N_GLUC - DN_GLUC	-18.5740	27.3099	8.6362	-38.1104	.9624	-2.151	9	.060
Pair 5	N_H_NH4 - DN_H_NH4	-1.9200	7.7028	2.4359	-7.4303	3.5903	-.788	9	.451
Pair 6	N_H_NO2 - DN_H_NO2	4.300E-02	7.602E-02	2.404E-02	-1.1381E-02	9.738E-02	1.789	9	.107
Pair 7	N_H_NO3 - DN_H_NO3	16.6450	5.2019	1.6450	12.9238	20.3662	10.119	9	**.000**

Appendix G. (cont.) Statistical test of physiological parameters to nitrate concentration in CRSS compared with CRSS with denitrification by *t*- test at day 45 and day 55.

Paired Samples Test at day 45

		Paired Differences					*t*	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	N_THCS - DN_THCS	.7230	1.5836	.5008	-.4098	1.8558	1.444	9	.183
Pair 2	N_PROT - DN_PROT	.1570	1.3253	.4191	-.7911	1.1051	.375	9	.717
Pair 3	N_PO - DN_PO	-448.8910	583.2097	184.4271	-866.0941	-31.6879	-2.434	9	**.038**
Pair 4	N_GLUC - DN_GLUC	-85.7720	87.9729	27.8195	-148.7040	-22.8400	-3.083	9	**.013**
Pair 5	N_H_NH4 - DN_H_NH4	4.5660	5.0770	1.6055	.9341	8.1979	2.844	9	**.019**
Pair 6	N_H_NO2 - DN_H_NO2	-7.5000E-02	.2989	9.453E-02	-.2888	.1388	-.793	9	.448
Pair 7	N_H_NO3 - DN_H_NO3	23.6900	2.6853	.8492	21.7691	25.6109	27.898	9	**.000**

Paired Samples Test at day 55

		Paired Differences					*t*	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	N_THCS - DN_THCS	-.9940	1.8796	.5944	-2.3386	.3506	-1.672	9	.129
Pair 2	N_PROT - DN_PROT	3.200E-02	.4094	.1295	-.2609	.3249	.247	9	.810
Pair 3	N_PO - DN_PO	-38.4660	258.7726	81.8311	-223.5808	146.6488	-.470	9	.649
Pair 4	N_GLUC - DN_GLUC	47.8780	87.1960	27.5738	-14.4983	110.2543	1.736	9	.117
Pair 5	N_H_NH4 - DN_H_NH4	-3.0090	5.0383	1.5932	-6.6132	.5952	-1.889	9	.092
Pair 6	N_H_NO2 - DN_H_NO2	3.300E-02	.3622	.1145	-.2261	.2921	.288	9	.780
Pair 7	N_H_NO3 - DN_H_NO3	19.2100	4.4262	1.3997	16.0437	22.3763	13.725	9	**.000**

Appendix G. (cont.) Statistical test of physiological parameters to nitrate concentration in CRSS compared with CRSS with denitrification by *t*-test at day 65 and day 74.

Paired Samples Test at day 65

		Paired Differences					*t*	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	N_THCS - DN_THCS	.9290	1.5436	.4881	-.1752	2.0332	1.903	9	.089
Pair 2	N_PROT - DN_PROT	-.1930	.7789	.2463	-.7502	.3642	-.784	9	.453
Pair 3	N_PO - DN_PO	73.1560	266.8444	84.3836	-117.7330	264.0450	.867	9	.408
Pair 4	N_GLUC - DN_GLUC	-8.3630	44.3496	14.0246	-40.0888	23.3628	-.596	9	.566
Pair 5	N_H_NH4 - DN_H_NH4	4.6610	12.2904	3.8866	-4.1310	13.4530	1.199	9	.261
Pair 6	N_H_NO2 - DN_H_NO2	.2240	.4925	.1557	-.1283	.5763	1.438	9	.184
Pair 7	N_H_NO3 - DN_H_NO3	28.5300	11.3059	3.5752	20.4422	36.6178	7.980	9	**.000**

Paired Samples Test at day 74

		Paired Differences					*t*	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	N_THCS - DN_THCS	-8.0000E-03	1.0847	.3430	-.7839	.7679	-.023	9	.982
Pair 2	N_PROT - DN_PROT	-.2160	.5917	.1871	-.6393	.2073	-1.154	9	.278
Pair 3	N_PO - DN_PO	34.0450	167.3583	52.9233	-85.6759	153.7659	.643	9	.536
Pair 4	N_GLUC - DN_GLUC	-41.8870	77.9598	24.6531	-97.6561	13.8821	-1.699	9	.124
Pair 5	N_H_NH4 - DN_H_NH4	-.5450	6.6471	2.1020	-5.3000	4.2100	-.259	9	.801
Pair 6	N_H_NO2 - DN_H_NO2	.2840	.5101	.1613	-8.0921E-02	.6489	1.761	9	.112
Pair 7	N_H_NO3 - DN_H_NO3	36.3280	8.0561	2.5475	30.5650	42.0910	14.260	9	**.000**

Appendix G. (cont.) Statistical test of physiological parameters to nitrate concentration in CRSS compared with CRSS with denitrification by *t*-test at day 216.

Paired Samples Test at day 216

		Paired Differences					*t*	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	N_THCS - DN_THCS	-.3900	2.0414	.9129	-2.9247	2.1447	-.427	4	.691
Pair 2	N_PROT - DN_PROT	-.1871	.7900	.3533	-1.1680	.7938	-.530	4	.624
Pair 3	N_PO - DN_PO	109.7866	482.5408	215.7988	-489.3669	708.9401	.509	4	.638
Pair 4	N_GLUC - DN_GLUC	-5.4615	3.6502	1.6324	-9.9938	-.9292	-3.346	4	**.029**
Pair 5	N_H_NH4 - DN_H_NH4	-6.5100	7.5216	3.3638	-15.8493	2.8293	-1.935	4	.125
Pair 6	N_H_NO2 - DN_H_NO2	.4200	.7257	.3245	-.4811	1.3211	1.294	4	.265
Pair 7	N_H_NO3 - DN_H_NO3	48.7320	16.6214	7.4333	28.0938	69.3702	6.556	4	**.003**

Appendix H. Statistical test of Oxygen consumption rate of *P. monodon* to ambient nitrate in CRSS

Oneway descriptive

Oxygen consumption rate in CRSS

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	9	3.1310	.8019	.2673	2.5147	3.7474	1.83	4.17
Treatment	9	3.4821	.2987	9.957E-02	3.2525	3.7117	3.12	3.90
Total	18	3.3066	.6142	.1448	3.0012	3.6120	1.83	4.17

Test of Homogeneity of Variances

Oxygen consumption rate in CRSS

Levene Statistic	df1	df2	Sig.
7.462	1	16	.015

ANOVA

Oxygen consumption rate in CRSS

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.555	1	.555	1.515	.236
Within Groups	5.858	16	.366		
Total	6.412	17			

BIOGRAPHY

Mr. Sarayut Onsanit was born on March 4, 1977 in Trang. He graduated with the primary education at Trangchristiansuksa School, secondary education at Wichienmatu School, Trang province and bachelor degree in Marine Science from Department of Marine Science, Faculty of Science and Fisheries Technology, Rajamangala Institute of Technology, Trang campus. During undergraduate study, In April-July 1998, he used to join with the International Field School in Marine Biology and Thai Culture of Rajamangala Institute of Technology, and Malaspina University Collage, Canada. After graduation, he was a researcher in The Domestic Black Tiger Shrimp broodstock commercial production Project of Charoen Pokphand Group Public Co., Ltd.

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In December 2003, he is a member of the Coastal Aquatic Feed Research Institute, Department of Fisheries, Ministry of Agricultural and Cooperatives.

Research Publications:

Onsanit, S., Powtongsook, S. and P., Menasveta. 2003. *Physiological response of Black Tiger Shrimp (Penaeus monodon) to Nitrate in Seawater*. Abstracts of Biothailand 2003, 17-20 July 2003, Thailand. 175.

Onsanit, S., Powtongsook, S., Ngampongsai, C. and P., Menasveta. 2004. *Physiological responses of Black Tiger Shrimp (Penaeus monodon) to Nitrate in The Closed Recirculating Seawater System*. Proceeding of the 5th National Symposium on Marine Shrimp, 29-30 March 2004, Bangkok, Thailand. 177-178.