Effects of Melamine and Cyanuric Acid on Growth Performance and Health Condition in Thai Economic Aquatic Animal Species

Nutt Nuntapong

A Thesis Submitted in Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Aquatic Science

Prince of Songkla University

2018

Thesis Title	Effects of Melamine and Cyanuric Acid on Growth Performance and Health Condition in Thai Economic Aquatic Animal Species
Author	Mr. Nutt Nuntapong
Major Program	Aquatic Science

Major Advisor

……..

(Assoc. Prof. Dr. Wutiporn Phromkunthong)

Examining Committee :

……..Chairperson

(Asst. Prof. Dr. Suphada Kiriratnikom)

……..Committee

(Assoc. Prof. Dr. Wutiporn Phromkunthong)

……..Committee

(Assoc. Prof. Dr. Karun Thongprajukaew)

……..Committee

(Asst. Prof. Dr. Naraid Suanyuk)

……..Committee

(Dr. Pornpimon Chuadaungpui)

The Graduate School, Prince of Songkla University, has approved this thesis as fulfillment of the requirements for the Degree of Doctor of Philosophy in Aquatic Science

……..

(Prof. Dr. Damrongsak Faroongsarng)

Dean of Graduate School

This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

…… Signature

(Assoc. Prof. Dr.Wutiporn Phromkunthong)

Major Advisor

…….Signature

(Mr.Nutt Nuntapong)

Candidate

I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

..... Signature
(Mr.Nutt Nuntapong)
Candidate

Thesis Title Effects of Melamine and Cyanuric Acid on Growth Performance and Health Condition in Thai Economic Aquatic Animal Species

Author Mr.Nutt Nuntapong

Major Program Aquatic Science

Academic Year 2018

ABSTRACT

The study consisted of three experiments to investigate the effects of melamine (MEL) and cyanuric acid (CYA) in three differences economic aquatic animal species of Thailand i.e. Asian seabass (*Lates calcarifer*), Pacific white shrimp (*Litopenaeus vannamei*) and hybrid *Clarias* catfish (*Clarias macrocephalus* (Günther) x *C. gariepinus* (Burchell)). An identical dosages of MEL and CYA were used in each experiment as follows: diet 1 (a control diet without MEL and CYA); diets 2-5 (with MEL and CYA at 2.5+2.5, 5+5, 7.5+7.5, or 10+10 g kg^{-1} diet); diet 6 (with only MEL at 10 g kg^{-1} diet); and diet 7 (with CYA alone at 10 g kg^{-1}diet).

In experiment 1, seven experimental isonitrogenous (40%) and isolipidic (12%) diets were formulated. Asian seabass (initial weight of 4.77±0.25 g) were fed three times a day to satiation at 8:00, 12:00 and 16:00 h for 12 weeks. It was obviously indicated that those fish received combined MEL-CYA: 7.5+7.5, 10+10 or MEL alone diets had poor growth and feed conversion ratio (FCR) ($P<0.05$). Abnormalities were observed in the liver and kidney of fish with combined MEL and CYA supplementation. The renal tubules of fish that were fed with diets 2-5 had golden-brown melamine-cyanurate crystals. Fish given only one type of supplementation (MEL or CYA alone) did not have such crystals in the kidneys. The highest MEL residue in fillet was detected in the fish ingested MEL alone (10 g kg^{-1} diet). Levels of heat shock protein (Hsp) 70 were elevated in the liver of fish that had ingested MEL/CYA, in combination or alone (diets 2-7) ($P<0.05$). There were no significant differences between the treatments ($P>0.05$) in the level of Hsp70 in the kidneys of the fish. High dosages of MEL-CYA induced the activities of catalase and glutathione peroxidase in liver and kidneys.

In experiment 2, seven experimental isonitrogenous (35%) and isolipidic (8%) diets were formulated. The shrimp with an initial body weight of 2.37±0.02 g were subjected to these diets for 10 weeks. The results indicated that all the diets with MEL and CYA singly or in combination had adverse effects on growth and nutrient utilization relative to the control diet ($P<0.05$). Total protease and trypsin activities were significantly lowered by all diets containing a combination of MEL-CYA or MEL alone ($P<0.05$). Blood parameters, including total hemocyte count (THC), phenoloxidase (PO) activity, nitroblue tetrazolium (NBT) reduction, and lysozyme activity, were significantly decreased ($P<0.05$) in shrimp receiving MEL alone (10 g kg^{-1} diet) and at high combination dosages (10+10 g kg^{-1} diet). Moreover, MEL and CYA induced oxidative stress, decreased antioxidant responses, increased lipid peroxidation and caused damaged to hepatopancreas.

In experiment 3, seven experimental isonitrogenous (35%) and isolipidic (7%) diets were formulated. The hybrid *Clarias* catfish with an initial body weight 6.00±0.02 g were randomly distributed into seven dietary treatments and each treatment consisted of 3 replications. Fish were fed twice daily to satiation at 8:00 and 16:00 h for 8 weeks. The results indicated that the supplementation of MEL and CYA had no significant effect on survival rate ($P<0.05$). However, body weight and specific growth rate (SGR) were significantly lower than the control group. There were no significant differences in FCR among the dietary treatments ($P>0.05$). The percentages of skin discoloration of fish were significantly increased ($P<0.05$) in treatment groups than control groups. Blood parameters, including white blood cell count (WBC) and NBT reduction were significantly decreased ($P<0.05$) in fish exposed to MEL and CYA. When the combination of MEL and CYA up to 7.5+7.5 and 10+10 g kg^{1} diet, catalase (CAT) and glutathione peroxidase (GPx) activities in liver and kidney were significantly higher ($P<0.05$) than the control groups. The renal tubules of fish that were fed with diets combination of MEL and CYA had gold-brown melamine-cyanurate crystals.

In conclusion, this study investigated the responses of three differences of economic aquatic species to MEL and CYA exposures either singly or in combination.

Results of the present work indicate that MEL and CYA had many toxic effects evidenced by growth reduction, changed activities of digestive and antioxidant enzymes, and severe histological damage to target organs i.e. kidney in fish and hepatopancreas in shrimp. It was expected that the results from this study can provide basic data for the further studies and it also provided knowledge to farmers for monitoring the contamination of melamine in aquatic diets in the future.

ชื่อวิทยานิพนธ์ ผลของเมลามีนและกรดไซยานูริคต่อการเจริญเติบโตและสุขภาพของสัตว์น้ำเศรษฐกิจของไทย

ผู้เขียน นายนัทท์ นันทพงศ์

สาขาวิชา วาริชศาสตร์

ปีการศึกษา 2561

บทคัดย่อ

การศึกษาครั้งนี้ประกอบด้วย 3 การทดลองโดยมีวัตถุประสงค์เพื่อศึกษาผลของเมลามีน (melamine, MEL) และกรดไซยานูริค (cyanuric acid, CYA) ในสัตว์น้ำที่มีความสำคัญทางเศรษฐกิจ 3 ชนิดของไทยคือ ปลากะพงขาว (*Lates calcarifer*) กุ้งขาว (*Litopenaeus vannamei*) และปลาดุกพันธุ์ผสม (*Clarias macrocephalus* (Günther) x *C. gariepinus* (Burchell)) โดยกำหนดให้อาหารทดลองที่ใช้สำหรับทั้ง 3 การทดลองมีปริมาณของ MEL และ CYA ที่ใกล้เคียงกันโดยกำหนดให้อาหารสูตรที่ 1 (อาหารควบคุมโดยไม่มี MEL และ CYA); อาหาร 2-5 (มี MEL และ CYA ที่อาหาร 2.5+2.5, 5+5, 7.5+7.5 และ 10+10 กรัมต่อกิโลกรัม); อาหารสูตรที่ 6 (MEL 10 กรัมต่อกิโลกรัม) และอาหารสูตรที่ 7 (CYA 10 กรัมต่อกิโลกรัม)

ในการทดลองที่ 1 ใช้สูตรอาหาร 7 สูตรโดยกำหนดให้มีปริมาณโปรตีนและไขมันที่ใกล้เคียงกัน (โปรตีน 40 เปอร์เซ็นต์ และ ไขมัน 12 เปอร์เซ็นต์ ตามลำดับ) โดยใช้ปลากะพงขาว (น้ำหนักเริ่มต้น 4.77 ± 0.25 กรัม) ปลาได้รับอาหารสามครั้งต่อวันโดยให้กินจนอิ่มเวลา 8:00, 12:00 และ 16:00 น. เป็นเวลา 12 สัปดาห์ จากการทดลองพบว่า ปลาที่ได้รับอาหารที่มี MEL-CYA: 7.5+7.5, 10+10 กรัมต่อกิโลกรัม หรือ MEL เพียงอย่างเดียว ส่งผลทางลบต่อการเจริญเติบโตและอัตราการเปลี่ยนอาหาร (FCR) ($P<0.05$) พบความผิดปกติในตับและไตของปลาที่ได้รับอาหารที่มีการเสริม MEL และ CYA โดยบริเวณท่อไตของปลาที่เลี้ยงด้วยอาหาร 2-5 พบผลึกสีน้ำตาลทอง (melamine-cyanurate crystals) ทั้งนี้ปลาที่ได้รับการเสริมเมลามีนและกรดไซยานูริคเพียงชนิดเดียวไม่มีผลึกดังกล่าวในไต พบการตกค้างของ MEL สูงสุดในเนื้อปลาที่กินอาหารเพียงอย่างเดียว MEL (10 กรัมต่อกิโลกรัม) ระดับ heat shock proteins (Hsp) 70 เพิ่มขึ้นในตับปลาที่ได้รับอาหารที่มี MEL และ CYA ทั้งในรูปแบบของการเสริมร่วมกันหรือเสริมเพียงรูปแบบเดียว (อาหาร 2-7) ($P<0.05$) ไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($P>0.05$) ในระดับ Hsp70 ในไตของปลา ปลาที่ได้รับ MEL และ CYA ในระดับสูงสุดส่งผลให้กิจกรรมของ catalase และ glutathione peroxidase ในตับและไตเพิ่มสูงขึ้น

ในการทดลองที่ 2 ใช้สูตรอาหาร 7 สูตรโดยกำหนดให้มีปริมาณโปรตีนและไขมันที่ใกล้เคียงกัน (โปรตีน 35 เปอร์เซ็นต์ และ ไขมัน 8 เปอร์เซ็นต์ ตามลำดับ) โดยใช้กุ้งที่มีน้ำหนักตัวเริ่มต้น 2.37±0.02 กรัม ระยะเวลาทดลอง 10 สัปดาห์ ผลการทดลองพบว่าอาหารทุกสูตรที่มี MEL และ CYA ทั้งในรูปแบบของการเสริมร่วมกันหรือเสริมเพียงอย่างเดียว ส่งผลทางลบต่อการเจริญเติบโตและประสิทธิภาพการใช้อาหารเมื่อเปรียบเทียบกับชุดควบคุม ($P<0.05$) กิจกรรมของเอนไซม์โปรตีเอสและทริปซินมีค่าลดลงอย่างมีนัยสำคัญทางสถิติ ($P<0.05$) ในทุกชุดการทดลองที่ได้รับ MEL ร่วมกับ CYA และ MEL เพียงอย่างเดียว ด้านองค์ประกอบเลือดพบว่า ปริมาณเม็ดเลือดรวม (total hemocyte count, THC), กิจกรรมของเอนไซม์ phenoloxidase (PO), nitroblue tetrazolium (NBT) reduction และกิจกรรมของไลโซไซม์ มีค่าลดลงอย่างมีนัยสำคัญทางสถิติ ($P<0.05$) ในกุ้งที่ได้รับ MEL อย่างเดียว (10 กรัมต่อกิโลกรัม) และในปริมาณที่สูง (10+10 กรัมต่อกิโลกรัม) นอกจากนี้ MEL และ CYA ยังก่อให้เกิดภาวะเครียดออกซิเดชั่น (oxidative stress) ลดประสิทธิภาพการทำงานของสารต้านอนุมูลอิสระ เพิ่มปฏิกิริยาออกซิเดชันของลิพิด (lipid oxidation) และสร้างความเสียหายต่อเซลล์ตับและตับอ่อน

ในการทดลองที่ 3 ใช้สูตรอาหาร 7 สูตรโดยกำหนดให้มีปริมาณโปรตีนและไขมันที่ใกล้เคียงกัน (โปรตีน 35 เปอร์เซ็นต์ และ ไขมัน 7 เปอร์เซ็นต์ ตามลำดับ) โดยใช้ปลาดุกพันธุ์ผสม ผสมที่มีน้ำหนักตัวเริ่มต้น 6.00 ± 0.02 กรัม แต่ละชุดการทดลองมี 3 ซ้ำ ปลาได้รับอาหารวันละสองครั้งในเวลา 8:00 น. และ 16:00 น. เป็นเวลา 8 สัปดาห์โดยให้กินจนอิ่ม จากการทดลองพบว่าการเสริม MEL และ CYA ไม่มีผลต่ออัตราการรอดตาย ($P<0.05$) อย่างไรก็ตามน้ำหนักตัวและอัตราการเจริญเติบโตจำเพาะ (specific growth rate, SGR) ต่ำกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ อัตราการเปลี่ยนอาหารสัตว์ไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($P>0.05$) เปอร์เซ็นต์ของการเปลี่ยนสีผิวของปลาเพิ่มขึ้นอย่างมีนัยสำคัญ ($P<0.05$) ในกลุ่มที่ได้รับ MEL และ CYA มากกว่ากลุ่มควบคุม พารามิเตอร์ของเลือด ได้แก่ จำนวนเม็ดเลือดขาว (WBC) และ NBT reduction ลดลงอย่างมีนัยสำคัญ ($P<0.05$) ในปลาที่ได้รับ MEL และ CYA เมื่อระดับของ MEL และ CYA สูงถึง 7.5 + 7.5 และ 10 +10 กรัมต่อกิโลกรัม กิจกรรมของ catalase (CAT) และ glutathione peroxidase (GPx) ในตับและไตมีค่าสูงกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ ($P<0.05$) ปลาที่ได้รับอาหารที่มีส่วนผสมของ MEL และ CYA มีผลึกสีทองตกค้างอยู่ คาดว่าผลจากการศึกษาครั้งนี้สามารถให้ข้อมูลพื้นฐานสำหรับการศึกษาต่อไปและให้ความรู้แก่เกษตรกรในการติดตามการปนเปื้อนเมลามีนในอาหารสัตว์น้ำในอนาคต

ACKNOWLEDGEMENTS

This study would have never been completed and made possible without the support and guidance from the following people...

First and foremost, I would like to express my sincere appreciation to my advisor, Associate Professor Dr. Wutiporn Phromkunthong for the continuous support during my Ph.D study, for accepting me as his student, for his patience, motivation, and for everything that this word “thank you” does not seem enough. I appreciate his endeavor to give me a motivation and dedication to the world of aquatic animal nutrition.

Besides my advisor, I would like to express my gratitude and thank the rest of my thesis committee as follows: Assistant Professor Dr. Suphada Kiriratnikom, Assistant Professor Dr. Naraid Suanyuk, Associate Professor Dr. Karun Thongprajukaew and Dr. Pornpimon Chuadaungpuifor their valuable suggestions and academic guidance.

At KS-AAHRC, it feels like home for me. Once again, I would like to thank Assistant Professor Dr. Naraid Suanyuk, Dr. Sunee Wanlem and Mr. Akkarawit Issaro for all their kindness, support when I meet the problems and encouragement at every step of my graduation.

I wish to acknowledge all those lecturers and staffs in the Department of Aquatic Science for all kindness and support. A special thank to Ms. Rattana Pocharuang for kindly help me to preparing the official documents.

My special thanks are extended to the staffs and students at KS-AAHRC, Starting with Ms. Chutima Klingklib, Mr. Anuvee Baka, Ms. Machalin Dangwetngam, Mr. Witsarut Choseng, Mr. Pattapong Saetoo and Mr. Taranan Kongkapun for kindly help during specimen and data collection.

For Dr. Pattarinee Klumdoung, my best friend, many thanks for always listening to me, supporting me and encouraging me.

Honestly, I would like to thank my family for supporting me and I am sorry to have kept you waiting for so long.

Finally, the financial support by National Research University (NRU) permitted me to undertake this study was much appreciated.

Nutt Nuntapong

TABLE OF CONTENTS

TABLE OF CONTENTS (cont.)

LIST OF TABLES

LIST OF TABLES (cont.)

LIST OF FIGURES

LIST OF FIGURES (cont.)

CHAPTER 1

1.1 Introduction

In aquaculture industry, feed cost represents more than 50 percent of operating costs in cultural farming. Among the essential nutrients, protein is the most expensive component of most aquaculture feeds, which significantly influence growth performance, product quality and health status of aquatic animal. So, dietary protein is an important factor for determining quality and value in aquatic feeds. However, in recent years, feed price has dramatically increased due to inadequate supply and rising price of raw materials used as protein sources especially, fish meal and soybean meal. Recently, the replacements of expensive protein sources with alternative protein sources derived from vegetable and other terrestrial sources without compromising growth performance or health status of animal are suitable for sustainable development. However, the major disadvantages of most alternative protein sources are low protein quality, less digestibility and imbalance of essential nutrients.

Unfortunately, unethical manufacturers are illegally adulterated some cheaper nitrogen-rich materials in the feeds or feed ingredients for increasing protein appearance. In the feed industry, protein content in a test sample can be obtained by determining the nitrogen (N) value of the sample multiplied by 6.25. Melamine is nitrogenous base-material containing 66% of nitrogen by mass. Therefore, adding of MEL in foodstuff increases total nitrogen content, which can be converted directly into protein, as also called “fake protein”. The contamination of farmed aquatic feed with melamine has already been reported in many countries.

In order to enhance knowledge of the toxicological profile, this research aims to study the effect of melamine, cyanuric acid and the interaction of these substances at various levels in diet on growth and health of economically important aquatic animal species. This study was focused on Asian seabass, hybrid *Clarias* catfish and Pacific white shrimp, which representing for finfish, scaleless fish and crustacean, respectively. It was expected that the results from this study can provide

basic knowledge for the further studies and it also provided data to farmers for monitoring the contamination of melamine in aquatic feeds in the future.

1.2 Objectives

The objectives of this study were:

1.2.1 To determine the effects of melamine and cyanuric acid on growth and feed efficiency of Thai aquatic valuable species.

1.2.2 To determine histological alterations, antioxidant enzyme related to stress in treated fish and shrimp compared to the control.

1.2.3 To determine melamine and cyanuric acid residue in edible parts of animal which may cause a negatively effects to the consumer.

CHAPTER 2

Review of literatures

2.1 Melamine

2.1.1 Structure and properties of melamine

Melamine (1,3,5-triazine-2,4,6-triamine) is an important raw material in the plastic industries (NTP, 1983; IARC, 1999; Baynes *et al*., 2008). Melamine was first described by German chemist, Justus Liebig in 1834. The name “melamine” is originated from German word “melamin” where it was created by combining the words “melam” (a derivative of ammonium thiocyanate) and “amine” (Bann and Miller, 1758). The appearance of melamine is fine white crystalline powder with chemical formula $C_3H_6N_6$ and a molecular weight of 126.12 g mol^{-1} (Rovina and Siddiquee, 2015). Melamine is a weak base (pK_b = 9) (Gautney *et al*., 1982). The polarity of this compound is influenced by the presence of three amine (NH_2) functional groups (Figure 2-1.), which are responsible for its solubility in water (Baynes *et al*., 2008). The solubility of melamine in water is 3.24 g L^{-1} at 19.9ºC, and increasing to 50.5 g L^{-1} at 99ºC (Gautney *et al*., 1982; Zidar *et al*., 2013). Melamine is widely used for the manufacture of dishware, laminates, coating materials, flame retardants, pesticides and fertilizer (Zhang and Horrocks, 2003; Nemil *et al*., 2005; Rovina and Siddiquee, 2015).

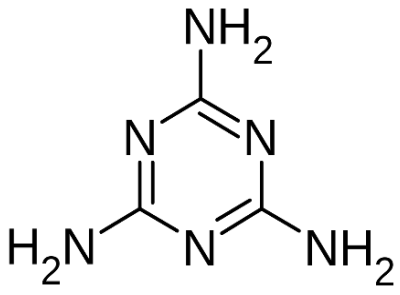

Figure 2-1. Structure of melamine

Source: Chen *et al*. (2006)

For industrial scale production, melamine is manufactured using urea as starting material (Figure 2-2) and the by-products ammeline, ammelide and cyanuric acid originate during this step under strong acidic and alkaline conditions (Figure 2-3 to 2-5) (IARC, 1999; Hau *et al*., 2009; Tyan *et al*., 2009; WHO, 2009).

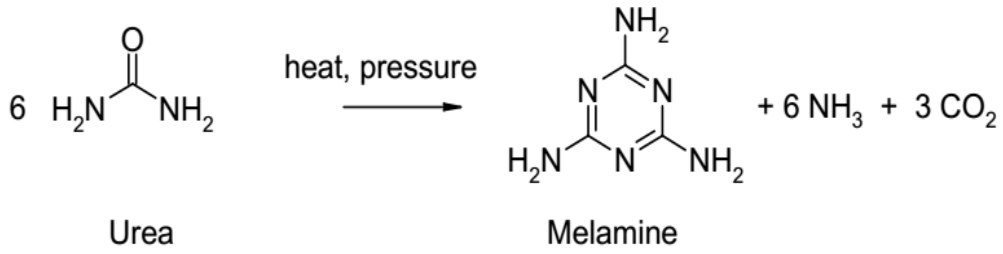

Figure 2-2. The reaction for the production of melamine from urea
Source: WHO (2009)

The reaction is typically performed in one or more stages using either a high-pressure or a low-pressure process. The high-pressure process (Figure 2-3) is performed in the liquid phase without a catalyst, at pressures of 90–150 bar and temperatures of 380–450ºC. In this step, urea is first decomposed to isocyanic acid and ammonia (Figure 2-3). The cyanuric acid is then reacted with ammonia to form melamine (WHO, 2009).

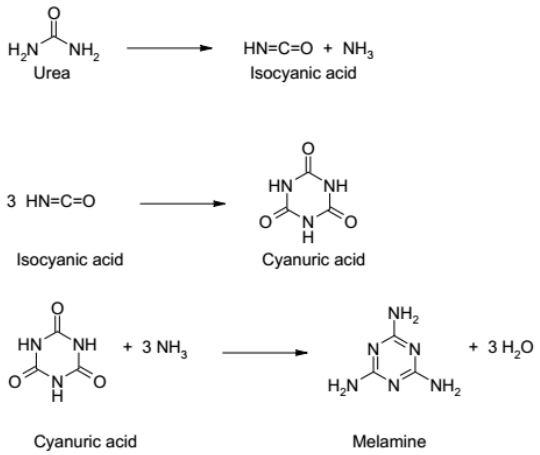

Figure 2-3. The high-pressure process of melamine production

Source: WHO (2009)

The low-pressure process (Figure 2-4) is carried out in the gas phase in the presence of a catalyst, such as modified aluminium oxide or aluminosilicate, at pressures of 1–10 bar and temperatures of 350–450ºC. As with the high-pressure process, urea is first transformed to isocyanic acid. In the second stage of the reaction, the isocyanic acid is converted on the catalyst to either cyanamide or carbodiimide (Equation 1-5), which are then converted to melamine (Figure 2-4).

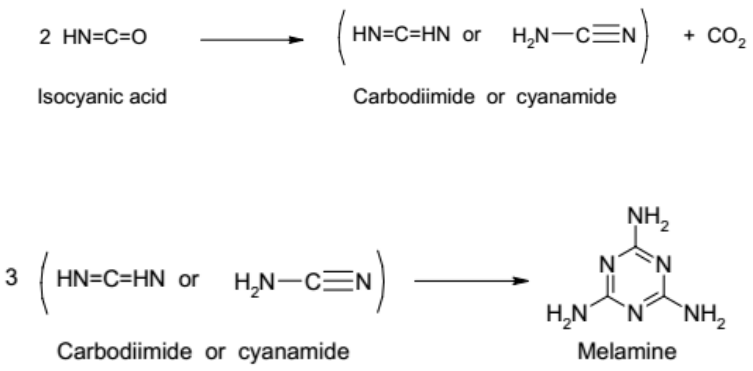

Figure 2-4. The low-pressure process of melamine production

Source: WHO (2009)

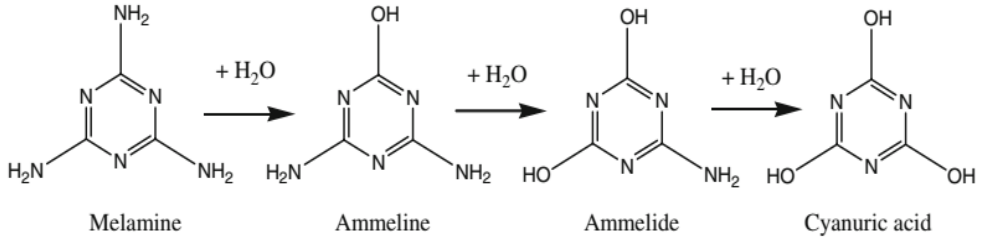

Figure 2-5. Formation and structures of melamine and its derivatives

Source: Tyan *et al*. (2009)

The details of melamine and itsderivatives are as follows: (WHO, 2009).

Cyanuric acid (1,3,5-triazine-2,4,6-triol, $C_3H_3N_3O_3$) is a derivative of melamine that occurs as a by-product in the melamine synthesis process. Cyanuric acid has been approved by the US Food and Drug Administration (US-FDA) for using as a non-protein nitrogen source or feed additive in ruminant (Karbiwnyk *et al*., 2009; Rovina and Siddiquee, 2015). In addition, sodium dichloroisocyanurate, a chlorinated derivative of cyanuric acid is widely used in swimming pools as a stabilizer for prevent unnecessary loss of chlorine (Patel and Jones, 2007).

Ammelide (6-amino-2,4-dihydroxy-1,3,5-triazine, $C_3H_4N_4O_2$) and ammeline (4,6-diamino-2-hydroxy-1,3,5-triazine, $C_3H_5N_5O$) are compounds in the monoamino and diaminooxytriazine groups. Ammeline, a derivative of melamine, is produced by reacting melamine with concentrated sulfuric acid (H_2SO_4) under high-thermal condition (190°C), or by the microbial degradation of melamine. Ammeline is used in the production of lubricants, however ammelide have no used data (WHO, 2009; Rovina and Siddiquee, 2015).

Structures and properties of melamine and its derivatives are given in Table 2-1.

Table 2-1. Structures and properties of melamine and its derivatives

Features and properties	Melamine	Cyanuric acid	Ammelide	Ammeline
Chemical formula	$C_3H_6N_6$	$C_3H_3N_3O_3$	$C_3H_4N_4O_2$	$C_3H_5N_5O$
Molecular weight (g mol^{-1})	126.12	129.07	128.09	127.10
Nitrogen content (% w/w)	66.6	32.6	43.7	55.1
Appearance	Fine white crystalline powder	White crystalline solid	White powder	White powder
Aqueous solubility (g L^{-1})	3.24	2.0	0.077	0.075
PK_a	5.35	6.88-13.5	-	9.65
PK_b	9.0	-	-	-
Log*P*	-0.17	0.61	0.35	-0.19

Source: WHO (2009); Baynes and Riviere (2010)

2.1.2 The importance of melamine as it relates to agriculture

During 1950-1960, Melamine is used in agriculture as a nitrogen-rich fertilizer for plants. However, melamine is more expensive than other nitrogen fertilizers such as urea. Furthermore, the nitrogen mineralization, a biological transformation of nitrogen to plant-available inorganic forms, of melamine is very slow. For this reason, melamine is not widely used as a fertilizer for plants (Hauck and Stephenson, 1964). Later in 1978, melamine is used as a source of non-protein nitrogen for livestock. However, some case study reported that melamine is slowly decayed and the final reaction in the animal is less completed than that of other nitrogen sources, such as urea (Colby and Mesler, 1958). Recently, melamine is not authorized by the US Food and Drug Administration (FDA) as a non-protein nitrogen source in animal feed (US-FDA, 2007; WHO, 2009).

2.1.3 Melamine production and marketing

Melamine business is expected to grow up every year. The main producers of melamine located in China and Europe such as Germany, Netherlands, Poland, Austria and Russia (Bizzari and Yokose, 2008). China is the largest market of melamine in terms of production and consumption, accounting for approximately 47% and 26% of global production and exports, respectively (MEEA, 2011; Xue *et al*., 2011a). As compared to countries in Europe and North America, the Chinese market for melamine production is more stable and less vulnerable to fluctuations, though recent slowdown of the Chinese economy may affect the local wood-based panel industry with its adhesive resins and laminates production sectors, which are considered the largest outlet for melamine application.

2.1.4 Report of melamine contamination in animal feed

According to the reports in 2007, outbreak of renal failure in large numbers of dogs and cats in the United States and Canada were linked to melamine contamination in feedstuffs which imported from China, including wheat gluten, rice protein, and corn gluten. Later, melamine contamination was also reported in wheat

gluten, corn meal, soybean meal, rice bran, rice protein concentrate, squid liver meal, swine feed, poultry feed and shrimp larval feed (US-FDA, 2007; Squadrone *et al*., 2010). In Thailand, Amornthewaphat (2009) reported the survey results of 41 feed mill companies, which were located in Bangkok, Pathum Thani, Ratchaburi, Samutsakorn, Samut Prakan, Chonburi, Saraburi, Lop Buri, Phetchaburi, Nakhon Pathom, Lamphun, Chiang Mai, Ubon Ratchathani and Songkhla. The survey results showed that 47.8 percent of feed mill companies had been detected melamine contaminated in feed ingredients at concentrations lower than limit of detection.

2.1.5 Methods for melamine examination in samples

Recently, the instruments and methods for melamine examination are developed for precise results. Major instruments and methods currently used for melamine determination in sample as follows: enzyme-linked immunosorbent assay (ELISA), spectrophotometric, GC (gas chromatography), HPLC (high performance liquid chromatography), LC (liquid chromatography), CE (capillary electrophoresis), UPLC (ultra performance liquid chromatography) and HILIC (hydrophilic interaction liquid chromatography).

Table 2-2 Summary of melamine and its derivatives contamination in feed samples.

Feed types	Sources	No. of sampling	Level of detection (ppm)			
			Melamine	Cyanuric acid	Ammelide	Ammeline
Swine feed	1	1	Nagative	NA	NA	NA
	2	6	Negative - 120	NA	NA	NA
	3	3	Negative – 83.4	6.6 - 22.5	Negative – 10.8	Negative – 43.2
	4	2	Nagative	NS	NS	NS
	5	1	Nagative	NS	NS	NS
	6	1	Positive	NS	NS	NS
Poultry feed	7	3	Nagative	Nagative	Nagative	Nagative
	8	14	Nagative	Nagative	Nagative	Nagative
	9	7	Nagative	2.11 – 2.63	13.9	Nagative
Fish feed	10	5	53 - 400	Nagative	2.46	Nagative

Positive = detected but unidentified; Nagative = undetected; NA = not analyzed; NS = not specified in the report

Source: US-FDA (2007)

Table 2-3 Instruments and methods for melamine analysis.

Methods	Samples	LOQ	LOD	Detection compound	References
ELISA	Wheat gluten,	10-250 ppm	10 pm	Melamine	ELISA
	Moist feed	2-50 ppm	2 ppm		AgraQuant® kit
	Dry feed	4-100 ppm	4 ppm		Romer Labs
	Dairy	2-50 and 10-250 ppm	2 and 10 ppm		
	Meat	0.01 ppm	0.05 ppm	Melamine	Wang *et al*. (2010)
spectrophotometric	Food, ingredients, dairy	-	-	Melamine	Rima *et al*. (2009)
	Dairy	0.04–3.5 ppm	0.04–3.5 ppm	Melamine	Liu *et al*. (2011)
GC-MS	Dairy product	0.001 ppm	0.0003 ppm	Melamine and its derivative	Li *et al*. (2009)
	Food, feed ingredients	10 ppm	2.38 ppm	Melamine	Squadrone *et al*. (2010)
	Egg	20 ppb	10 ppb	Melamine	Xia *et al*. (2009)

LOQ, Limit of Quantification: the minimum concentration that can be quantified.

LOD, Limit of Detection: the concentration or minimum weight that can be measured at a certain confidence level.

ELISA: enzyme-linked immunosorbent assay, GC/MS: gas chromatography–mass spectrometry.

Table 2-3 (cont.) Instruments and methods for melamine analysis.

Methods	Samples	LOQ	LOD	Detection compound	References
GC-MS-MS	Dairy	0.05-0.005 ppm	0.002 ppm	Melamine and its derivative	Miao *et al*. (2009)
	Milk powder	0.5 ppb	0.2 ppb	Melamine	Tzing and Ding (2010)
		1 ppb	0.5 ppb	Cyanuric acid	
UPLC-MS/MS	Egg	10 ppb	5 ppb	Melamine	Xia *et al*. (2009)
reversed phase HPLC	Dairy product	60 ppb	18 ppb	Melamine	Sun *et al*. (2010)
LC-EIS- MS/MS	Milk powder	0.5 ppm	0.1 ppm	Melamine	Ibáñez *et al*. (2009)
LC/MS-MS	Soil and plant	0.8-4.4 ppb	0.2-1.3 ppb	Melamine	Ge *et al*. (2011)
HILIC-UV	Dairy	0.003 ppm	0.005–32 ppm	Melamine	Zheng *et al*. (2012)

LOQ, Limit of Quantification: the minimum concentration that can be quantified.

LOD, Limit of Detection: the concentration or minimum weight that can be measured at a certain confidence level.

GC-MS/MS: gas chromatograph–mass spectrometer/mass spectrometer, UPLC-MS/MS: ultra performance liquid chromatography mass spectrometer/mass spectrometer, reversed phase HPLC: reversed phase high performance liquid chromatography, LC-ESI-MS/MS: liquid chromatography-electrospray ionization-tandem mass spectrometry, LC/MS-MS: liquid chromatograph–mass spectrometer/mass spectrometer, HILIC-UV: hydrophilic interaction chromatography-ultraviolet.

2.2 Toxicology, metabolism and mechanism of action of melamine in combination with or without cyanuric acid

2.2.1 Background

Sugita *et al*. (1990) cited by Buur *et al*. (2008) reported that melamine is a highly polar basic compound, which can be distributed through the various animal organs via water system. In addition, melamine has octanol: water partition coefficient (log_{Pow}) is -2.03 (log_{Pow} is the coefficient of dispersion of the substance in the water layer and the lipid layer). After ingestion, melamine is absorbed through the gastrointestinal tract into the bloodstream, but this substance is not metabolized in the liver (like other amino acids), and it is readily excreted as unchanged form by the kidneys (Smith *et al*., 1994).

2.2.2 The relationship between melamine and cyanuric acid in animals

Melamine or cyanuric acid singly is generally mild to moderately toxic for animal. However, the combination of melamine and cyanuric acid is highly toxic and forms a crystalline residue in the tissue by hydrogen bonding between melamine and cyanide. Melamine-cyanurate crystals are often found in the kidneys of animal, which are a major cause of kidney failure (Prior *et al*., 2013; Reimschuessel *et al*., 2010). Stine *et al*. (2011) reported that piglets received combination of melamine and cyanuric acid at doses of 0+0, 1+1, 3.3+3.3, 10+10, 33+33 and 100+100 mg kg^{-1} body weight per day. The results from this study indicated that melamine-cyanurate crystals were found in renal medulla and renal cortex in the renal tubule. However, the crystals were not detected in pig that receiving with melamine alone at a concentration of 200 mg kg^{-1} of body weight.

2.2.3 Cases study in aquatic animal

2.2.3.1 Scaleless fish (catfish)

Janlek *et al*. (2009) reported abnormalities of hybrid *Clarias* catfish receiving melamine in diet at the levels of 0.5-3 percent for 8 weeks. The results showed that, poor growth, abnormalities skin color (Figure 2-6) and histophatological change

were recorded in fish fed diets containing melamine. The levels of melamine in the diets had strongly correlated to melamine residues in fish flesh. Melamine residues were found between 63-450 mg kg^{-1} of sample in fish flesh after 8 weeks of feeding trial.

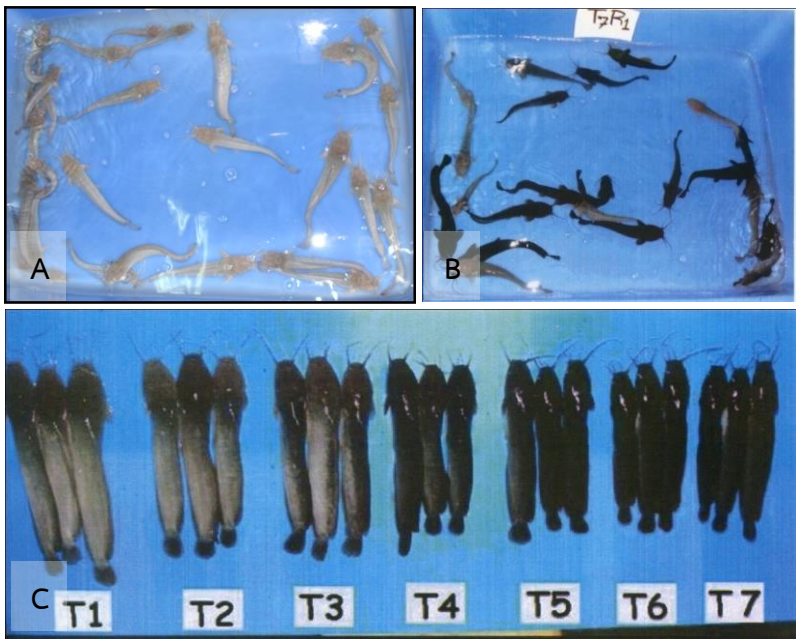

Figure 2-6. Abnormalities skin color of hybrid *Clarias* catfish fed with melamine contaminated feed. Fish fed control diet had a normal appearance (A: control) compared to those fed a diet containing melamine (B: 3 percent and (C): T2-T7, Melamine 0.5-3 percent, respectively)

Source: Janlek *et al*. (2009)

Xue *et al*. (2011b) studied the effects of melamine (MEL) on growth performance and skin color of darkbarbel catfish (*Pelteobagrus vachelli*). Experimental diet with 0.2, 0.5 and 1% MEL supplementation were given to juvenile darkbarbel catfish with an initial weight of 14.30±0.10 g for 8 weeks. The results showed that, specific growth rate (SGR) was significantly lower than that of the control group. Darkbarbel catfish fed the diets with 0.5 and 1% MEL showed the abnormal skin color, which significantly paler than control fish (Figure 2-7).

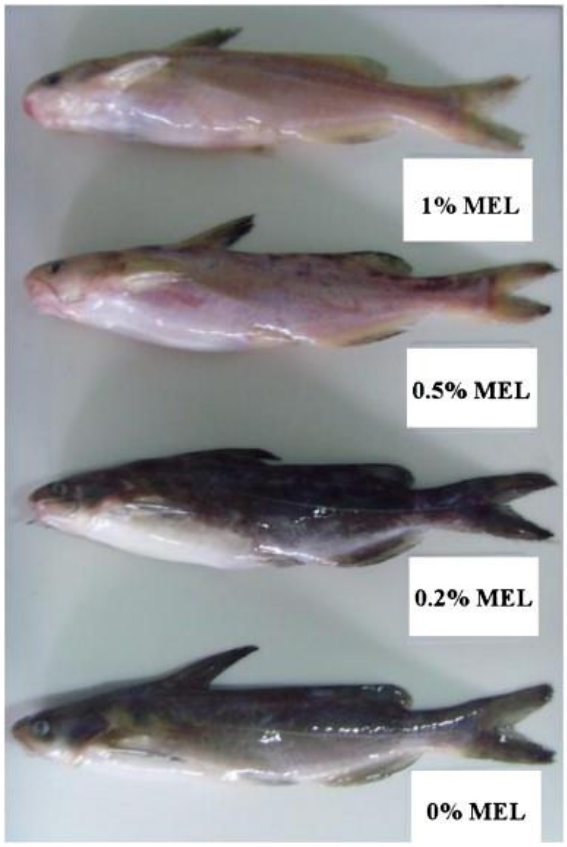

Figure 2-7. Abnormalities skin color of darkbarbel catfish fed with melamine contaminated feed.

Source: Xue *et al.* (2011b)

Pirarat *et al*. (2012) studied the effects of melamine and cyanuric acid in walking catfish*, Clarias batrachus* at concentrations of 5,000 and 20,000 mg kg^{-1} for melamine or cyanuric acid alone and at concentrations of 2,500 and 10,000 mg kg^{-1} for mixing. The results indicated that fish exposed to melamine and cyanuric acid developed darkening of the skin as early as 3 days post feeding (Figure 2-8). Inflammation of the kidney tissue as well as severe renal death and melamine-cyanurate were found in renale tubules of the kidney of fish that receiving only combined dosage of melamine and cyanuric acid (Figure 2-9). Alanine transaminase, aspartate transaminase, creatinine and uric acid, indicator of liver and kidney functions were

related to the crystal-associated granulomatous inflammation in the liver and kidney of treated fish.

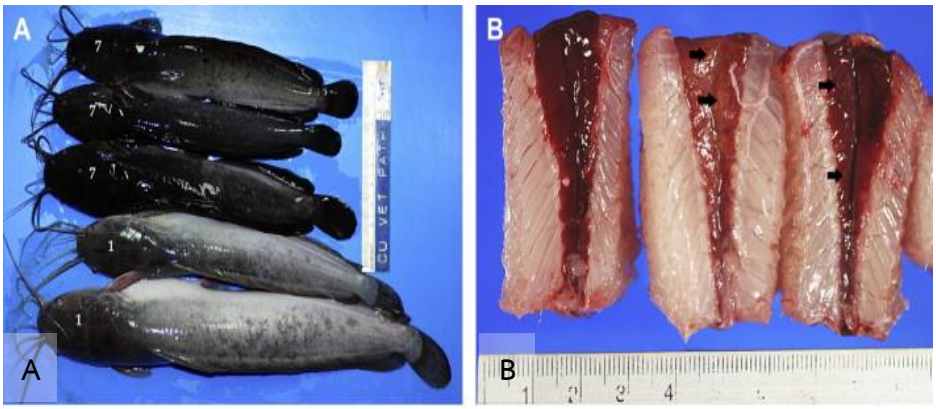

Figure 2-8. (A) Clinical signs of walking catfish after exposed melamine and cyanuric acid for 8 weeks. (B) Kidneys from fish fed on control diet (left side), 1% melamine + 1% cyanuric acid (centre) and 0.25% melamine + 0.25% cyanuric acid (right side).

Source: Pirarat *et al*. (2012)

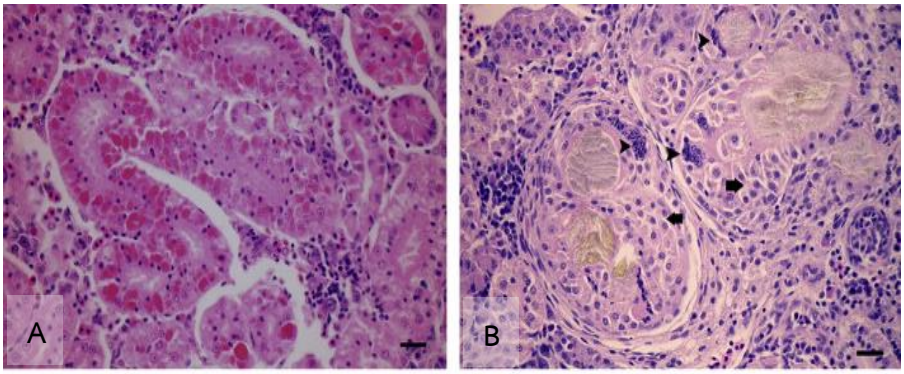

Figure 2-9. Histophatological changes in the kidney of walking catfish after exposed melamine and cyanuric acid for 8 weeks. (A) Kidney from fish fed with 2% melamine showing degeneration of the renal tubular epithelium. (B) Kidney from fish fed with 1% melamine + 1% cyanuric acid showing crystals with in degenerate or necrotic renal tubules.

Source: Pirarat *et al*. (2012)

2.2.3.2 Fin fish

Xue *et al*. (2011a) studied pharmacokinetic parameters for melamine alone or blend with cyanuric acid in rainbow trout (*Oncorhynchus mykiss*). The results from this study indicated that, half-life ($t_{1/2}$) of melamine in rainbow trout was 32.2–32.9 h. Withdrawal of cyanuric acid was much more rapid than that of MEL with much lower $t_{1/2}$ of 7.92 h

Phromkunthong *et al*. (2013) studied toxicity of melamine in red tilapia (*Oreochromis niloticus* (L.) × *O. mossambicus* (Peters)) in concentrations of 0.5-3%. The results indicated that, dietary melamine had an adverse effect on the growth, feeds efficiently and fish performance. Moreover, clinical signs such as fin erosion, anorexia, sluggish swimming behaviour, and abnormalities color of skin were found in this study (Figure 2-10). Melamine residue in the fish flesh had positively correlate to dietary melamine concentration in the diets. Histopathological alterations were evident in the kidney, liver and gills of fish exposed to melamine.

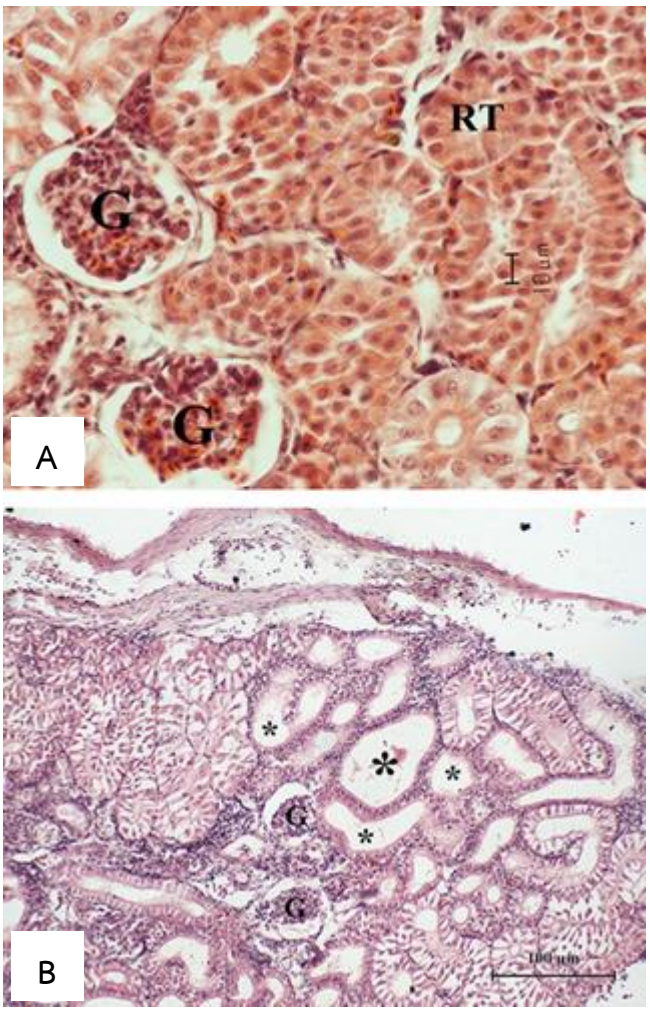

Figure 2-10. Photomicrographs of kidney in red tilapia which were exposed feeds containing different levels of melamine. (A) Normal appearance of renal tubules in control fish. (B) Enlargement of renal tubules (asterisk) occurred in the kidney of the fish fed with 30 g melamine kg^{-1} feed.

Source: Phromkunthong *et al*. (2013)

Phromkunthong *et al*. (2015) studied the adverse effects of diaetary melamine and cyanuric acid in red tilapia. The results indicated that, he renal tubules of fish ingested MEL-CYA combination had melamine–cyanurate crystals. Dietary melamine and cyanuric acid induced the activity of catalase in liver and the activity of glutathione peroxidase in liver and kidneys. The renal tubules of fish ingested MEL-CYA combination had melamine–cyanurate crystals.

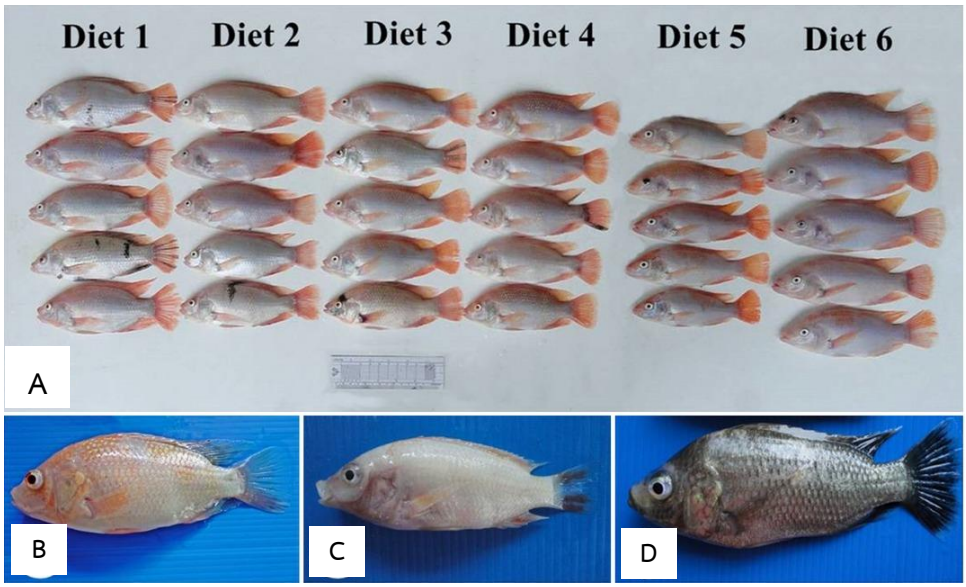

Figure 2-11. Changes in the external features of red tilapia which were exposed melamine and cyanuric acid feeds for 8 weeks. (A) T1, control fish received melamine-free feed, T2–T7, fish received feeds incorporated with melamine at 5, 10, 15, 20, 25 and 30 g kg^{-1} feed, respectively. (B) Normal appearance and body colour of fish fed on control feed. Fish that received melamine-containing feeds had deformities such as depigmentation (C) and darkening (D) of skin.

Source: Phromkunthong *et al*. (2015)

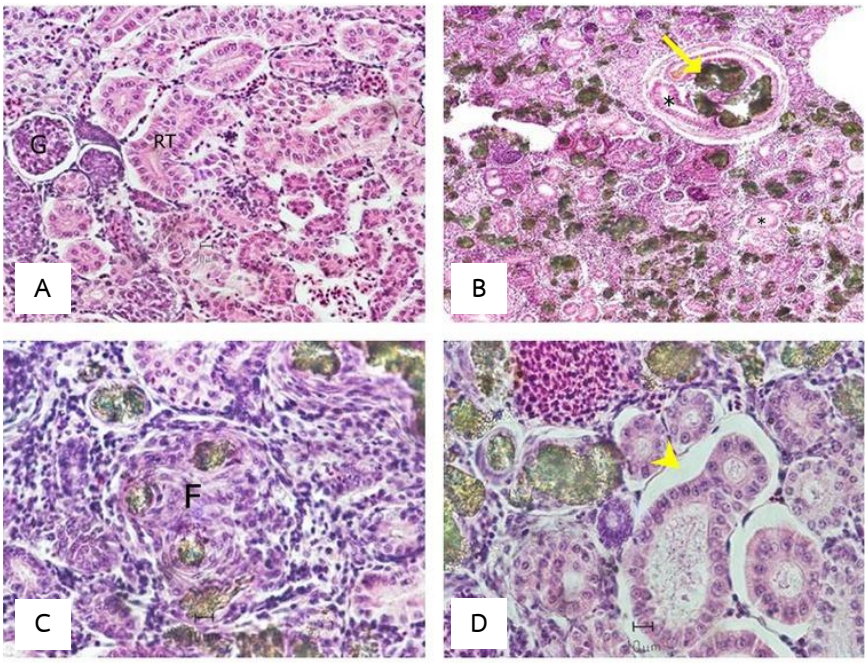

Figure 2-12. Photomicrographs of kidney of red tilapia. (A) Control group showing. (B) MEL-CYA combination groups (2.5+2.5) had crystals in renal tubule (yellow arrow). (C) renal tubules and tubular epithelial cells containing bundles of actin filaments (F) (400×) were detected in fish fed on combined MEL-CYA groups (2.5+2.5). (D) Large space between renal tubules and tissue (yellow arrowhead) (400×) was found in fish fed on combined MEL-CYA groups (7.5+7.5).

Source: Phromkunthong *et al*. (2015)

2.2.3.3 Crustacean

Limsuwan *et al*. (2009) reported abnormalities of white shrimp after received diet with melamine and cyanuric acid derived from feed ingredients. The feed contained melamine between 100-120 mg kg^{-1} and Cyanuric acid 80-100 mg kg^{-1}. The commercial diets for white shrimp that was produced from companies and free from melamine and cyanuric acid served as control. The results showed that, shrimp fed with melamine found abnormalities when observed with naked eye. The swelling of hepatopancreas was bigger than normal and found many crystals in the antennal gland.

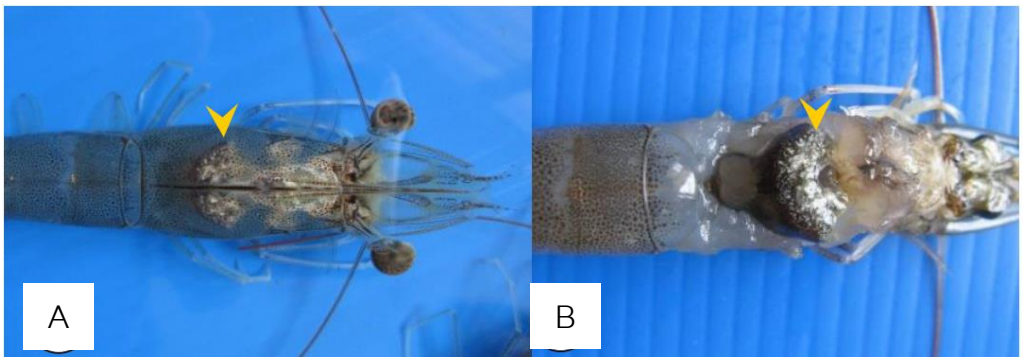

Figure 2-13. Water sac syndrome or big head syndrome were detected in shrimp exposed to MEL and CYA contamination (A) Whitish spots/patches (arrow) observed from shrimp after received melamine and cyanuric acid for 60 days. (B) White spots/patches on hepatopancreas after removing carapace (arrow).

Source: Limsuwan *et al*. (2009)

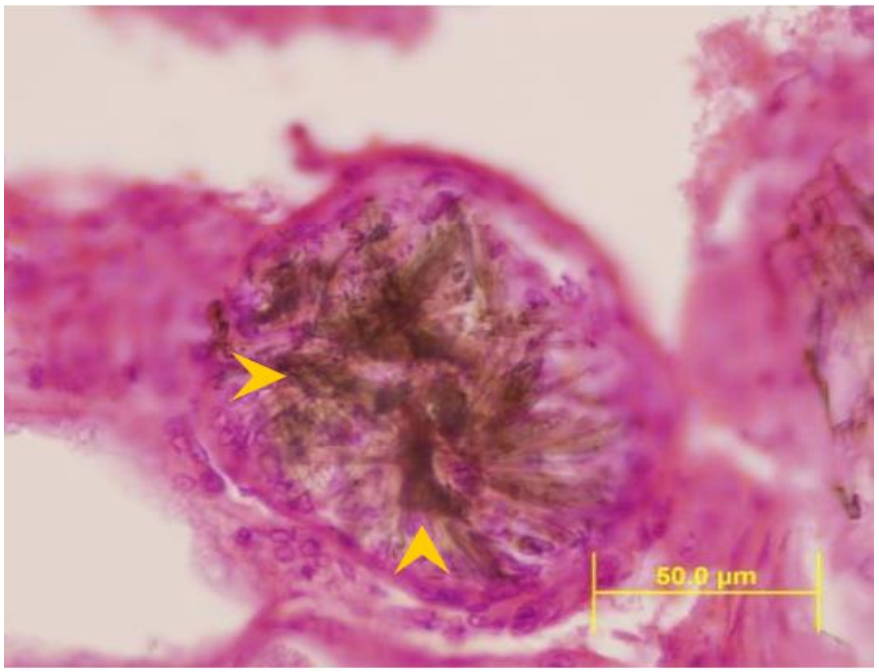

Figure 2-14. Higher magnification of the anternal gland showed fragmented dense melamine/cyanuric acid crystals (arrows) (H&E, bar = 50 µm)

Source: Limsuwan *et al*. (2009)

References

Amornthewaphat, N. 2009. Status of animal feed industries in Thailand. *In*: proceedings of 47th Kasetsart University Annual Conference: Animals, Kasetsart University, Bangkok, 17-20 March 2009.

Bann, B. and Miller, S.A. 1758. Melamines and derivatives of melamine. Chem. Rev. 58, 131-172.

Baynes, R.E. and Riviere, J.E. 2010. Risks associated with melamine and related triazine contamination of food. Emerg. Health Threats J. 3, 1-10.

Baynes, R.E., Smith, G., Mason, S.E., Barrett, E., Barlow, B.M. and Riviere, J.E. 2008. Pharmacokinetics of melamine in pigs following intravenous administration. Food Chem. Toxicol. 46, 1196-1200.

Bizzari, S. and Yokose, K. 2008. Melamine. *In*: Chemical Economics Handbook. Menlo Park, CA: SRI Consulting Incorporation.

Buur, J.L., Baynes, R.E. and Riviere, J.E. 2008. Estimating meat withdrawal times in pigs exposed to melamine contaminated feed using a physiologically based pharmacokinetic model. Regul. Toxicol. Pharmacol. 51, 324-331.

Chen, C., Yeh, C.W. and Chen, J.D. 2006. Syntheses, structures and thermal properties of two new copper (II) melamine complexes. Polyhedron 25, 1307-1312.

Colby, R.W. and Mesler, R.J.Jr. 1958. Ruminant feed compositions. U.S. Patent No. 2,819,968, 1958.

Gautney, J., Kim, Y.K. and Hatfield, J.D. 1982. Melamine: A regenerative SO_2 absorbent. J. Air Pollut. Control Assoc. 32, 260-265.

Ge, J., Zhao, L.W., Liu, C.Y., Jiang, S., Lee, P.W. and Liu, F. 2011. Rapid determination of melamine in soil and strawberry by liquid chromatography–tandem mass spectrometry. Food Control 22, 1629-1633.

Hau, A.K., Kwan, T.H. and Li, P.K. 2009. Melamine toxicity and the kidney. J. Am. Soc. Nephrol. 20, 245-250.

Hauck, R.D. and Stephenson, H.F. 1964. Fertilizer nitrogen sources, nitrification of triazine nitrogen. J. Agric. Food Chem. 12, 147-151.

IARC (International Agency for Research on Cancer). 1999. Some chemicals that cause tumours of the kidney, or urinary bladder in rodents and some other substances. IARC Monogr. Eval. Carcinogen. Risks. Hum. 73, 329-338.

Ibáñez, M., Sancho, J.V. and Hernández, F. 2009. Determination of melamine in milk-based products and other food and beverage products by ion-pair liquid chromatography–tandem mass spectrometry. Anal. Chim. Acta 649, 91-97.

Janlek, P., Boonyaratpalin, M. and Phromkunthong, W. 2009. Effect of melamine on growth performance, blood components and histological changes in hybrid catfish, *Clarias macrocephalus* (Gunther) × *Clarias gariepinus* (Burchell). Thai Fish. Gaz. 62, 331-340 (in Thai).

Karbiwnyk, C.M., Andersen, W.C., Turnipseed, S.B., Storey, J.M., Madson, M.R., Miller, K.E., Gieseker, C.M., Miller, R.A., Rummel, N.G. and Reimschuessel, R. 2009. Determination of cyanuric acid residues in catfish, trout, tilapia, salmon and shrimp by liquid chromatography-tandem mass spectrometry. Anal. Chim. Acta. 637, 101-111.

Li, J., Qi, H.Y. and Shi, Y.P. 2009. Determination of melamine residues in milk products by zirconia hollow fiber sorptive microextraction and gas chromatography–mass spectrometry. J. Chromatogr. 1216A, 5467-5471.

Limsuwan, C., Chuchird, N., Prasertsri, S., Wongmaneeprateep, S., Laisutisan, K., Wiriyapattanasub, P. and Limhang, K. 2009. Gross and histopathological changes of Pacific white shrimp (*Litopenaeus vannamei*) experimentaly induced with contaminated of melamine plus cyanuric acid in pelleted feed. In: Proceedings of 47th Kasetsart University Annual Conference: Fisheries, Kasetsart University, Bangkok, 17-20 March 2009.

Liu, Y., Deng, J., An, L., Liang, J., Chen, F. and Wang, H. 2011. Spectrophotometric determination of melamine in milk by rank annihilation factor analysis based on pH gradual change-UV spectral data. Food Chem. 126, 745-750.

MEEA (The Ministry of Energy & Energy Affairs). 2011. Downstream Gas Industry. Annual Report 2011. Port of Spain: MEEA.

Miao, H., Fan, S., Wu, Y.N., Zhang, L., Zhou, P.P., Li, J.G., Chen, H.J. and Zhao, Y.F. 2009. Simultaneous determination of melamine, ammelide, ammeline, and cyanuric acid in milk and milk products by gas chromatography-tandem mass Spectrometry. Biomed. Environ. Sci. 22, 87-94.

Nemil, G., Yildiz, S.and Gezer, E.D. 2005. Effects of melamine raw paper weight, varnish type and the structure of continuous pressed laminate (CPL) on the physical, mechanical properties and decay resistance of particleboard. Int. Biodeterior. Biodegrad. 56, 166-172.

NTP (National Toxicology Program). 1983. Carcinogenesis Bioassay of Melamine (CAS NO. 108-78-1) in F344/N Rats and B6C3F1 mice (feed study). Natl. Toxicol. Program Tech. Rep. Ser. 245, 1-171.

Patel, K. and Jones, K. 2007. Analytical method for the quantitative determination of cyanuric acid as the degradation product of sodium dichloroisocyanurate in urine by liquid chromatography mass spectrometry. J. Chromatogr. 853B, 860-863.

Phromkunthong, W., Choochuay, P., Kiron, V., Nuntapong, N. and Boonyaratpalin, M. 2015. Pathophysiological changes associated with dietary melamine and cyanuric acid toxicity in red tilapia. J. Fish Dis. 38, 161-173.

Phromkunthong, W., Nuntapong, N., Boonyaratpalin, M. and Kiron, V. 2013. Toxicity of melamine, an adulterant in fish feeds: experimental assessment of its effects on tilapia. J. Fish Dis. 36, 555-568.

Pirarat, N., Katagiri, T., Chansue, N., Ponpornpisit, A., Endo, M. and Maita, M. 2012. The pathological effects of melamine and cyanuric acid in the diet of walking catfish (*Clarius batrachus*). J. Comp. Pathol. 147, 259-266.

Prior, T.J., Armstrong, J. A., Benoit, D. and Marshall, K.L. 2013. The structure of the melamine-cyanuric acid co-crystal. Cryst. Eng. Comm. 15, 5838-5843.

Reimschuessel, R., Evans, E.R., Stine, C.B., Hasbrouck, N., Mayer, T.D., Nochetto, C. and Gieseker, C.M. 2010. Renal crystal formation after combined or sequential oral administration of melamine and cyanuric acid. Food Chem. Toxicol. 48, 2898-2906.

Rima, J., Abourida, M., Xu, T., Cho, K. and Kyriacos, S. 2009. New spectrophotometric method for the quantitative determination of melamine using Mannich reaction. J. Food Compos. Anal. 22, 689-693.

Rovina, K. and Siddiquee, S. 2015. A review of recent advances in melamine detection techniques. J. Food Compost. Anal. 43, 25-38.

Smith, J.L., Wishnok, J.S. and Deen, W.M. 1994. Metabolism and excretion of methyamines in rats. Toxicol. Appl. Pharmacol. 125, 296-308.

Squadrone, S., Ferro, G.L., Marchis, D., Mauro, C., Palmegiano, P., Amato, G., Poma Genin, E. and Abete, M.C. 2010. Determination of melamine in feed: Validation of a gas chromatography–mass spectrometry method according to 2004/882/CE regulation. Food Control 21, 714-718.

Stine, C.B., Reimschuessel, R., Gieseker, C.M., Evans, E.R., Mayer, T.D., Hasbrouck, N.R., Tall, E., Boehmer, J., da Costa, G.G. and Ward, J.L. 2011. A no observable adverse effects level (NOAEL) for pigs fed melamine and cyanuric acid. Regul. Toxicol. Pharmacol. 60, 363-372.

Sugita, T., Ishiwata, H. and Yoshihira, K. 1990. Release of formaldehyde and melamine from tableware made of melamine-formaldehyde resin. Food. Addit. Contam. 7, 21-27.

Sun, F., Ma, W., Xu, L., Zhu, Y., Liu, L. and Peng, C. 2010. Analytical methods and recent developments in the detection of melamine. Trends Anal. Chem. 29, 1239-1249.

Tyan, Y., Yang, M., Jong, S.B., Wang, C. and Shiea, J. 2009. Melamine contamination. Anal. Bioanal. Chem. 395, 729-733.

Tzing, S.H. and Ding, W.H. 2010. Determination of melamine and cyanuric acid in powdered milk using injection-port derivatization and gas chromatography–tandem mass spectrometry with furan chemical ionization. J. Chromatogr. 1217A, 6267-6273.

US-FDA (U.S. Food, Drug Administration). 2007. Interim Melamine and Analogues Safety/Risk Assessment Peer Review Report. United States Food and Drug Administration: Washington, DC.

Wang, Z., Ma, X., Zhang, L., Yang, W., Gong, L., He, P. and Li, Z. 2010. Screening and determination of melamine residues in tissue and body fluid samples. Anal. Chim. Acta 662, 69-75.

WHO (World Health Organization). 2009. Toxicological and health aspects of melamine and cyanuric acid: report of a WHO expert meeting in collaboration with FAO. WHO: Ottawa.

Xia, X., Ding, S., Li, X., Gong, X., Zhang, S., Jiang, H., Li, J. and Shen, J. 2009. Validation of a confirmatory method for the determination of melamine in egg by gas chromatography–mass spectrometry and ultra-performance liquid chromatography–tandem mass spectrometry. Anal. Chim. Acta 651, 196-200.

Xue, M., Qin, Y., Wang, J., Qiu, J., Wu, X., Zheng, Y. and Wang, Q. 2011a. Plasma pharmacokinetics of melamine and a blend of melamine and cyanuric acid in rainbow trout (*Oncorhynchus mykiss*). Regul. Toxicol. Pharmacol. 61, 93-97.

Xue, J., Ai, Q., Mai, K., Xu, W., Yang, Y. and Liufu, Z. 2011b. Effects of melamine on growth performance and skin color of darkbarbel catfish (*Pelteobagrus vachelli*). Aquaculture 320, 142-146.

Zhang, S. and Horrocks, A.R. 2003. A review of flame retardant polypropylene fibres. Prog. Polym. Sci. 28, 1517-1538.

Zheng, X.L., Yu, B.S., Li, K.X. and Dai, Y.N. 2012. Determination of melamine in dairy products by HILIC-UV with NH_2 column. Food Control 23, 245-250.

Zidar, J., Pavlin, M., Mihelic, I., Ogorelec, P., Plavec, J. and Mavri, J. 2013. Stability and reactivity of progressively methylolated melamine derivatives. Comput. Theor. Chem. 1006, 85-91.

CHAPTER 3

A study on growth, histopathology and oxidative stress in Asian seabass on diets with various loadings of melamine and cyanuric acid adulterants

3.1 Abstract

This study was conducted to investigate the effects of of melamine (MEL) and cyanuric acid (CYA) at different doses on growth, histopathology and oxidative stress of Asian seabass (*Lates calcarifer* Bloch). Diet 1 (without supplementation of MEL and CYA); diets 2-5 (with MEL and CYA at inclusion levels 2.5+2.5, 5+5, 7.5+7.5 and 10+10 g kg^{-1} diet); diet 6 (with only MEL at 10 g kg^{-1} diet); and diet 7 (with only CYA at 10 g kg^{-1} diet) were formulated. Results from this study indicated that average body weight, specific growth rate (SGR) and feed conversion ratio (FCR) in fish fed the diet with combined MEL-CYA: 7.5+7.5, 10+10 or MEL alone diets were significantly different to control group ($P<0.05$). Significant histological alterations of liver and kidney were observed in fish fed with combined MEL and CYA supplementation. Gold-brown melamine-cyanurate crystals were detected in the renal tubules of fish fed with combined MEL and CYA supplementation. Fish that were fed either one of the chemical did not have such crystals in the kidneys. MEL was highest in the fillets of fish fed MEL alone (10 g kg^{-1} diet). The expression of heat shock protein (Hsp) 70 in the liver presented a significant difference in fish supplemented with MEL/CYA, in combination or alone (diets 2-7) ($P<0.05$), whereas there were no significant differences ($P>0.05$) in the level of Hsp70 in the kidneys between the treatments and control group. The activities of catalase and glutathione peroxidase in liver and kidneys were significantly increased with dietary MEL-CYA supplementation at high doses.

3.2 Introduction

The toxicity of melamine (MEL) has been reported widely in humans and animals after an outbreak of renal failure in dogs and cats in North America in 2007

(Brown *et al*., 2007; Cianciolo *et al*., 2008; Reimschuessel and Puschner, 2010). Since MEL was detected in food and animal feeds, World Health Organization (WHO) and U.S. Food and Drug Administration (US-FDA) began assessing the risk and safety levels to comprehend the effects that ingestion of contaminated edible tissues can have on human health (Reimschuessel *et al*., 2010; US-FDA, 2007; WHO, 2008). Melamine (1,3,5-triazine-2,4,6-triamine) is a nitrogen rich molecule that is used to produce many consumer products, and it has a high about 67% content of nitrogen by molecular weight (Bischoff, 2011). Therefore, the addition of MEL in foodstuff can increase the apparent protein content, as feed protein concentration is traditionally measured by analysis of total nitrogen content (US-FDA, 2007). A toxicity study of MEL in hybrid catfish [*Clarias macrocephalus* (Günther) × *Clarias gariepinus* (Burchell)] showed the adverse effects of this toxic substance on growth and blood components, and histological alterations (Janlek *et al*., 2009). In addition, MEL also presented negative effects on growth and dorsal skin melanin content in darkbarbel catfish, *Pelteobagrus vachelli* (Richardson) (Xue *et al*., 2011). Phromkunthong *et al*. (2013) found that red tilapia ingesting MEL at high dosages had poor growth performance and feed efficiency. Furthermore, histopathological alterations were detected in the kidney, liver and gills of the melamine treated fish. The accumulation of MEL residues in fish reflected its dietary levels.

An incident of melamine and cyanuric acid (CYA) contamination has been found in feed ingredients (Karbiwnyk *et al*., 2012; Stine *et al*., 2012). Data on toxicity of this combination in aquatic animals are particularly scarce. Even though MEL and CYA at low concentrations are known to be relatively nontoxic when taken individually, some recent research indicates the potential of toxic interaction for the MEL and CYA combination (Pirarat *et al*., 2012; Puschner *et al*., 2007; Reimschuessel *et al*., 2008). Several studies of renal failure reported in pets and farm animals, including aquatic species and ruminants that received MEL and CYA at high dosages, found crystals in the kidneys, similar to the pathological signs of renal failure (Brown *et al*., 2007; Nilubol *et al*., 2009; Puschner *et al*., 2007; Reimschuessel *et al*., 2008; Sun *et al*.,

2010; Thompson *et al*., 2008). To understand the effects of MEL and its derivative (CYA) more clearly, Phromkunthong *et al*. (2015) studied the effects of MEL or CYA and MEL-CYA combination on tilapia, through histopathological observations and using stress biomarkers. They also found that the combination of MEL and CYA induces crystal nephropathy. The purpose of our current study was to assess the toxicity of MEL and CYA alone and in combination when fed to Asian seabass, to describe its effects on pathophysiological changes or stress biomarkers, and to evaluate the bioaccumulation of the toxic chemicals in edible tissues to assess the safety of Asian seabass as food. Because the protein content of animal feed affects its price, there is incentive to add MEL or CYA to the feed. However, singly or in combination these could either harm the animals, or become a food safety problem. The food safety risk is highest with animals that are farmed and significantly consumed as food. Each such species individually provides a pathway and poses a risk, with varying characteristics in animal health effects and in transmission to humans. For food safety, these different pathways should be individually assessed. Ideally we would like to find a species that will not transmit, either because it is sensitive to the contamination, or because the profile of accumulation prevents it. Alternatively, we need to know if some species are a particularly high food safety risk. This paper is the second in a series that assesses such pathways in the aquaculture of Thailand. The first one assessed tilapia, while this one focuses on seabass. The results show significant differences between these two species in their transmission characteristics.

3.3 Materials and methods

3.3.1 Experimental set-up and fish

Healthy fingerling of Asian seabass, *Lates calcarifer* Bloch (4.77±0.25 g, mean±SD) were obtained from the National Institute of Coastal Aquaculture (NICA), Songkhla, Thailand and maintained in dechlorinated freshwater in a 1,000 L fiberglass tank at the Kidchakan Supamattaya Aquatic Animal Health Research Center (KS-AAHRC), Department of Aquatic Science, Faculty of Natural Resources, Prince of Songkla University, Songkhla, Thailand for 4 weeks. Before initiating the experiment, all

the fish were fed until satiation with MEL/CYA non-supplemented diet (diet 1, see Table 1) during the acclimatization period. A total of 504 fish with average weight of 8.62±0.01 g (mean±SD) were randomly allocated to 28 glass aquaria (184 L) with 18 fish per aquaria. Each experimental diet was hand-fed 3 times a day at 8:00, 12:00 and 16:00 h to visual satiation. Feces and uneaten feed of each tank was siphoned daily, which was replaced by dechlorinated freshwater from a storage concrete pond. During the experiment, the ranges of water temperature was 27-28°C, pH was 7.5-7.7, and the dissolved oxygen was higher than 6.0 mg L^{-1}. All the previous water quality parameters are within the acceptable range for seabass rearing as recommended in Thai agricultural commodity and food standard TACFS 7412-2007 (2007).

3.3.2 Preparation of diets

MEL (99.5% purity) used in this study was supplied by Chang Chun Petrochemical Co. Ltd., Taiwan. CYA (98 % purity) was obtained from Sigma-Aldrich (St Louis, MO, USA). The seven experimental diets were: control diet (without supplementation of MEL or CYA), and in the other diets the MEL-CYA supplementations (in g kg^{-1} feed) were 2.5+2.5, 5+5, 7.5+7.5, 10+10, 10+0 and 0+10. Fishmeal, soybean meal and shrimp head meal were used as the protein source (Table 3-1). The 3 mm diameter pellets were prepared at KS-AAHRC using a Hobart mixer (Model A200T, USA) and dried for 4 h in a hot air oven at 60°C, and stored at -20 °C until used. All experimental diets were analyzed in triplicate for proximate composition at KS-AAHRC using the procedures of AOAC (1995) (Table 3-2). Nitrogen content (N x 6.25) was determined by Kjeldahl method using Kjeltec protein analyzer (Kjeltec™ 8100, FOSS, Tecator, Sweden). Crude lipid was determined using Soxhlet method (Soxtec System HT1043, Sweden). Ash was was determined by muffle furnace (Gallenkamp Box Furnace, UK) at 550 °C for 6 h. Moisture was determined by drying the samples at 105°C until constant weight. MEL and CYA content in the diets were determined by LC-MS/MS (Central Laboratory (Thailand) Co Ltd., Bangkok, Thailand) using the standard

methods of US-FDA LIB No.4422 with HILIC chromatography (Smoker & Krynitsky, 2008).

3.3.3 Sampling and growth monitoring

Asian seabass were individually weighed at the start for all treatments. During the 12-week feeding period, fish were manually fed to apparent satiation three times daily at 08:00, 12:00 and 16:00 h. Daily feed intake for each group was recorded fortnightly. At the end of the 12-week period, the fish were starved for 12 h and then individually weighed after anesthetizing with eugenol (1:20,000) (Index-education Co Ltd., Thailand). The specific growth rate (SGR) **and** feed conversion ratio (FCR) were calculated for each tank according to the following formulae:

Specific growth rate (SGR) (% day^{-1})

= 100 [Ln(Mean final body weight, g) - Ln(Mean initial body weight, g)] / /duration (84 days)

Feed conversion ratio (FCR)

= dry feed intake (g) / [final biomass (g)-initial biomass (g)+biomass of the dead fish (g)]

3.3.4 Samples for proximate composition, MEL/CYA content of fish

Initial samples of 10 fish were collected and stored at -20°C, until determining the whole body proximate compositions. At the end of the feeding trial, twelve fish from each treatment group (3 fish per replicate tank) were randomly collected for whole body proximate analysis. Twenty fish from each treatment group (5 fish per replicate tank) were sampled to determine MEL and CYA contents in fillet. The fillets of were rinsed with distilled water, dried in an oven at 60°C for 72 h and homogenizing the sample with a mortar and pestle. The ground samples were placed in polyethylene bags and stored in a freezer at -20 °C until analyzed.

Table 3-1. Formulations and proximate compositions of the experimental diets.

Ingredients composition (g kg^{-1} diet)	Diets (MEL-CYA, g kg^{-1} diet)						
	Diet 1 (Control)	Diet 2 (2.5+2.5)	Diet 3 (5+5)	Diet 4 (7.5+7.5)	Diet 5 (10+10)	Diet 6 (10+0)	Diet 7 (0+10)
Fishmeal	380	380	380	380	380	380	380
Soybean de-hulled	110	110	110	110	110	110	110
Shrimp head meal	100	100	100	100	100	100	100
Wheat flour	190	190	190	190	190	190	190
Rice flour	89.8	89.8	89.8	89.8	89.8	89.8	89.8
Wheat gluten	37	37	37	37	37	37	37
Vitamin & Mineral premix[1]	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Fish oil : soybean oil (1:1)	60	60	60	60	60	60	60
Mono-calcium phosphate	10	10	10	10	10	10	10
Choline chloride	1	1	1	1	1	1	1
Inositol	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Antioxidant (BHT)	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Microcrystalline Cellulose	20	15	10	5	0	10	10
Melamine (MEL)[2]	0	2.5	5	7.5	10	10	0
Cyanuric acid (CYA)[3]	0	2.5	5	7.5	10	0	10

[1] Vitamin & Mineral premix deliver the following in unit kg^{-1} diet: Retinal (A) 8,000 IU; Cholecalciferol (D3) 1,500 IU; Tocopherol (E) 100 mg; Menadione sodium bisulfite (K3) 5 mg; Thiamine (B1) 10 mg; Riboflavin (B2) 15 mg; Pyridoxine (B6) 15 mg; Cobalamin (B12) 0.02 mg; Niacin 80 mg; Calcium pantothenate 40 mg; Ascorbic acid (C) 150 mg; Biotin 0.5 mg; Folic acid 4 mg; Cu 5 mg; Fe 30 mg; Zn 40 mg; Mn 25 mg; Co 0.05 mg; I 1 mg; Se 0.25 mg.

[2] Melamine : Chang Chun Petrochemical CO.,LTD, Taipei Taiwan (purity 99.5%).

[3] Cyanuric acid (98%) : Sigma, ALDRICH CO., LTD.

Table 3-2. Proximate compositionof the experimental diets. (g kg^{-1} as dry matter basis, analyses of three batches of diet given as mean±SD)

Ingredients (g kg^{-1} diet)	Diets (MEL+CYA, g kg^{-1} diet)						
	Diet 1 (Control)	Diet 2 (2.5+2.5)	Diet 3 (5+5)	Diet 4 (7.5+7.5)	Diet 5 (10+10)	Diet 6 (10+0)	Diet 7 (0+10)
Nitrogen content (N×6.25)	407.75±1.03	434.16±2.13	442.01±1.03	449.44±1.03	472.63±4.38	446.62±3.06	435.29±0.33
Crude lipid	120.62±1.09	122.62±6.05	115.07±0.96	109.29±0.13	118.10±1.14	107.82±2.20	116.72±0.16
Crude ash	128.84±2.62	133.14±1.92	138.07±5.59	130.67±3.76	122.19±0.54	131.22±0.64	134.90±2.54
Gross energy (MJ kg^{-1} diet)	16.37	16.37	16.37	16.37	16.37	16.37	16.37
MEL content[1]	0	2.3	4.5	7.3	9.7	10.1	0
CYA content[1]	0	2.2	4.2	7.1	9.5	0	9.6

[1] LC-MS/MS using a modified method of US-FDA LIB No. 4422 with HILIC chromatography (Smoker and Krynitsky, 2008)

3.3.5 Histological study

Twelve fish from each treatment group (3 fish from each replicate tank) were sampled for the histological examination. At this dissection, liver and kidney samples were fixed in Bouin's fixative solution. After 24 h, the tissue samples were dehydrated in graded ethanol series, and embedded in paraffin according to standard methods. Transverse sections (3 μm) were prepared using sliding microtome, mounted onto slides and later were stained with hematoxylin and eosin (H&E) (Bancroft, 1967). Stained sections were examined with an Olympus PROVIS AX-70 microscope (Olympus Corporation, Japan) and images were captured with an Olympus DP71 digital camera (Olympus Corporation, Japan).

3.3.6 Determination of stress enzyme and Hsp70 in liver and kidneys

3.3.6.1 Sample preparation

Eight fish per treatment were sacrified with a lethal overdose of eugenol. Livers and kidneys were immediately dissected on an ice-chilled plate and then all the samples were kept in liquid nitrogen for further analysis. Each organ was individually weighted and homogenized in chilled acetate buffer, pH 7.5, plus 20% protease inhibitor (Amresco, USA). The homogenates were centrifuged at 12,879 × *g* for 10 min at 4°C and the supernatants were collected and used to determine the enzyme activity and Hsp70 level. Soluble protein concentration of supernatant in each sample was determined by the Bradford method (Bradford, 1976) with bovine serum albumin (Sigma, USA) as the standard.

3.3.6.2 Determination of Hsp70

The supernatant of each sample (as mentioned above) was gently mixed with SDS loading dye and boiled at 95°C for 5 min. After cooling in an ice bath, the total protein concentration of each sample was determined following the method of Bradford (1976). The mixture was loaded on a 12% polyacrylamide gel at a constant total protein concentration of 60 μg per lane, following the modified method of Köhler *et al*. (2009). Separated proteins were transferred to nitrocellulose membrane (Santa Cruz Biotechnology, USA) using semi-dry blotting (Thermo Scientific, USA). The membrane

was then incubated with 50% horse serum in TBS (50 mM Tris pH 7.5, 150 mM NaCl) for blocking non-specific protein binding for 1 h, and then washed in TBS for 5 min. The membrane was incubated overnight with diluted primary antibody against Hsp70 (mouse anti-human IgG, Thermo Scientific, USA). After washing in TBS for 5 min, the membrane was incubated with peroxidase coupled secondary antibody (goat anti-mouse IgG, Dianova, Germany) for 2 h. After repeated washing for 5 min in TBS, 4-chloro-1-naphthol was used to visualize the antibody-detected protein bands. Finally, the intensities of the Hsp70 bands were determined by image analysis using ImageJ software (NIH, Bethesda, MD).

3.3.6.3 Enzymes assays

For determination of catalase (CAT) and glutathione peroxidase (GPx) activities were measured using commercial assay kits (Cayman, USA) with absorbance readings performed in a microplate reader according to the manufacturer's protocol. The specific enzyme activity was calculated and expressed as U mg protein^{-1}.

3.3.7 Determination of serum biochemistry

At the end of feeding trial, the blood sample from eight fish per treatment were randomly sampled by caudal venipuncture, and each blood sample was divided into two portions. The first blood portion was transfered into sterilized micro-centrifuge tube containning 1% ethylenediaminetetraacetic acid (EDTA) as an anticoagulant in order to determine red blood cells, white blood cells, hematocrit (Blaxhall and Daisley, 1973) and hemoglobin concentration (Cyanmet-haemoglobin) (Larsen and Snieszko, 1961). The second blood portion was centrifuged at 2,236 × *g,* for 10 min, at 4°C, and the serum was collected and stored at -80 °C for analysis of blood urea nitrogen (BUN) and serum electrolytes (Na^+, Cl^- and K^+) using a Beckman CX-3 delta automatic analyzer (Beckman Coulter, Inc., CA, USA) following the method as described by Chayanunnukul *et al*. (2007). Serum lysozyme activity was determined based on turbidimetric measurement of the lysozyme-sensitive gram-positive bacterium *Micrococcus lysodeikticus* (Sigma, USA) folowing the method as described by Demers and Bayne (1997). Serum protein content was determined by the method of Bradford (1976).

3.3.8 Statistical analysis

Statistical analyses were performed using SPSS version 11.5 for Windows. The data was tested for normality by Shapiro-Wilks test and homogeneity of variance was tested by Levene's *F* test. Independent-samples t-test was conducted to compare significant differences between the treatment group means and the control group, followed by Duncan's multiple range test. Differences were considered statistically significant at $P<0.05$.

3.4 Results

3.4.1 Growth and feed efficiency

The results of growth performance and feed efficiency are presented in Table 3-3. At the end of the 12 weeks feeding trial, the average body weight and specific growth rates (SGR) of fish fed combination of MEL/CYA diets (diets 2-5) or MEL alone (diet 6) were significantly diferences than that of fish fed the control diet ($P<0.05$). Feed conversion ratio (FCR) were significantly diferences in fish fed diets containing 7.5+7.5 and 10+10 g kg^{-1} diet MEL-CYA (diets 4 and 5, respectively) or MEL alone at 10+0 g kg^{-1} diet (diet 6) ($P<0.05$). No significant difference was observed in the survival rates between the actual treatment groups and the control group ($P>0.05$; Table 3-3).

3.4.2 Proximate composition and melamine and cyanuric acid content in fillet

Results of the whole-body proximate compositions of fish are presented in Table 3-4. The dry matter content of fish fed diets supplemented with 7.5+7.5 or 10+10 g kg^{-1} MEL-CYA (diets 4 and 5, respectively), or MEL or CYA alone (diets 6 and 7), was significantly differences than those of fish fed control diet (diets 1) ($P<0.05$). Significant differences were found in the nitrogen content of fish fed diets supplemented with 10+10 g kg^{-1} MEL-CYA (diets 5) and MEL alone (diets 6) compared with fish fed control diet (diet 1). Fish that received diets with MEL-CYA (diets 2-7) had a significantly lower lipid content than those of fish fed control diet ($P<0.05$). There was no significant differences in ash contents of whole body among all treatments ($P>0.05$). The nitrogen content in the fish fillet was the highest in fish fed diets supplemented with 10+10 g kg^{-1}

MEL-CYA (diets 5) ($P<0.05$). The melamine concentration in fish fillet was the highest in fish fed MEL alone (diet 6) (Table 3-4). CYA was not detected in the fillet of fish from any of the actual treatments (Table 3-4).

Table 3-3. Growth, feed efficiency and survival of Asian seabass on diets with various levels of MEL and CYA over 12 weeks (Values are given as mean±SD; n = 4 replicate tanks per dietary treatment; each replicate had 18 fish)

Parameters	Diets (MEL-CYA, g kg $^{-1}$diet)						
	Diet 1 (Control)	Diet 2 (2.5+2.5)	Diet 3 (5+5)	Diet 4 (7.5+7.5)	Diet 5 (10+10)	Diet 6 (10+0)	Diet 7 (0+10)
Survival (% cumulative)							
Week 0-12	98.61±2.78 (71/72)	93.06±6.99 (67/72)	91.67±7.17 (66/72)	95.83±8.33 (69/72)	90.28±13.13 (65/72)	97.22±5.56 (70/72)	93.06±5.32 (67/72)
Average body weight (g fish^{-1})							
Week 0	8.62±0.01	8.62±0.00	8.62±0.00	8.62±0.01	8.62±0.01	8.62±0.02	8.62±0.00
Week 12	86.35±5.31	76.52±5.62*	76.38±4.35*	73.51±4.13*	75.69±0.89*	36.12±4.10*	84.48±1.84
Specific growth rate, SGR (% day^{-1})							
Week 0-12	2.74±0.07	2.60±0.09*	2.60±0.07*	2.55±0.07*	2.59±0.02*	1.70±0.14*	2.72±0.03
Feed conversion ratio, FCR							
Week 0-12	1.22±0.03	1.29±0.10	1.26±0.08	1.30±0.05*	1.35±0.02*	1.57±0.12*	1.20±0.06
Estimated MEL and/or CYA dose over the exposure period (mg kg-1BWday^{-1})							
Week 0-12	-	77.22	138.14	212.27	292.91	242.75	316.08

Statistically significant differences (P<0.05) between a particular treatment group and control.

Survival count / initial count is given in parentheses for each treatment.

Table 3-4. Proximate composition of whole fish, protein content in fillet, and melamine and cyanuric acid residues in fillet of Asian seabass on diets with various levels of MEL and CYA over 12 weeks (on dry matter basis)

Parameters	Diets (MEL-CYA, g kg^{-1} diet)						
	Diet 1 (Control)	Diet 2 (2.5+2.5)	Diet 3 (5+5)	Diet 4 (7.5+7.5)	Diet 5 (10+10)	Diet 6 (10+0)	Diet 7 (0+10)
Whole fish (g kg^{-1}) (mean±SD; n=3 fish from each of the 4 replicate tanks)							
Dry matter	241.0±1.01	231.6±1.02	231.2±157	224.8±0.50*	220.7±1.21*	209.8±2.30*	223.1±0.76*
Nitrogen content	654.9±0.30	666.0±1.59	669.0±1.76	663.1±2.57	672.6±1.01*	666.5±0.38*	661.4±2.14
Lipid	186.0±0.59	145.0±2.14*	142.2±0.65*	137.2±1.26*	133.0±1.35*	130.1±0.87*	129.4±0.63*
Ash	164.1±2.64	174.7±1.17	161.4±1.28	195.0±0.60	186.3±0.13	165.2±0.90	181.9±2.98
Fillet (g kg^{-1}) (mean±SD; n=3 fish from each of the 4 replicate tanks)							
Nitrogen content	800.2±1.07	823.4±0.64	840.9±1.24	852.6±2.01	861.8±0.10*	843.1±1.32	821.9±0.51
Melamine residue (mg kg^{-1}) (pooled sample; n = 20 fish from each treatment)							
Fillet	nd	27.96	15.47	13.54	10.40	114.33	nd
Cyanuric acid residue (mg kg^{-1}) (pooled sample; n = 20 fish from each treatment)							
Fillet	nd	nd	nd	nd	nd	nd	nd

Asterisks indicate statistically significant differences ($P<0.05$) between an actual treatment group and the control group

nd = non detectable

3.4.3 Histopathology

The trunk kidney of fish fed on the control diet (dier 1) showed normal appearance with reddish-brown color (Figure 3-1a), while the trunk kidney of fish fed combination of MEL/CYA diets (diet 2-5) exhibited to yellow-green (Figure 1b). Observations under light microscopy of wet mount slides presented the fish that fed combination of MEL/CYA diets (diets 2-5) had golden-brown crystal spherulites in kidney tissue (Figure 3-1b, bottom right inset). In addition, golden-brown crystal and discoloration of trunk kidney were not found in kidneys of fish fed diets containing MEL alone (diet 6) or CYA alone (diet 7) (Figure 3-1c, 3-1d, respectively).

Histological examination of trunk kidney in fish fed the control diet (diet 1) exhibited normal renal tubules and and well defined glomeruli in the renal hematopoietic tissue (Figure 3-2a). Histopathology recorded for the kidneys of fish fed on MEL-CYA combination diets exhibited severity correlated with exposure concentration. Significant histopathological alterations, including tubular dilation, Irregular golden-brown or needle-like shape crystals were found in several areas throughout the affected kidneys from 48 fishes (100% of fish cases) that received diets containing a MEL-CYA combination (diets 2–5) (Figure 3-2b). From a subjective evaluation of the photomicrographs, even though the number of crystals was not counted, the amount of crystals in a given area of the kidney increased with increasing exposure concentration. Furthermore, actin-filament-bundle formation in renal tubular epithelial cells and deformation of renal tubules were detected. In severe cases, golden-brown crystals were detected within renal tubular degeneration (Figure 3-2c). Enlarged renal tubules were also detected in the kidneys from fish that received diets containing a MEL-CYA combination (diets 2–5) (Figure 3-2d). Multifocal melano-macrophages were detected in fish fed diets containing MEL alone (diet 6) (Figure 3-2e) or CYA alone (diet 7) (Figure 3-2f). Most kidneys of fish fed on CYA alone had spaces between renal tubules and tissues, and the tubular architecture and epithelium throughout the proximal and distal convoluted tubules were affected by CYA. It was noted that no golden-brown crystals were detected in the kidneys of fish fed with MEL alone (diet 6) or CYA alone (diet 7).

The liver tissues of Asian seabass fed the control diet had normal cellular structure with ordinary spherical nuclei. Furthermore, cell boundaries of hepatocytes from this treatment group were noticeable (Figure 3-3: a). However, the livers of fish received diets containing a MEL-CYA combination (diets 2-5) presented cell necrosis characterized by increased cytoplasmic eosinophilia and formation of pyknotic nuclei of some hepatocytes. With these treatments, regular shaped nuclei located in the central region of the hepatocytes were detected along with small and pycnotic ones. In addition, focal necrosis and cellular vacuolization were found side by side with normal hepatocytes (Figure 3-3: c and Figure 3-3: d). The dramatic change in the liver of fish fed the diet containing MEL-CYA 10+0 g kg^{-1} was vacuolization of the hepatocytes which almost covered the inspected area (Figure 3-3: e). Similar to the histopathological signs which noted in fish fed diets containing a MEL-CYA combination (diets 2-5), pyknotic nuclei and increased cytoplasmic eosinophilia of some hepatocytes were detected in the fish fed diet with CYA alone (diet 7) (Figure 3-3: f).

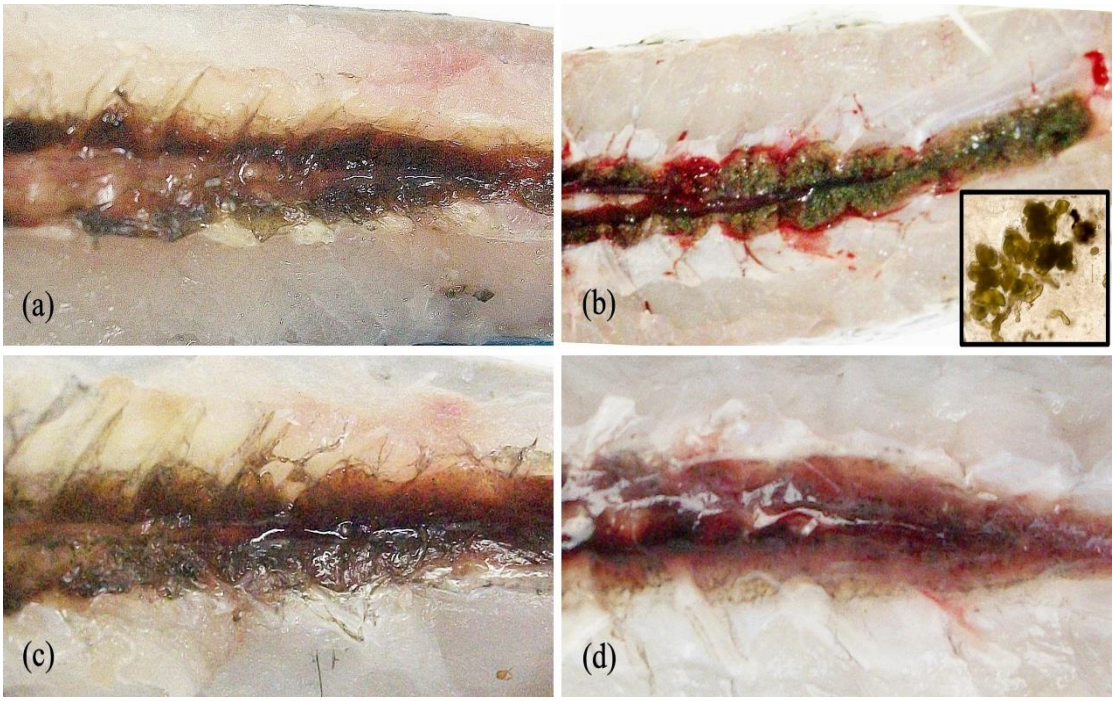

Figure 3-1. Adverse effects of dietary MEL and/or CYA appeared in the kidneys of seabass that ingested the toxic substances. (a) Kidney of a fish fed on the control diet had normal red color. (b) The kidney color changed to yellow-green with exposure to combined MEL and CYA (10+10 g kg^{-1} diet). The bottom right inset in Figure 1(b) shows gold-brown crystal spherulites after combined MEL-CYA treatment, observed with light microscopy. ((c), (d)) Kidneys of fish fed on diets 6 and 7 that contained MEL alone (c) or CYA alone (d) show normal characteristics without discoloration.

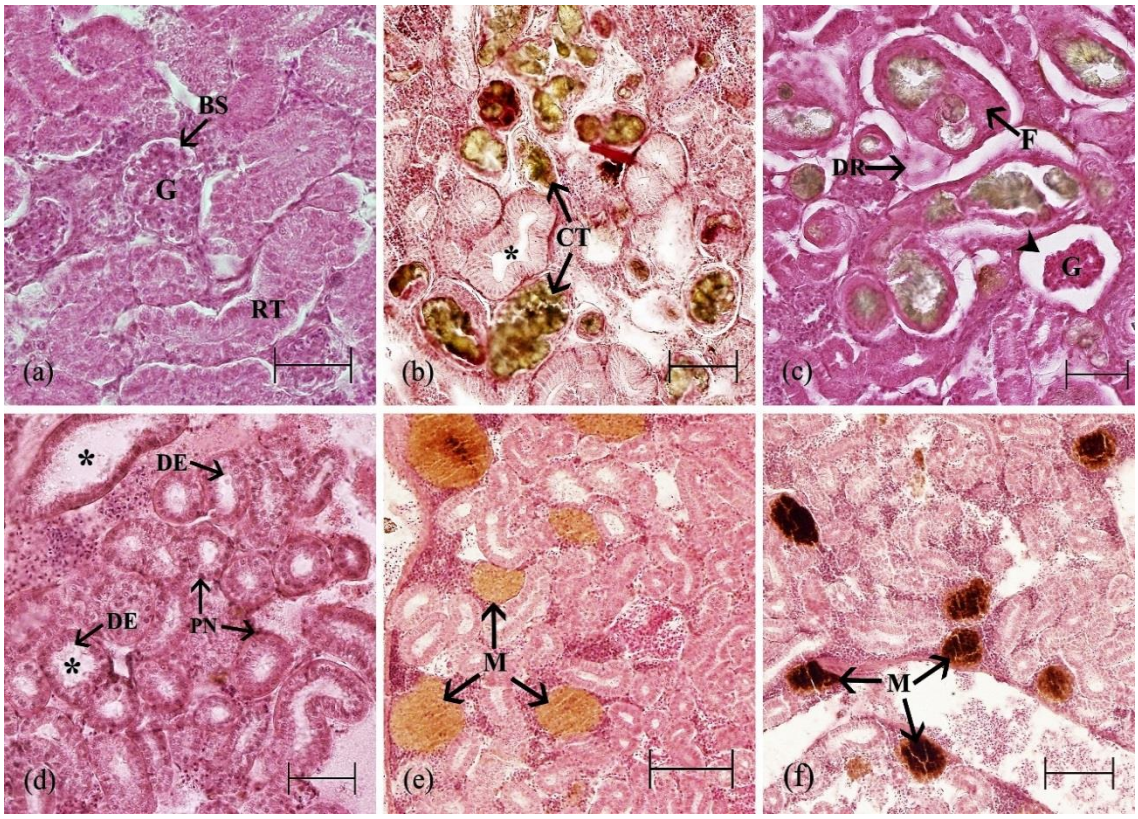

Figure 3-2. Photomicrographs showing pathologies of kidneys in Asian seabass on diets containing various levels of MEL and/or CYA. (a) Normal appearance of renal tubules (RT), glomerulus (G) and Bowman's space (BS) in the control group (400×; Scale bar = 50 μm). (b) Trunk kidney from fish fed on a MEL-CYA combination (10+10 g kg^{-1} diet) showing irregular golden-brown crystals (CT) in the renal tubules and presenting tubular dilation (asterisk) (200×; Scale bar = 100 μm). (c) Tubular epithelial cells containing bundles of actin filaments (F) were detected in fish fed on combination of MEL-CYA. In this severe case the kidney shows crystals, degenerated renal tubules (DR) and shrinkage of glomerulus (G) with expansion of the cavity (arrowhead) (400×; Scale bar = 50 μm). (d) An enlargement of the renal tubules and the tubular dilation (asterisk) including degenerated tubular epithelium (DE) and pyknotic nuclei (PN) in the kidney of fish receiving MEL alone (400×; Scale bar = 50 μm). (e) and (f) Melanomacrophage (M) were found in fish fed on a high dose of MEL alone (diet 6) (e) (200×; Scale bar = 100 μm) and with CYA alone (diet 7) (f) (200×; Scale bar = 100 μm).

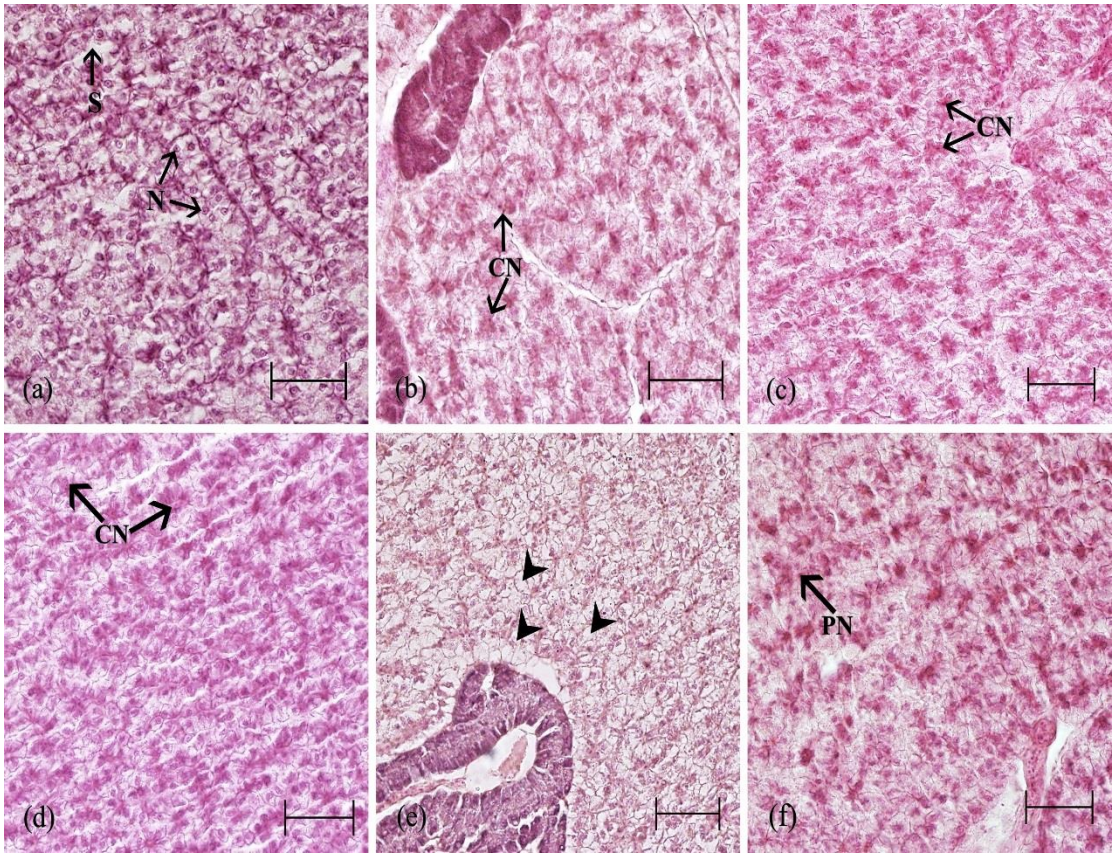

Figure 3-3. Photomicrographs showing pathologies of liver in Asian seabass fed on diets containing various levels of MEL and/or CYA (a) Histology of a normal liver from fish fed on the control diet, nucleus (N) and sinusoid space (S). (400×; Scale bar = 50 μm). (b) Photomicrograph of a liver section of fish fed on MEL-CYA: 2.5+2.5 g kg^{-1} diet shows cell necrosis (CN) including pyknotic nuclei and loss of cellular boundaries (400×; Scale bar = 50 μm). Similar symptoms were found with the treatments MEL-CYA: 7.5+7.5 and 10+10 g kg^{-1} diet ((c) and (d), respectively, 400×; Scale bar = 50 μm). (e) Vacuolization was a distinctive feature observed in fish fed on MEL-CYA: 10+0 g kg^{-1} diet (arrow head) (400×; Scale bar = 50 μm). (f) Pyknotic nuclei were detected in the hepatocytes of fish fed on diet 7 with MEL-CYA 0+10 g kg^{-1} diet (400×; Scale bar = 50 μm).

3.4.4 Stress-70 proteins (Hsp70) and antioxidant enzyme activities

The expression of stress-70 protein (Hsp70) is given in Figure 3-4. The expression of Hsp70 increased significantly in the liver of fish fed on MEL-CYA diets (diets 2-7) ($P<0.05$), whereas expression of Hsp70 in the kidney were not significantly influenced by the dietary treatments ($P>0.05$) (Figure 3-4).

The results of the antioxidant enzyme activities in liver and kidneys are given in Figure 3-5 and 3-6. Catalase (CAT) activity in the liver was increased in fish fed diets containing a MEL-CYA combination (diets 2–5) ($P<0.05$), whereas the CAT activity in the kidney was significantly influenced only by diets 4 and 5 ($P<0.05$) (Figure 3-5). Glutathione peroxidase (GPx) activity in the liver was higher in fish fed diets containing 10+10 g kg^{-1} diet MEL-CYA (diets 5) and MEL alone (diet 6) ($P<0.05$) (Figure 3-6). GPx activity in kidneys of fish fed diets containing 5+5, 7.5+7.5 and 10+10 g kg^{-1} diet MEL-CYA (diets 3, 4 and 5, respectively) was elevated ($P<0.05$) (Figure 3-6).

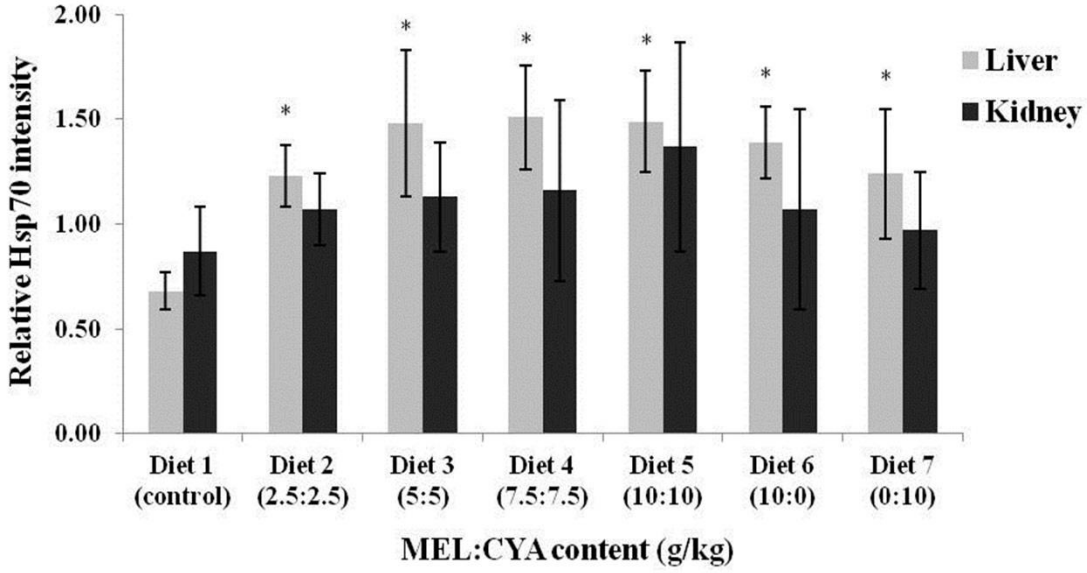

Figure 3-4. Expression of Hsp70 in the liver and kidneys of seabass after the various dietary treatments.

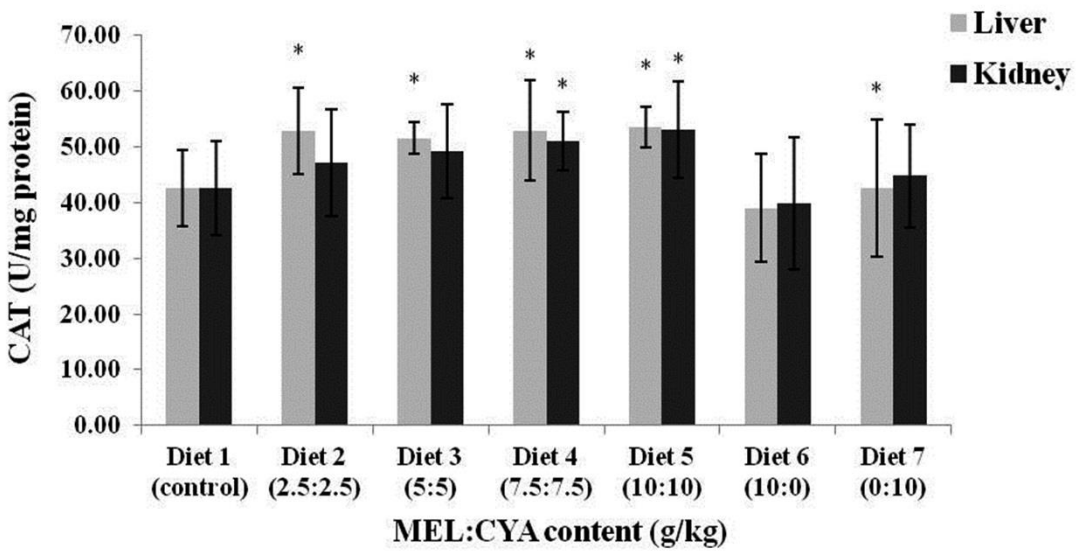

Figure 3-5. CAT activity in liver and kidneys of seabass after the various dietary treatments.

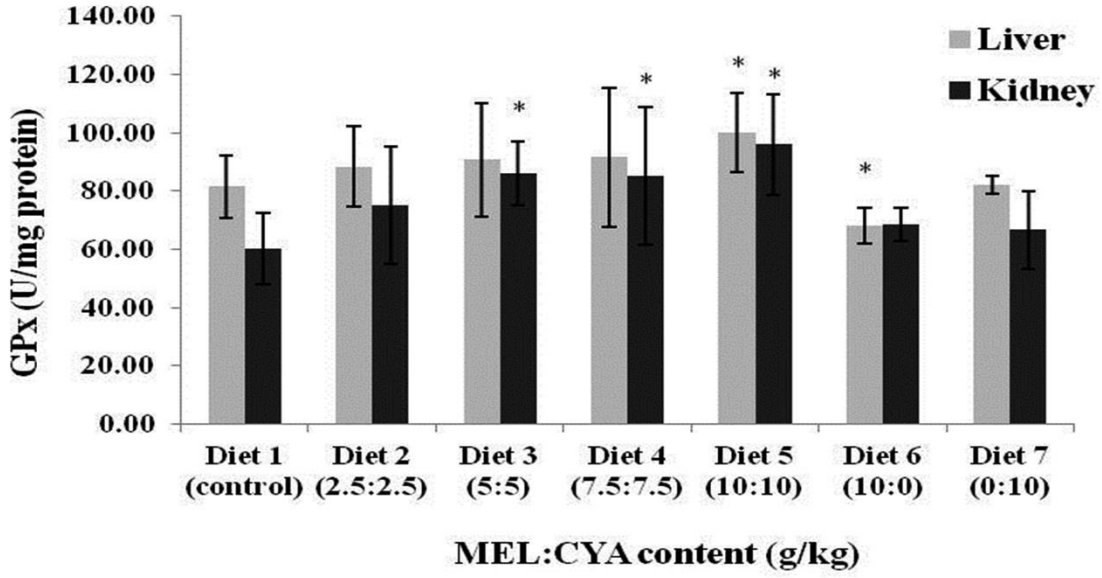

Figure 3-6. GPx activity in liver and kidneys of seabass after the various dietary treatments.

3.4.5 Blood chemistry components

The effects of dietary MEL-CYA on blood parameters are given in Table 3-5. Red blood cell and white blood cell counts, hemoglobin and hematocrit were significantly lowered in fish fed diets incorporating MEL-CYA from 5+5 to 10+0 g kg^{-1} diet ($P<0.05$). Na^{+} and Cl^{-} ions in serum were elevated in fish fed diets incorporating MEL-CYA from 7.5+7.5 g kg^{-1} diet onwards ($P<0.05$) (Table 3-5). Serum protein was increased with MEL-CYA 10+10 g kg^{-1} and MEL alone diet ($P<0.05$) (Table 3-5). The lysozyme activity of fish fed diets supplemented with MEL-CYA at different concentrations (diets 2-7) was significantly differences than those of fish fed control diet ($P<0.05$) (Table 3-5).

Table 3-5. Blood parameters of Asian seabass on diets with various levels of MEL and CYA over 12 weeks.

Parameters	Diets (MEL-CYA, g kg^{-1}diet)						
	Diet 1 (Control)	Diet 2 (2.5+2.5)	Diet 3 (5+5)	Diet 4 (7.5+7.5)	Diet 5 (10+10)	Diet 6 (10: 0)	Diet 7 (0+10)
Complete blood count (mean±SD; n=2 fish from each of the 4 replicate tanks)							
Red blood cells (x10^9 cell mL^{-1})	4.04±0.11	3.98±0.30	3.59±0.31*	3.58±0.43*	3.50±0.30*	3.10±0.37*	3.98±0.72
White blood cells (x10^7 cell mL^{-1})	5.35±1.40	4.63±1.22	3.87±0.64*	3.83±0.39*	2.59±0.86*	2.12±0.92*	2.21±0.28*
Hemoglobin (g dL^{-1})	11.68±0.69	11.25±1.55	10.17±1.12*	10.68±0.68*	10.60±0.83*	8.90±0.42*	11.45±0.75
Hematocrit (%)	58.62±9.93	53.58±7.36	48.72±5.05*	48.24±3.13*	50.25±6.98*	41.62±2.26*	52.83±5.62
Blood Chemistry Components (mean±SD; n=2 fish from each of the 4 replicate tanks)							
Blood Urea Nitrogen, BUN (mg%)	6.40±1.56	6.20±0.57	7.40±0.30	7.83±1.10	6.75±0.49	7.35±1.34	6.40±0.28
Serum sodium, Na^+ (mmol L^{-1})	176.45±2.19	175.60±0.28	175.10±3.11	172.45±0.49*	170.75±0.78*	171.35±2.76*	170.00±4.53*
Serum potassium, K^+ (mmol L^{-1})	1.72±0.84	2.26±0.52	1.44±0.01	1.27±0.21	1.66±0.27	2.23±0.69	4.80±0.47*
Serum chloride, Cl^- (mmol L^{-1})	150.05±0.78	152.27±1.88	152.30±0.98	147.20±1.51*	144.83±2.95*	147.05±2.19*	153.50±5.66
Serum protein (mg mL^{-1})	74.60±8.67	80.93±7.29	76.84±12.36	79.38±6.88	88.32±11.72*	87.69±9.44*	79.44±11.21
Lysozyme activity (U min^{-1})	22.13±3.94	25.41±3.06*	25.58±3.07*	32.38±5.03*	26.53±4.42*	31.87±4.44*	32.45±4.71*

Asterisks indicate statistically significant differences ($P<0.05$) between a particular treatment group and the control group

3.5 Discussion

Recently, farmed fish for human consumption are fed compound diets specially formulated for their nutritional needs. Unadulterated and properly formulated diets prepared using pure natural ingredients are expected to deliver the essential nutrients to the animals, which gets simplified into high quality products. However, adulterants in the feedstuffs remain a critical problem, and there are recent reports on MEL and CYA adulterants in aquatic feeds (Andersen *et al*., 2008; Karbiwnyk *et al*., 2009; Stine *et al*., 2012). The Rapid Alert System for Food and Feed (RASFF) recently published on adulteration of animal feeds (organic soy expeller) originating from China. These feeds were delivered to France, Germany and the United Kingdom, and had MEL concentrations in the range 1.6-410 mg kg^{-1} (European Commission 2008; INFOSAN 2008). MEL was not the only adulterant in these cases, another derivative compound, CYA, was also detected (Phromkunthong *et al*., 2015). Therefore, WHO (2008) approved a tolerated daily intake (TDI) of 0.2 mg kg^{-1} of body weight for MEL and 1.5 mg kg^{-1} of body weight for CYA for humans. Furthermore, Codex Alimentarius Commission (2010) recommended that the MEL concentration in animal feed should not exceed 2.5 mg kg^{-1} diet. Nowadays, MEL or CYA may be appear in animal feeds at concentrations above that recommendation, and report on the adulteration by MEL or CYA are restricted. Studies on the toxicity of MEL in combination with CYA and their metabolism, absorption and distribution in animals are still limited. There are few reports describing the dose response relationship of MEL in combination with CYA administration in the diets of aquatic animals. The data reported here can also help risk monitors dealing with probable feed-linked contamination in aquatic food.

In our previous study, Phromkunthong *et al*. (2015) reported that the SGR of red tilapia was significantly lower in fish that received MEL alone in the diet relative to control, whereas no significant effects were found with combined MEL-CYA in the diet. Likewise, in the present study, the SGR of Asian seabass receiving MEL alone was significantly lower than those of fish fed with control diet, demonstrating that these fish are more sensitive to MEL than mammals. For Asian seabass even a 2.5 g kg^{-1} diet supplementation of MEL and CYA each in the diet, here equivalent to the average daily

intake of 77 mg kg^{-1} of body weight per day, reduced the growth performance of seabass. In comparison, tilapia was less sensitive to MEL and CYA, as its growth was not significantly affected by these contaminants in combinations of 2.5+2.5 - 7.5+7.5 g kg^{-1} diet each (Phromkunthong *et al*., 2015). Further, the results from this study clearly indicate that the supplementation of high doses of MEL-CYA from 7.5+7.5 to 10+10 g kg^{-1} diet (equivalent average daily intakes of 212 to 293 mg kg^{-1} of body weight per day, respectively) negatively affected FCR. This is similar to the results obtained from our previous studies where MEL alone and combination of MEL-CYA were toxic to red tilapia (Phromkunthong *et al*., 2013; 2015). However, it is demonstrated that growth performance and FCR of red tilapia received combined MEL-CYA were not significantly different from the control in our previous study (Phromkunthong *et al*., 2015). Only the tilapia receiving MEL alone suffered in growth performance and FCR. This suggests, since combined MEL-CYA negatively affected the growth performance of Asian seabass, that this Asian seabass is more sensitive to MEL-CYA than the red tilapia. Our results confirm those in the studies of Liu *et al*. (2009) and Xue *et al*. (2011) evaluating the toxicity of MEL to Japanese seabass (*Lateolabrax japonicu*) and to darkbarbel catfish (*Pelteobagrus vachelli*), respectively. However, no irregular findings, such as skin discoloration or other anomalies, were recorded in the current study on MEL or its derivative as feed adulterants.

In the present study, the nitrogen contents of the whole body and the fillet of fish significantly increase with MEL-CYA 10+10 g kg^{-1}diet, and with MEL alone (equivalent average daily intakes 293 and 243 mg kg^{-1} of body weight per day). The highest amount of MEL residue was detected in fillet of fish fed with MEL alone at 10 g kg^{-1}diet. Similar results were noted in rainbow trout that had lower MEL residue in muscle when receiving combination of high-dose MEL and CYA than with only MEL in the diet (Pacini *et al*., 2013). It is noted that CYA residues were not found in the fillet of fish regardless of treatment. This differs from our previous study for tilapia (Phromkunthong *et al*., 2015) where high amount of CYA residue in the fillet was detected with CYA 10 g kg^{-1}diet (equivalent average daily intake 316 mg kg^{-1} of body weight per day). This demonstrates that Asian seabass excretes CYA more efficiently than tilapia. The growth performance and FCR effects of this treatment

confirm this hypothesis. It is generally noticed that the crystal nephropathy associated with the co-administration of MEL and CYA causes renal dysfunction at dosages much lower than the lethal doses of MEL or CYA alone. The WHO (2008) recommended a TDI of 0.2 mg $kg^{-1}$of body weight for MEL and 1.5 mg kg^{-1} of body weight for CYA for humans. Examining the abovementioned TDIs, the MEL residue levels in the fillet of fish were possibly toxic to humans. Furthermore, the decreased lipid content in the whole-body of fish fed with diets containing MEL-CYA confirms this, according to the results of our previous studies (Phromkunthong *et al*., 2013; 2015). Similarly, Deng *et al*. (2010) reported that decreased lipid content in the liver of tilapia could be generated by high oxidative stress triggered by aflatoxin B1 exposure. This could be possible to the utilization of lipids as the major energy source during the detoxification pathways (Marie *et al*., 2012; Zheng *et al*., 2011).

The blood biochemical parameters are significant for health status, toxicology, and bio-monitoring of fish (Li *et al*., 2010; McDonald and Milligan, 1992). In this study, blood parameters including blood cell counts, hemoglobin and hematocrit significantly decreased in fish fed with MEL-CYA from 5+5 g kg^{-1} diet (equivalent average daily intake 138 mg kg^{-1} of body weight per day) onwards. Especially the white blood cell counts decreased with the CYA at 10 g kg^{-1}diet, suggesting it as a useful indicators of these contaminants in diet for Asian seabass. The decrease in red blood cell counts of fish fed MEL-CYA combination diets is resulted by the damage to erythrocyte membranes (Rai *et al*., 2014). Moreover, the erythrocyte hemolytic is induced by MEL-CYA particles is a complex process, as demonstrated by the sigmoidal shape of hemoglobin release. A sufficient amount of substances require to accumulate on the erythrocyte membrane before hemoglobin release occurs (Wang *et al*., 2010). Haney *et al*. (1992) suggested that when red blood cells are hemolyzed by the leukocytosis activity in erythrocytic anemia, with subsequent erythroblastosis, the red blood cell, the hemoglobin and the hematocrit decrease. Furthermore, our present study demonstrates that a combination of MEL and CYA, administered in the Asian seabass diet at levels of 7.5+7.5, 10+10 and MEL alone at 10 g kg^{-1}diet, decreased Na^+ and Cl^- ion in the serum, while K^+ increased with CYA alone at 10 g kg^{-1}diet. The electrolyte balance was disrupted with associate to Na^+, Cl^- or K^+

changes. Öner *et al*. (2008) reported reduced Na^+ levels in serum of freshwater fish, *O. niloticus* after exposed to Ag. The electrolyte imbalance are possibly linked to dysfunction of the renal tubules, whereby electrolytes are excreted in excess in the urine (Barham *et al*., 1980). Increased serum K^+ may be explained as an indication of the response in intracellular acid-base balance to pathological response to stress (Wedemeyer *et al*., 1990). Further, Puschner *et al*. (2007) reported an increase in serum ion gap of cats that received a combination of MEL and CYA, whereas they did not find abnormal changes in Na^+, Cl^- or K^+ in the serum. Elevated serum protein was recorded in fish fed with MEL-CYA 10+10 g kg^{-1}diet and with MEL alone. This is similar to the results of Saravanan *et al*. (2010) who reported that fish exposed to gamma-hexachlorocyclohexane, also known as lindane had increased plasma protein levels relative to control group. In addition, Teixeira *et al*. (2010) reported a significant differenc in the plasma protein of tilapias exposured to high-thermal stress. Lysozyme (N-acetylmuramide glycanhydrolase) is a nonspecific immune modulator against pathogen infections and toxic substances, and its activity increases in fish blood after exposure to harmful substances (Alexander and Ingram, 1992). Serum lysozyme activity in this study increased with all actual treatments as might be estimated.

The survival of fish in all the treatments in this study was 90-98% and did not significantly differ between treatments despite treatment specific renal damage. Serum biochemistry demonstrated that the BUN levels did not significantly differ between the treatments, and this finding agrees with previous results on walking catfish (*Clarius batrachus*) (Pirarat *et al*., 2012).

The 70 kilodalton heat shock proteins (Hsp70) family, are constitutively expressed in cells under normal or unstressed conditions and function as molecular chaperones and protein folding, to maintain other proteins from forming inappropriate aggregates (Kammenga *et al*., 2000; Sørensen *et al*., 2003). Aside from this function, the Hsps are also associated in the general protection of stressed cells (Basu *et al*., 2002; Iwama *et al*., 1999). In general, the Hsps are constitutively display even in non-stressed cells as they have important functions in normal cell regulation process (Köehler *et al*., 2009). Many studies have reported that on exposure of cells and organisms to stressors the cells

produce Hsp70 so that its increased level would protect them against cellular damage caused by oxidative stress (Gupta *et al*., 2010; Köehler *et al*., 2007; Padmini and Rani, 2008; Taleb *et al*., 2008). Hsps has been intensively studied in model organisms including fish by inducing them to stress under laboratory conditions. However, few Hsps studies include nutrition and feeding experiments where the fish is exposed to toxic substances. The results in our study demonstrate that the liver produced Hsp70 in response to MEL/CYA administration, whereas it was not detected in the kidney. These results agree with the findings of Phromkunthong *et al*. (2015), where Asian seabass seems to have organ-specific responses to toxic substances in its diet.

Case reports on adulterant-induced stress with oral administration of contaminants, such as MEL/CYA in aquatic feeds, are scarce. Therefore, in this study we used an antioxidant enzyme as a relevant biomarker for detecting cellular responses against toxic substances. Asian seabass on combined MEL-CYA diets had higher catalase activity in the liver, and treatment effects were also found in the kidneys of fish fed with combined MEL-CYA 7.5+7.5 and 10+10 g kg^{-1} diet. Furthermore, increased GPx activity was recorded in the liver of fish fed with combined MEL-CYA 10+10 g^{-1}kg diet, and this activity was increased also in the kidneys of fish fed with MEL-CYA 5+5, 7.5+7.5 and 10+10 g kg^{-1}diet, suggesting that these contaminants elevated oxidative stress of the fish. Generally, evaluation of the antioxidant enzyme activity indicates that a excessive amount of free radicals have to be eliminated (Pacini *et al*., 2013; 2014; Ross *et al*., 2001). Therefore, the increased CAT and GPx activities indicate attempts to combat free radicals and to prevent oxidative damage (Winston and Di Giulio, 1991). Triazines may generate hydrogen peroxide (H_2O_2) that can cause oxidative stress in exposed tissues. Altered activities of CAT may counter the free radicals induced by MEL–CYA diets. However, the enzyme analyses were operated with the kidneys of fish already used for crystal examination, so that the alterations in CAT activity may be a response to the tissue damage by the crystals (Pacini *et al*., 2014). The results of this study are similar to the study by Phromkunthong *et al*. (2015). In addition, this study similar to the report by Rai *et al*. (2014), where they present that MEL induces cell responses to oxidative stress in fishes. However, with MEL alone in the diet, GPx activity in

the liver was significantly decreased. We speculate that GPx activity might be inhibited by high toxic levels of these contaminants, levels above the tolerance range, which could partly clarify the higher MEL residue and the poorer survival rate in the treatment with MEL alone (Phromkunthong *et al*., 2015).

In this study, melamine-cyanurate crystals were detected in the kidneys of fish that fed diets supplemented with a combination of MEL-CYA, and the melamine-cyanurate crystals were distributed widely in almost all the renal tubules. The the melamine-cyanurate crystals have also been detected in the other internal organs in walking catfish, including heart, liver, spleen and the corpuscle of Stannius, and they may be related with a granulomatous inflammatory response (Pirarat *et al*., 2012). Phromkunthong *et al*. (2015) also reported crystals in the kidneys of red tilapia exposed with a combination of MEL and CYA, similar to the results in cats that induced renal failure (Puschner *et al*., 2007). These spherulites cause intratubular obstruction and subsequent acute renal dysfunction (Conger *et al*., 1976; Dobson *et al*., 2008; Reimschuessel *et al*., 2008). In addition, a combination of factors, such as inflammation, tubular obstruction by proteinaceous material secondary to casts, apoptosis, and obstruction by the crystals themselves, is necessary for the development of acute renal failure (Puschner *et al*., 2007). In both mammals and fish, renal failure can generally occur within the first few days of exposure to MEL and CYA (Dobson *et al*., 2008; Kim *et al*., 2010; Puschner *et al*., 2007; Reimschuessel *et al*., 2008). The incidence of lesions increased with dose, but no crystals were detected in the kidneys of Asian seabass that received MEL or CYA alone (diets 6 and 7). The results in our study are similar to the studies conducted in rainbow trout (Pacini *et al*., 2014), walking catfish (Pirarat *et al*., 2012) and red tilapia (Phromkunthong *et al*., 2015), acknowledging some differences between the species. On the other hand, Reimschuessel *et al*. (2010) reported that 3.33% (1/30) of fish received with MEL alone had renal crystals that were similarly recorded in the MEL-CYA exposed animals. They also found crystals in the kidney of pig receiving MEL alone. There are few reports of crystal formation in the kidneys of animals when MEL or CYA were given orally administered alone. It is demonstrated that the induction of renal failure induced by insoluble complex of melamine-cyanurate crystals did not affect the survival of

Asian seabass in this study. The fish might have eliminated some of the nitrogenous waste products through the gills without burdening the kidney, as noted by Pirarat *et al*. (2012). Thus, the potentiality of fish to tolerate renal damage may be better than of most mammals (Reimschuessel *et al*., 2008). The results of the present study are similar to the finding of Phromkunthong *et al*. (2015).

Dietary MEL and CYA induced hepatotoxicity in Asian seabass in this present study. Pirarat *et al*. (2012) found that the administration of high combined doses of MEL and CYA elevated serum alanine transaminase and aspartate transaminase in walking catfish, indicating liver dysfunction. Similarly, Liu *et al*. (2009) reported that feeding 10 g kg^{-1} diet of MEL increased serum alkaline phosphatase in Japanese seabass, whereas alanine transaminase and aspartate aminotransferase activities in serum were not affected. The results in our present study corroborate the findings from Phromkunthong *et al*. (2013) where lesions in liver occured in red tilapia receiving MEL in the diet. In addition, Neerman *et al*. (2004) also found hepatocyte necrosis and increased alanine transaminase activity in mice after injection with melamine dendrimer at 40 mg kg^{-1} of body weight. It is noted that liver alterations of fish in our present study were found in the fish with all the actual treatments, whether receiving MEL-CYA either individually or in combination. Studies on the effects of MEL and CYA on liver of teleost fish are rare. The alteration in the liver of fish by the diet containing MEL alone at 10 g kg^{-1} diet was vacuolation of hepatocytes. Similar changes were found in red tilapia with an orally administered of MEL (5 g kg^{-1} diet) (Phromkunthong *et al*., 2013), and the toxicity of MEL may be species specific in fishes.

3.6 Conclusions

The results of this study indicated that the dietary combination of MEL and CYA, or MEL alone at a comparatively high dose level, had adversely effects in Asian seabass. Furthermore, MEL residue was detected in fillet of fish in these all treatments too. CYA alone in the diet affected feed efficiency as well as liver and kidney histopathology of the fish. The combination of MEL and CYA induced melamine-cyanurate crystals in the renal tubule. HSP70 and antioxidant enzyme activities elevated

in fish receiving a diet with combined dose of MEL and CYA. The results from this study can be used for a risk assessment of MEL and CYA in Asian seabass.

3.7 References

Alexander, J.B. and Ingram, G.A. 1992. Noncellular nonspecific defense mechanisms of fish. Annu. Rev. Fish Dis. 2, 249-279.

Andersen, W.C., Turnipseed, S.B., Karbiwnyk, C.M., Clark, S.B., Madson, M.R., Gieseker, C.M., Miller, R.A., Rummel, N.G. and Reimschuessel, R. 2008. Determination and confirmation of melamine residues in catfish, trout, tilapia, salmon, and shrimp by liquid chromatography with tandem mass spectrometry. J. Agric. Food Chem. 56, 4340-4347.

AOAC (Association of Official Analytical Chemists). 1995. Official methods of analysis of AOAC International. 2 vols. sixteenth ed. Association of Analytical Communities: Arlington, VA.

Bancroft, J.D. 1967. Histochemical Techniques. first ed. Butterworths: London.

Barham, W.T., Smit, G.L. and Schoonbee, H.J. 1980. The haematological assessment of bacterial infection in rainbow trout, *Salmo gairdneri* Richardson. J. Fish Biol. 17, 275-281.

Basu, N., Todgham, A.E., Ackerman, P.A., Bibeau, M.R., Nakano, K., Schulte, P.M. and Iwama, G.K. 2002. Heat shock protein genes and their functional significance in fish. Gene 295, 173-183.

Bischoff, K. 2011. Melamine and cyanuric acid. *In*: Gupta, R.C. (ed.), Reproductive and Developmental Toxicology. Academic Press/Elsevier: Amsterdam, pp. 367-372.

Blaxhall, P.C. and Daisley, K.W. 1973. Routine haematological methods for use with fish blood. J. Fish Biol. 5, 771-781.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of micro-gram quantities of protein using the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Brown, C.A., Jeong, K.S., Poppenga, R.H., Puschner, B., Miller, D.M., Ellis, A.E., Kang, K.I., Sum, S., Cistola, A.M. and Brown, S.A. 2007. Outbreaks of renal failure associated with melamine and cyanuric acid in dogs and cats in 2004 and 2007. J. Vet. Diagn. Invest. 19, 525-531.

Chayanunnukul, W., Apirakthunyakorn, P. and Musigavon, P. 2007. A comparison of serum electrolytes in commercial and self-prepared reagents using a Beckman CX-3. Songkla Med. J. 25, 295-301.

Cianciolo, R.E., Bischoff, K., Ebel, J.G., Van Winkle, T.J., Goldstein, R.E. and Serfilippi, L.M. 2008. Clinicopathologic, histologic, and toxicologic findings in 70 cats inadvertently exposed to pet food contaminated with melamine and cyanuric acid. J. Am. Vet. Med. Assoc. 233, 729-737.

Codex Alimentarius Commission. 2010. Food and Agriculture Organization of the United Nations and World Health Organisation, Rome, Italy, ALINORM 10/33/41, pp. 69.

Conger, J.D., Falk, S.A., Guggenheim, S.J. and Burke, T.J. 1976. A micropuncture study of the early phase of acute urate nephropathy. J. Clin. Invest. 58, 681-689.

Demers, N.E. and Bayne, C.J. 1997. The immediate effects of stress on hormones and plasma lysozyme in rainbow trout. Dev. Comp. Immunol. 21, 363-373.

Deng, S.X., Tian, L.X., Liu, F.J., Jin, S.J., Liang, G.T., Yang, H.J., Du, Z.Y. and Liu, Y.J. 2010. Toxic effects and residue of aflatoxin B1 in tilapia (*Oreochromis niloticus* x *O. aureus*) during long-term dietary exposure. Aquaculture 307, 233-240.

Dobson, R.L.M., Motlagh, S., Quijano, M., Cambron, R.T., Baker, T.R., Pullen, A.M., Regg, B.T., Bigalow-Kern, A.S., Vennard, T., Fix, A., Reimschuessel, R., Overmann, G., Shan, Y. and Daston, G.P. 2008. Identification and characterization of toxicity of contaminants in pet food leading to an outbreak of renal toxicity in cats and dogs. Toxicol. Sci. 106, 251-262.

European Commission. 2008. The Rapid Alert System for Food and Feed (RASFF) Annual Report 2008. Office for Official Publications of the European Communities: Luxembourg.

Gupta, S.C., Sharma, A., Mishra, M., Mishra, R.K. and Chowdhuri, D.K. 2010. Heat shock proteins in toxicology: How close and how far?. Life Sci. 86, 377-384.

Haney, D.C., Hursh, D.A., Mix, M.C. and Winton, J.R. 1992. Physiological and hematological changes in chum salmon artificially infected with erythrocytic necrosis virus. J. Aquat. Anim. Health. 4, 48-57.

INFOSAN (The International Food Safety Authorities Network). 2008. Melamine-contaminated products, China. (Emergency Alert Update No.11). International Food Safety Authorities Network: Geneva.

Iwama, G.K., Vijayan, M.M., Forsyth, R.B. and Ackerman, P.A. 1999. Heat shock proteins and physiological stress in fish. Am. Zool. 39, 901-909.

Janlek, P., Boonyaratpalin, M. and Phromkunthong, W. 2009. Effect of melamine on growth performance, blood components and histological changes in hybrid catfish, *Clarias macrocephalus* (gunther) x *Clarias gariepinus* (burchell). Thai Fish. Gaz. 62, 331-340. (in Thai).

Kammenga, J.E., Dallinger, R., Donker, M.H., Köhler, H.R., Simonsen, V., Triebskorn, R. and Weeks, J.M. 2000. Biomarkers in terrestrial invertebrates for ecotoxicological soil risk assessment. Rev. Environ. Contam. T. 164, 93-147.

Karbiwnyk, C.M., Andersen, W.C., Turnipseed, S.B., Storey, J.M., Madson, M.R., Miller, K.E., Gieseker, C.M., Miller, R.A., Rumme,l N.G. and Reimschuessel, R. 2009. Determination of cyanuric acid residues in catfish, trout, tilapia, salmon and shrimp by liquid chromatography-tandem mass spectrometry. Anal. Chim. Acta. 637, 101-111.

Karbiwnyk, C.M., Williams, R.R., Andersen, W.C., Turnipseed, S.B., Madson, M.R., Miller, K.E. and Reimschuessel, R. 2012. Bioaccumulation of cyanuric acid in edible tissues of shrimp following experimental feeding. Food Addit. Contam. Part A: Chem. Anal. Control Expo. Risk Assess. 27, 1658-1664.

Kim, C.W., Yun, J.W., Bae, I.H., Lee, J.S., Kang, H.J., Joo, K.M., Jeong, H.J., Chung, J.H., Park, Y.H. and Lim, K.M. 2010. Determination of spatial distribution of melamine-cyanuric acid crystals in rat kidney tissue by histology and imaging

matrixassisted laser desorption/ionization quadrupole time-of-flight mass spectrometry. Chem. Res. Toxicol. 23, 220-227.

Köhler, H.R., Lazzara, R., Dittbrenner, N., Capowiez, Y., Mazzia, C. and Triebskorn, R. 2009. Snail phenotypic variation and stress proteins: do different heat response strategies contribute to Waddington's widget in field populations?. J. Exp. Zool. B Mol. Dev. Evol. 312, 136-147.

Köhler, H.R., Sandu, C., Scheil, V., Nagy-Petrica, E.M., Segner, H., Telcean, I., Stan, G. and Triebskorn, R. 2007. Monitoring pollution in river Mureş, Romania, Part III: biochemical effect markers in fish and integrative reflection. Environ. Monit. Assess. 127, 47-54.

Larsen, H.N. and Snieszko, S.F. 1961. Comparison of various methods of determination of haemoglobin in trout blood. Prog. Fish Cult. 23, 8-17.

Li, Z.H., Velisek, J., Zlabek, V., Grabic, R., Machova, J., Kolarova, J. and Randak, T. 2010. Hepatic antioxidant status and hematological parameters in rainbow trout, *Oncorhynchus mykiss*, after chronic exposure to carbamazepine, Chem. Biol. Interact. 183, 98-104.

Liu, H.Y., Zhang, W., Xue, M., Wu, X.F., Zheng, Y.H., Guo, L.Y. and Sheng, H.J. 2009. Acute toxicity study for melamine on Japanese seabass (*Lateolabrax japonicus*). Acta Hydrobiol. Sin. 33, 157-163. (in Chinese).

Marie, B., Huet, H., Marie, A., Djediat, C., Puiseux-Dao, S., Catherine, A., Trinchet, I. and Edery, M. 2012. Effects of a toxic cyanobacterial bloom (*Planktothrix agardhii*) on fish: insights from histopathological and quantitative proteomic assessments following the oral exposure of medaka fish (*Oryzias latipes*). Aquat. Toxicol. 114-115, 39-43.

McDonald, D.G. and Milligan, C.L. 1992. Chemical properties of the blood. *In*: Hoar, W.S., Randall, D.J., Farrell, A.P., (Eds.), Fish physiology Vol. XII, Part B, The Cardiovascular System. Academic Press: San Diego, pp. 56-113.

Neerman, M.F., Zhang, W., Parrish, A.R. and Simanek, E.E. 2004. *In vitro* and *in vivo* evaluation of a melamine dendrimer as a vehicle for drug delivery. Int. J. Pharm. 281, 129-132.

Nilubol, D., Pattanaseth, T., Boonsri, K., Pirarat, N. and Leepipatpiboon, N. 2009. Melamine- and cyanuric acid-associated renal failure in pigs in Thailand. Vet. Pathol. 46, 1156- 1159.

Öner, M., Atli, G. and Canli, M. 2008. Changes in serum biochemical parameters of freshwater fish *Oreochromis niloticus* following prolonged metal (Ag, Cd, Cr, Cu, Zn) exposures. Environ. Toxicol. Chem. 27, 360-366.

Pacini, N., Dörr, A.J., Elia, A.C., Scoparo, M., Abete, M.C. and Prearo, M. 2014. Melamine-cyanurate complexes and oxidative stress markers in trout kidney following melamine and cyanuric acid long-term co-exposure and withdrawal. Fish Physiol. Biochem. 40, 1609-1619.

Pacini, N., Prearo, M., Abete, M.C., Brizio, P., Dörr, A.J., Reimschuessel, R., Andersen, W., Gasco, L., Righetti, M. and Elia, A.C. 2013. Antioxidant responses and renal crystal formation in rainbow trout treated with melamine administered individually or in combination with cyanuric acid. J. Toxicol. Environ. Health A. 76, 491-508.

Padmini, E. and Rani, M.U. 2008. Impact of seasonal variation on HSP70 expression quantitated in stressed fish hepatocytes. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 151, 278-285.

Phromkunthong, W., Nuntapong, N., Boonyaratpalin, M. and Kiron, V. 2013. Toxicity of melamine, an adulterant in fish feeds: experimental assessment of its effects on tilapia. J. Fish Dis. 36, 555-568.

Phromkunthong, W., Choochuay, P., Kiron, V., Nuntapong, N. and Boonyaratpalin, M. 2015. Pathophysiological changes associated with dietary melamine and cyanuric acid toxicity in red tilapia. J. Fish Dis. 38, 161-173.

Pirarat, N., Katagiri, T., Chansue, N., Ponpornpisit, A., Endo, M. and Maita, M. 2012. The Pathological Effects of Melamine and Cyanuric Acid in the Diet of Walking Catfish (*Clarius batrachus*). J. Comp. Pathol. 147, 259-266.

Puschner, B., Poppenga, R.H., Lowenstine, L.J., Filigenzi, M.S. and Pesavento, P.A. 2007. Assessment of melamine and cyanuric acid toxicity in cats. J. Vet. Diagn. Invest. 19, 616-624.

Rai, N., Banerjee, D. and Bhattacharyya, R. 2014. Urinary melamine: Proposed parameter of melamine adulteration of food. Nutrition 30, 380-385.

Reimschuessel, R., Evans, E., Stine, C.B., Hasbrouck, N., Mayer, C.D., Nochetto, C. and Gieseker, C.M. 2010. Renal crystal formation after combined or sequential oral administration of melamine and cyanuric acid. Food Chem. Toxicol. 48, 2898-2906.

Reimschuessel, R., Gieseker, C.M., Miller, R.A., Ward, J., Boehmer, J., Rummel, N., Heller, D.N., Nochetto, C., De Alwis, G.K., Bataller, N., Andersen, W.C., Turnipseed, S.B., Karbiwnyk, C.M., Satzger, R.D., Crowe, J.B., Reinhard, M.K., Roberts, J.F. and Witkowski, M.R. 2008. Evaluation of the renal effects of experimental feeding of melamine and cyanuric acid to fish and pigs. Am. J. Vet. Res. 69, 1217-1228.

Reimschuessel, R. and Puschner, B. 2010. Melamine toxicity-stones vs. crystals. J. Med. Toxicol. 6, 468-469.

Ross, S.W., Dalton, D.A., Kramer, S. and Christensen, B.L. 2001. Physiological (antioxidant) responses of estuarine fishes to variability in dissolved oxygen. Comp. Biochem. Physiol. C: Comp. Pharmacol. 130, 289-303.

Saravanan, M., Prabhu Kumar, K. and Ramesh, M. 2011. Haematological and biochemical responses of freshwater teleost fish *Cyprinus carpio* (Actinopterygii: Cypriniformes) during acute and chronic sublethal exposure to lindane. Pestic. Biochem. Phy. 100, 206-211.

Smoker, M. and Krynitsky, A.J. 2008. Interim Method for Determination of Melamine and Cyanuric Acid Residues in Foods using LC-MS/MS: Version 1.0. US FDA/CFSAN Laboratory Information Bulletin LIB No. 4422. US-FDA: Maryland.

Sørensen, J.G., Kristensen, T.N. and Loeschcke, V. 2003. The evolutionary and ecological role of heat shock proteins. Ecol. Let. 6, 1025-1037.

Stine, C.B., Nochetto, C.B., Evans, E.R., Gieseker, C.M., Mayer, T.D., Hasbrouck, N.R. and Reimschuessel, R. 2012. Depletion of melamine and cyanuric acid in serum from catfish *Ictalurus punctatus* and rainbow trout *Oncorhynchus mykiss*. Food Chem. Toxicol. 50, 3426-3432.

Sun, N., Shen, Y. and He, L. 2010. Histopathological Features of the Kidney after Acute Renal Failure from Melamine. N. Engl. J. Med. 362, 662-664.

Taleb, M., Brandon, C.S., Lee, F.S., Lomax, M.I., Dillmann, W.H. and Cunningham, L.L. 2008. Hsp70 inhibits aminoglycoside-induced hair cell death and is necessary for the protective effect of heat shock. JARO 9, 277-289.

Teixeira, C.P., Barros, M.M., Pezzat co, L.E., Fernandes, Jr, A.C., Koch, J.F.A. and Padovani, C.R. 2012. Growth performance of Nile tilapia, *Oreochromis niloticus*, fed diets containing levels of pyridoxine and haematological response under heat stress. Aquacult. Res. 43, 1081-1088.

Thai agricultural commodity and food standard TACFS 7412-2007. 2007. National Bureau of Agricultural Commodity and Food Standards, Ministry of Agriculture and Cooperatives. “Good Aquaculture Practices for Seabass” in the Royal Gazette Vol.124 Section 78D, Dated 29 June B.E. 2550 (2007).

Thompson, M.E., Lewin-Smith, M.R., Kalasinsky, V.F., Pizzolato, K.M., Fleetwood, M.L, McElhaney, M.R. and Johnson, T.O. 2008. Characterization of melamine-containing and calcium oxalate crystals in three dogs with suspected pet food-induced nephrotoxicosis. Vet. Pathol. 45, 417-426.

US-FDA (U.S. Food and Drug Administration). 2007. Interim Melamine and Analogues Safety/Risk Assessment Peer Review Report. United States Food and Drug Administration: Washington, DC.

Wang, C., Qin, X., Huang, B., He, F. and Zeng, C. 2010. Hemolysis of human erythrocytes induced by melamine-cyanurate complex. Biochem. Biophys. Res. Commun. 402, 773-777.

Wedemeyer, G.A., Barton, B.A. and Mc Leay, D.J. 1990. Stress and aclimatation. *In*: Schreck, C.B., Moyle, P.B. (Eds.), Methods for Fish Biology. American Fisheries Society: Bethesda, MD, pp. 451- 489.

WHO (World Health Organization. 2008. Expert meeting to review toxicological aspects of melamine and cyanuric acid. Ottawa Canada, 1 - 4 December 2008. WHO: Geneva.

Winston, G.W. and Di Giulio, R.T. 1991. Prooxidant and antioxidant mechanisms in aquatic organisms. Aquat. Toxicol. 19, 137-161.

Xue, J., Ai, Q., Mai, K., Xu, W., Yang, Y. and Liufu, Z. 2011. Effects of melamine on growth performance and skin color of darkbarbel catfish (*Pelteobagrus vachelli*). Aquaculture 320, 142-146.

Zheng, J.L., Luo, Z., Chen, Q.L., Liu, X., Liu, C.X., Zhao, Y.H. and Gong, Y. 2011. Effect of waterborne zinc exposure on metal accumulation, enzymatic activities and histology of *Synechogobius hasta*. Ecotox. Environ. Saf. 74, 1864-1873.

CHAPTER 4

Dietary exposure to melamine and cyanuric acid induced growth reduction, oxidative stress and histopathological changes of hepatopancreas in Pacific white shrimp

4.1 Abstract

Adverse effects of melamine (MEL) and cyanuric acid (CYA) have been reported in several animal species, but regarding shrimp only limited information is available on these substances alone or in combination. Therefore, this study examined the toxicological effects of dietary MEL and CYA individually and in combination on growth performance, feed utilization, immune responses, oxidative stress, and histopathological changes of hepatopancreas in Pacific white shrimp. Seven isonitrogenous (350 g kg^{-1}) and isolipidic (80 g kg^{-1}) diets were formulated, namely diet 1 (a control diet without MEL and CYA); diets 2-5 (with MEL and CYA at 2.5+2.5, 5+5, 7.5+7.5 and 10+10 g kg^{-1} diet); diet 6 (with only MEL at 10 g kg^{-1} diet) and diet 7 (with CYA alone at 10 g kg^{-1} diet). The shrimp with initial body weight 2.37±0.02 g were fed with experimental diets for 10 weeks. The results of this study indicate that all the diets with MEL and CYA individually or in combination had adverse effects on growth and feed utilization relative to the control diet ($p<0.05$). Total protease and trypsin specific activities in hepatopancreas were significantly lowered by all diets containing MEL ($p<0.05$). Hemolymph immunoparameters, including total hemocyte count (THC), phenoloxidase (PO) activity, respiratory burst, and lysozyme activity, were significantly decreased ($p<0.05$) in shrimp fed MEL+CYA at high combination dosages (10+10 g kg^{-1} diet) and MEL alone (10 g kg^{-1} diet). Moreover, MEL and CYA induced oxidative stress in the hepatopancreas, which can cause severe damage to the cells of hepatopancreas, decreased antioxidant responses and increased lipid peroxidation.

4.2 Introduction

Recently, feed contamination is a worldwide serious issue with regard to consumer health, economic losses, and reliability of food products (Maule et al. 2007; Pettersson 2012; O'Keefe and Campabadal 2015). In general, melamine (MEL) and its derivatives including cyanuric acid (CYA), ammeline (AMN), and ammelide (AMD), are commonly used as the starting materials for the production of thermoplastic materials, such as tableware, particle board, laminating resins, coating materials, and flame retardant equipments (Casu et al. 1997; Roviello et al. 2015). Furthermore, the US Food and Drug Administration (US-FDA 2007) reported illegal addition of MEL and CYA to animal feed in many countries. MEL is a nitrogenous material with 66.6% of nitrogen by mass. Therefore, adulteration by the illegal addition of of MEL in a feedstuff increases the total nitrogen content typically determined by the Kjeldahl method to estimate the protein content, so MEL is also referred to as a "fake protein" (US-FDA 2007; Reimschuessel et al. 2008). Thus, protein-rich ingredients are the critical targets for MEL adulteration that has been evidenced in many high-protein feedstuffs, such as fishmeal, soybean meal, gluten (from cereal grains), squid meal, and shrimp meal; and also contamination of pets and aquatic feeds for farmed fish and shrimp has been documented (Ehling et al. 2007; WHO 2009). Adverse effects of MEL and its derivatives have been reported in different fish species; these included growth reduction, immunosuppression, dysfunctional behavior, hepatotoxicity and nephrotoxicity (Reimschuessel et al. 2010; Xue et al. 2011; Pirarat et al. 2012; Pacini et al. 2013; Phromkunthong et al. 2013; 2015a; 2015b; Mahardika et al. 2017). Thus, feed adulteration with MEL and CYA is one of the critical concerns in aquaculture industries. To date, due to the toxic effects of MEL and CYA and other adverse health effects impacting consumers, many countries have banned the use of MEL and CYA in animal feeds and incorporated these substances into a list of food additives and substances prohibited in animal feeds. In European countries, the maximum acceptable level of MEL in feed has been set at 2.5 mg/kg (European Commission 2013). In 2016, the Department of Livestock Development, Thailand has banned dried squid meal imported

from Vietnam after laboratory tests reported the presence of CYA (Byrne 2016), and there is evidence that MEL or CYA are still being used illegally in some area of the world.

Pacific white shrimp (*Litopenaeus vannamei*) is commercially important species in the aquaculture sector (World Bank 2013). This species has high global demand due to good taste and high nutritional content (FAO 2016). For producing high quality shrimp, the feed used in shrimp production system is a major key to success. In recent years, industrial shrimp feed production has dramatically focused on the alternative protein sources without negative effects on growth performance, production cost and health status of the shrimp (Amaya et al. 2007; Carvalho et al. 2016). Shrimp feed needs to have complete and high protein content to enhance growth and nutrient utilization, so it is a possible target for adulteration by MEL and its derivatives (Karbiwnyk et al. 2010). To date, there are a few case reports of MEL and its derivatives adulteration in shrimp feed. Lightner et al. (2009) reported diagnostic test results on black tiger shrimp, *Penaeus monodon,* and Pacific white shrimp from India and Indonesia. Golden to greenish-brown needle-like crystals were found in the antennal glands of shrimp, a probably adverse effect from MEL and CYA exposure. In contrast, JAVMA (2007) reported that MEL has been intentionally incorporated into shrimp feeds as binding agent in the USA, to improve water stability. However, after an MEL incident in 2007, its use as a pellet binding agent was discontinued.

To date, a few reports have examined the toxic effects of MEL and CYA to crustaceans, especially the penaeid shrimp (Lightner et al. 2009; Karbiwnyk et al. 2010). Hence, this present study examined the effects of MEL and CYA supplementation, individually or in combination, in practical diet for Pacific white shrimp. The effects on growth performance, feed utilization, immune responses, oxidative stress, and histopathological changes of hepatopancreas were evaluated. This study improves the understanding of Pacific white shrimp responses to MEL and CYA exposures.

4.3 Materials and methods

4.3.1 Experimental conditions and setup

This study was conducted at Kidchakan Supamattaya Aquatic Animal Health Research Center (KS-AAHRC), Department of Aquatic Science, Faculty of Natural Resources, Prince of Songkla University. Natural sea water with 30‰ salinity was provided by commercial shrimp farm located in Singhanakhon District, Songkhla Province, Thailand, stocked in a 7-m^3 concrete pond. After disinfection, sea water was diluted with de-chlorinated freshwater to 15‰ brackish water that was used for the entire experiment. During the 10-weeks of feeding trial, the water quality parameters including temperature, DO, alkalinity, and pH were monitored for suitability to a marine shrimp culture according to Thai Agricultural Standards TAS 7401-2009 (2009). The feeding trial was conducted in 200 L rectangular fiberglass tanks. Each tank was provided with an aerator and water draining system, and was covered with a plastic net to prevent escape of the shrimp.

4.3.2 Test shrimp and feeding

Pacific white shrimp, *L. vannamei* (0.5-1.0 g average weight) were acclimated in a 3-m^3 indoor concrete pond with 15‰ brackish water and fed with the control diet (diet 1 without MEL or CYA) four times daily for 3 weeks. At the initiation of feeding experiment, 18 shrimp with an average body weight of 2.37 ± 0.02g were randomly stocked into 35 fiberglass tanks (containing 150-L 15‰ brackish water). Each tank was then randomly assigned to one of five replicates of the seven dietary treatments. The shrimp were fed experimental diets to apparent satiation four times daily at 8:00, 12:00, 16:00 and 20:00, for 10 weeks. The care and handling of the white shrimp were performed according to the guidelines of the Canadian Council on Animal Care (CCAC 2005) and with Thai Agricultural Standards TAS 7401-2009 (2009).

4.3.3 Diet preparation

Seven isonitrogenous (350 g kg^{-1} crude protein) and isolipidic (80 g kg^{-1} crude lipid) experimental diets were formulated to meet the nutritional requirements of

Pacific white shrimp. Fish meal, soybean meal and corn gluten were used as the major protein sources. MEL (99.5% purity) was supplied by Chang Chun Petrochemical Co. Ltd., Taiwan. CYA (98% purity) was purchased from Sigma-Aldrich (MO, USA). The experimental diets are detailed in Table 4-1. Diet 1 without MEL or CYAsupplementation; MEL and CYA were added (g kg^{-1} diet) at levels 2.5+2.5 (diet 2), 5+5 (diet 3), 7.5+7.5 (diet 4), 10+10 (diet 5), 10+0 (diet 6), and 0+10 (diet 7), respectively. The diets were processed at the KS-AAHRC using Hobart mixer A-200 (OH, USA). All the dry ingredients were finely ground through a 30-mesh seive and weighed. After mixing for fifteen minutes, lipid sources (fish oil, soybean oil, and lecithin) and distilled water (400 mL kg^{-1} feed) were added with continuous mixing. The mixture was pelleted (3-mm diameter) and dried to below 10% moisture. The dry pellets were then packed in low-density polyethylene bags and stored in a freezer at -20^{o}C. Proximate compositions of experimental diets were analyzed according to the standard methods of AOAC (1995). The proximal compositions are presented in Table 4-2. MEL and CYA content in the diets were determined by LC-MS/MS using a modified method of US-FDA LIB No. 4422 with HILIC chromatography (Smoker and Krynitsky 2008), as described in previous study (Phromkunthong et al. 2013; 2015a).

Table 4-1. Ingredients compositions of the experimental diets

Ingredients (g kg^{-1} diet)	Diets (MEL+CYA, g kg^{-1} diet)						
	Diet 1 (Control)	Diet 2 (2.5+2.5)	Diet 3 (5+5)	Diet 4 (7.5+7.5)	Diet 5 (10+10)	Diet 6 (10+0)	Diet 7 (0+10)
Fish meal	200	200	200	200	200	200	200
De-hulled soybean meal	250	250	250	250	250	250	250
Squid liver meal	30	30	30	30	30	30	30
Corn gluten	50	50	50	50	50	50	50
Canola meal	28.5	28.5	28.5	28.5	28.5	28.5	28.5
Wheat flour	320	320	320	320	320	320	320
Wheat gluten	30	30	30	30	30	30	30
Fish oil	15	15	15	15	15	15	15
Soybean oil	1	1	1	1	1	1	1
Lecithin	20	20	20	20	20	20	20
Vitamin & mineral premix[1]	2	2	2	2	2	2	2
Di-calcium phosphate	22	22	22	22	22	22	22
Choline chloride	1	1	1	1	1	1	1
Inosital	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Melamine (MEL)[2]	0	2.5	5	7.5	10	10	0
Cyanuric acid (CYA)[3]	0	2.5	5	7.5	10	0	10
Microcrystalline cellulose	30	25	20	15	10	20	20

[1] Vitamin and mineral premixed (unit kg^{-1} feed): Retinal (A) 7,000 IU; Cholecalciferol (D3) 3,000 IU; Tocopherol (E) 1,500 mg; Menadione sodium bisulfite (K3) 30 mg; Thiamine (B1) 25 mg; Riboflavin (B2) 20 mg; Pyridoxine (B6) 25 mg; Cobalamin (B12) 0.02 mg; Niacin 100 mg; pantotenic acid 80 mg; Ascorbic acid (C) 200 mg; Biotin 1 mg; Folic acid 10 mg; Cu 25 mg; Fe 30 mg; Mn 30 mg; I 1 mg; Co 0.2 mg; Zn 100 mg; Se 0.35 mg

[2]Melamine: Chang Chun Petrochemical Co., Ltd., Taipei Taiwan (purity 99.5%)

[3]Cyanuric acid (98%): Sigma, Aldrich Co., Ltd.

Table 4-2. Proximate composition of the experimental diets (g kg^{-1} as dry matter basis, analyses of three batches of diet)

Ingredients (g kg^{-1} diet)	Diets (MEL+CYA, g kg^{-1} diet)						
	Diet 1 (Control)	Diet 2 (2.5+2.5)	Diet 3 (5+5)	Diet 4 (7.5+7.5)	Diet 5 (10+10)	Diet 6 (10+0)	Diet 7 (0+10)
Nitrogen content (N×6.25)	367.97±1.46	381.12±2.12	399.78±0.75	415.51±2.32	437.01±1.38	411.53±1.15	387.67±0.91
Crude lipid	77.71±1.33	78.24±2.12	75.95±1.47	77.70±0.82	78.47±0.13	73.90±1.75	75.43±1.34
Crude ash	65.17±1.43	63.90±0.52	70.24±2.11	67.88±2.70	63.09±4.01	65.81±1.29	67.90±1.52
Gross energy (MJ kg^{-1} diet)	15.86	15.86	15.86	15.86	15.86	15.86	15.86
MEL content[1]	0	2.3	4.5	7.3	9.7	10.1	0
CYA content[1]	0	2.2	4.2	7.1	9.5	0	9.6

[1] LC-MS/MS using a modified method of US-FDA LIB No. 4422 with HILIC chromatography (Smoker and Krynitsky, 2008)

4.3.4 Growth performance

At the beginning and at the end of the dietary treatment trial, the shrimp in each tank were counted and bulk weighed after immersing in low temperature water (22^{o}C) to reduce their physical activity. The mean final weight, number of shrimp, and feed intake were recorded. for each tank to calculate growth parameters, feed utilization and survival according to Kim et al. (2011) as follows:

Weight gain (g) = final body weight (g) - initial body weight (g)

Average daily gain (ADG, g shrimp^{-1} day^{-1}) = [final body weight (g) - initial body weight (g)] / days

Specific growth rate (SGR, % day^{-1}) = [(ln final body weight (g) - ln initial body weight (g)) / days] × 100

Feed conversion ratio (FCR) = feed consumption (g) / weight gain (g)

Feed intake (FI, g shrimp^{-1} day^{-1}) = [feed consumption (g) / number of shrimp] / days

Survival (%) = 100 × (final count of shrimp) / (initial count of shrimp)

4.3.5 Chemical composition analyses

At the beginning of the experiment, 20 shrimp were sampled and they were stored at -20°C until analysis of the initial proximate composition. At the end of the 10-weeks feeding trial, 15 shrimp per treatment group were randomly sampled for analyze the whole body composition. Moisture, nitrogen content, crude lipid and ash content in the whole body of shrimp were analyzed following the standard methods of AOAC (1995). Briefly, Dry matter was determined by drying the samples in hot air oven (Memmert UF110, Germany) at 105°C until constant weight. Nitrogen content (N x 6.25) was determined by Kjeldahl method using Kjeltec protein analyzer (Kjeltec^{TM} 8100, FOSS, Tecator, Sweden). Crude lipid was determined by methylene chloride extraction using Soxhlet system (Soxtec^{TM} 8000, China). Ash was measured by combustion in a muffle furnace (Gallenkamp Box Furnace, UK) at 550°C for 6 h.

4.3.6 Hemolymph analysis and immunological response

At the final sampling at week 10, hemolymph from individual shrimp (n=10/test group) was collected without the use of anticoagulants, after the final weights of the shrimp in all treatment groups were measured. Haemolymph was taken from the base of the 3rd walking leg of each shrimp, using a 1 mL syringe with 25gauge needle, and then immediately placed into sterile micro-centrifuge tubes. Soluble protein concentration in the supernatant was determined according to the method of Bradford (1976) using bovine serum albumin (BSA, Sigma-Aldrich) as a standard. Hemolymph parameters related to immune functions were analyzed by the methods described below.

4.3.5.1 Total hemocyte count (THC)

THC was determined according to the method of Supamattaya et al. (2005). Briefly, 50 µL of haemolymph was diluted with 450 µL pre-cooled (4°C) trypan blue solution (0.5% trypan blue in 2.6% NaCl) in sterile micro-centrifuge tubes. Hemocytes were counted using a haemocytometer (Bright-Line™, NY, USA) under a compound microscope (Olympus CH30, Tokyo, Japan) and recorded as the number of cells (total hemocytes mL^{-1}).

4.3.5.2 Phenoloxidase (PO) activity

PO activity was determined according to the method of Liu et al. (2004) with minor modifications. Briefly, haemolymph (100 µL) was mixed with an equal volume of cacodylate (CAC) buffer (10mM sodium cacodylate, 0.45M NaCl, 10mM $CaCl_2$, 0.26M $MgCl_2$, pH 7.4) in sterile micro-centrifuge tube and flushed with liquid nitrogen. The glaced mixture was homogenized using sterile plastic pestle, and centrifuged (Avanti™30, Beckman Coulter, CA, USA) at 12879×*g* for 10 min at 4 °C. The supernatant (25 µL) was collected and transferred to each well of a flat-bottomed 96-well plate. An enzyme reaction was started by adding 25 µL trypsin (Sigma-Aldrich, Buchs, Switzerland) (1 mg mL^{-1} in CAC buffer) and the well plate was incubated at 25°C for 2 minutes. After incubation, 50 µl of L-dihydroxyphenylalanine (L-DOPA, Sigma-Aldrich, Shanghai, China) (3 mg mL^{-1} in CAC buffer) and 150 µl of CAC buffer were added. The optical density at 490 nm was measured in kinetic mode every minute for a total of 30 minutes using a microplate spectrophotometer ($PowerWave_x$, Bio-Tek Instruments, VT,

USA). One unit of PO activity was defined as increasing the absorbance by 0.001/min, and the results are reported as unit min^{-1} mg $protein^{-1}$.

4.3.5.3 Respiratory burst activity

Respiratory burst activity was estimated following the method of Hsu and Chen (2007). Briefly, hemolymph (100 µL) was incubated with nitro-blue tetrazolium (NBT, Sigma-Aldrich, MO, USA) solution (0.3% in 1.5% NaCl). The mixture was discarded and the pellet was fixed with absolute methanol, and washed three times with 100 µL 70% methanol. After air drying in laminar air flow, formazan was dissolved by the addition of 120 µL 2 M KOH and 140 µL dimethyl sulfoxide (DMSO). The optical density at 630 nm was measured using a microplate reader, and is expressed as NBT-reduction in 100 µL haemolymph.

4.3.5.4 Lysozyme

Lysozyme activity was measured by its ability to clearance of target bacterial suspension during incubation. The suspension of 0.02% lyophilized *Micrococcus lysodeikticus* (Sigma-Aldrich, MO, USA) in 50 mM phosphate buffer saline at pH 6.2 was used as the substrate. Lysozyme activity was determined following the method of Shen et al. (2010). Briefly, hemolymph (25 µL) was mixed with 175 µL of the substrate in a flat-bottomed 96-well plate. The reaction was carried out at room temperature, and the absorbance at 490 nm was measured in kinetic mode every minute for a total of 10 minutes, using a microplate reader. One unit of lysozyme activity was defined as reducing the absorbance by 0.001 per min (Ellis 1990), and results are reported in unit mL^{-1}.

4.3.6 Antioxidant enzyme analysis

Antioxidant enzyme activities of catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD), were determined at end of the feeding trial. At the same time with hemolymph collection, the gill and the hepatopancreas of the same individual were dissected rapidly in an ice-cooled petri dish, and were then transferred to cryogenic vials (Corning®, Mexico City, Mexico) that were immediately stored in liquid nitrogen. Prior to analysis, the target tissue was individually homogenized (1:4, 1:2 w/v

for hepatopancreas and gill tissues, respectively) in ice-cold acetate extraction buffer (4 mM magnesium acetate, 80 mM potassium acetate, 20 mM Hepes, pH 7.5, 20% protease inhibitor, Amresco), and was then centrifuged at 12879×*g* at 4 °C for 15 min. The supernatant was transferred into a sterile micro-centrifuge tube for enzyme activities assay. Soluble protein content in the samples was determined by the method of Bradford (1976) using bovine serum albumin (Sigma-Aldrich, MO, USA) as the protein standard.

CAT activity was measured based on formaldehyde production from reacting the tissue extract with hydrogen peroxide (H_2O_2) (Trasviña-Arenas et al. 2013). Formaldehyde concentration was measured from colored complexes it forms with chromogenic agent, Purpald® (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole, Sigma-Aldrich, WI, USA), and was measured at 540 nm. The CAT activity was calculated using a standard curve calibrated with various known levels of formaldehyde concentrations (r^2=0.9993) and the results are expressed in unit min^{-1} mg $protein^{-1}$.

GPx and SOD activities were measured using a colorimetric assay kits (Cayman Chemical Company Inc., MI, USA). These enzyme activities were measured according to the assay protocols. Specific activities of the enzymes are expressed in unit min^{-1} mg $protein^{-1}$.

Ten shrimp per treatment group were euthanized by immersion in cold water until no movement of their appendages was observed. The shrimp were wiped with blotting paper to remove excess water and then individually weighed. The hepatopancreas was immediately collected and weighed to determine the hepato-somatic index (HSI) as follows:

HSI = 100 × [hepatopancreas weight (g)/shrimp body weight (g)]

Lipid peroxidation (LPO) in the hepatopancreas was measured as thiobarbituric acid reactive substances (TBARS) level, based on the reaction between the 2-thiobarbituric acid (TBA) and malondialdehyde (MDA). Measurements of LPO were performed according to the method as described by Senphan and Benjakul (2012). Hepatopancreas (~0.3 g) (n=10/feed group) were blended and gently mixed

with TBA solution (0.375% (w/v) TBA (Sigma-Aldrich, Steinheim, Germany), 15% (w/v) trichloroacetic acid (TCA) and 0.25M HCl). The homogenized mixtures were heated in a 95°C shaking water bath for 10 min, and were immediately cooled to room temperature with running tap water, and centrifuged at 4800×*g* for 10 min at 4 °C. The optical density of the samples was measured by a microplate reader at 532 nm. MDA concentration in each sample was calculated according to the eight-point standard curve (r^2=0.9997) for 1,1,3,3-tetramethoxypropane (TEP) (Sigma-Aldrich, Steinheim, Germany), and the results are expressed in nanomole (nmol) of MDA per gram of wet weight tissue.

4.3.7 Digestive enzyme analysis

The activities of digestive enzymes in shrimp hepatopancreas were measured at the end of feeding trial. The hepatopancreas (n=10/treatment) was dissected, weighed and homogenized with 50 mM Tris–HCl (pH 7.5). The homogenized mixture was then centrifuged at 12879×*g* at 4 °C for 15 min. For the different enzyme analyses, the supernatant was aliquoted in triplicate into cryogenic vials, to determine total protease, trypsin, and amylase. The vials were kept at -70 °C until analysis.

Total proteinase activity was estimated as suggested by Ávila-Villa et al. (2012). Briefly, sample extract (20 µL) was mixed with 500 µL of 2% azocasein (Sigma-Aldrich, MO, USA) and 240 µL of 50 mM Tris–HCl buffer (pH 7.5) in a test tube. The mixture was incubated at 25°C for 30 min. The reaction was stopped by adding 500 µL of 20% TCA and then the sample was centrifuged at 6500×*g* for 5 min. The optical density of the sample was measured using a microplate reader at 450 nm. The activity is expressed in Unit mg $protein^{-1}$. One unit of protease activity was defined based on the amount of enzyme that catalyzes the release of the azo dye, causing the change in absorbance at 450 nm of 0.001 per minute under assay conditions.

Trypsin activity was measured following the method of García-Carreño and Haard (1994) with some modification. Benzoil-Arg-*p*-nitroanilide (BAPNA) (Sigma-Aldrich, Tokyo, Japan) was used as the substrate. The reaction was started by mixing sample extract (30 µL) with 320 µL of 0.1 mM BAPNA. After incubating at 37°C for 20 min, the reaction was stopped by adding 50 µL of 20% TCA, and the mixture was then

centrifuged at 6500×*g* for 5 min. The absorbance was measured in a microplate reader at 410 nm. Trypsin activity was estimated as [(Abs410nm/min × volume (mL) of reaction mixture × dilution factor) / (8800 × volume (mL) of enzyme × mg protein of the sample extract)], where the constant 8800 is the extinction coefficient of *p*-nitroanilide at 410 nm under assay condition. The activity is expressed in Unit mg protein^{-1}. One unit of trypsin activity was defined as 1 µmol of *p*-nitroanilide released per minute under the specified conditions.

Amylase activity was measured with the 3,5-dinitrosalicylic acid (DNS) method (Rick and Stegbauer 1974) using 1% (w/v) starch solution as substrate. First, the sample extract (100 µL) was mixed with 100 µL of substrate and 1.8 mL phosphate buffer (0.1 M, pH 7) in a test tube. The mixture was incubated at 37 °C for 30 min. After incubation, the reaction was stopped by adding 2 mL DNS (Sigma-Aldrich, Bangalore, India), and then the sample was heated in boiling water for 5 min. After cooling with running tap water, the reaction mixture was diluted with distilled water and the optical density of the sample was measured at 540 nm. Amylase activity was calculated using a standard curve calibrated with various known levels of maltose. One unit of enzyme activity was defined as 1 mg of maltose liberated in 1 min at 37^{o}C.

4.3.8 Histological examination

At the end of the feeding trial, ten shrimp per treatment group were selected after euthanasia. Shrimp heads were cut in half lengthwise, and then immediately preserved in Davidson's fixative (Lightner et al. 2009). After fixation, samples were embedded in tissue embedding cassette and then were processed by automatic tissue processors (Leica, Nussloch, Germany). The samples were embedded into paraffin blocks and cut to 3-µm thickness with a sliding microtome (R. Jung AG, Heidelberg, Germany). The sections were stained with haematoxylin and eosin (H&E) (Bancroft 1967; Humason 1979). After staining, the sections were examined using light microscope (Olympus CH30, Olympus Corporation, Tokyo, Japan), and then a selected area in the stained section was imaged with an Olympus DP71 digital camera (Olympus Corporation, Tokyo, Japan).

4.3.9 Statistical analysis

The data are presented as mean ± standard deviation (SD). The statistical analyses used SPSS version 11.5 for Windows. One-way analysis of variance (ANOVA) was assessed for comparison between groups, and the means were compared with LSD post hoc method. Mean differences were considered statistically significant at $p<0.05$.

4.4 Results

4.4.1 Growth performance and feed efficiency

Survival rate of shrimp fed the control diet was significantly higher than that of shrimp fed the MEL alone diet ($p<0.05$), but these were not significantly different from the other treatments ($p>0.05$) (Table 4-3). Furthermore, the diets including MEL or CYA (singly or in combination) did not cause any obvious external abnormality. However, the FCR was significantly higher ($p<0.05$) in shrimp fed the diets 2-7 compared to those fed the control diet (Table 4-3). Shrimp receiving high doses of MEL (alone or with CYA, diets 4-6) had significantly ($p<0.05$) lower feed consumption than that of shrimp fed the control diet. The growth performances are shown in Table 4-3. The diets 2-7 gave significantly ($p<0.05$) lower final body weight, ADG, and SGR than the control treatment.

4.4.2 Proximate composition of whole shrimp body

The proximate composition of shrimp (whole body) is presented in Table 4-4. At the end of feeding trial, shrimp fed with the experimental diets, either individually or combined at various dose levels of MEL-CYA, had significant ($p<0.05$ differences on the whole body composition, including dry matter, crude protein, and crude lipid, deviating from the control group. It was noted that protein in the shrimp carcass was significantly ($p<0.05$) elevated whereas lipid content was significantly ($p<0.05$) reduced by the actual dietary treatments. However, crude ash was not significantly ($p>0.05$) different between the treatments.

Table 4-3. Growth parameters and nutrient utilization of Pacific white shrimp fed experimental diets for 10 weeks

Parameters	Diets (MEL+CYA, g kg^{-1} diet)						
	Diet 1 (Control)	Diet 2 (2.5+2.5)	Diet 3 (5+5)	Diet 4 (7.5+7.5)	Diet 5 (10+10)	Diet 6 (10+0)	Diet 7 (0+10)
Survival (% cumulative)							
Week 0-10	95.00±2.89	88.33±6.38	87.92±3.70	90.67±3.65	90.67±5.96	84.67±2.98	90.00±3.33
Average body weight (g $shrimp^{-1}$)							
Week 0	2.37±0.02	2.37±0.02	2.37±0.02	2.37±0.01	2.37±0.01	2.37±0.02	2.37±0.02
Week 10	13.65±0.43	11.62±0.34*	11.69±0.05*	11.33±0.68*	11.38±0.29*	10.65±0.43*	12.44±0.12*
Average daily gain (g $shrimp^{-1}$ day^{-1})							
Week 0-10	0.15±0.01	0.13±0.00*	0.13±0.01*	0.12±0.01*	0.12±0.00*	0.11±0.01*	0.14±0.00*
Specific growth rate (% day^{-1})							
Week 0-10	2.46±0.07	2.27±0.05*	2.31±0.08*	2.23±0.09*	2.24±0.04*	2.18±0.09*	2.30±0.15*
Feed conversion ratio							
Week 0-10	1.31±0.04	1.54±0.05*	1.41±0.03*	1.43±0.03*	1.43±0.05*	1.58±0.15*	1.48±0.07*
Feed intake (g $shrimp^{-1}$ day^{-1})							
Week 0-10	0.22±0.01	0.22±0.02	0.20±0.03	0.17±0.02*	0.17±0.03*	0.16±0.04*	0.21±0.02

Mean±S.D. from five replicates. Statistically (*) significant differences ($P<0.05$) between a particular treatment group and control.

Table 4-4. Whole body chemical composition of Pacific white shrimp fed experimental diets for 10 weeks

Parameters	Diets (MEL+CYA, g kg^{-1} diet)						
	Diet 1 (Control)	Diet 2 (2.5+2.5)	Diet 3 (5+5)	Diet 4 (7.5+7.5)	Diet 5 (10+10)	Diet 6 (10+0)	Diet 7 (0+10)
Whole body composition (g kg^{-1} wet weight basis) (mean±SD; n=3 shrimp from each of the 5 replicate tanks)							
Dry matter	245.33±0.21	248.70±0.92*	239.30±0.95*	231.23±0.21*	224.70±0.30*	221.83±0.85*	235.47±0.55*
Nitrogen content (N ×6.25)	181.78±0.19	202.75±0.70*	203.63±0.50*	195.25±1.18*	195.45±0.45*	192.08±1.07*	193.40±0.89*
Crude lipid	23.31±0.46	17.91±0.40*	17.48±0.18*	15.20±0.28*	13.93±0.45*	13.38±0.20*	17.12±0.58*
Crude ash	23.50±0.40	23.41±0.36	23.38±0.56	23.66±1.26	24.13±0.42	23.37±0.39	23.66±0.74

Mean±S.D. from five replicates. Statistically (*) significant differences ($P<0.05$) between a particular treatment group and control.

4.4.3 Hemolymph analysis and immunological response

Hemolymph analyses for the different treatment groups are presented in Table 4-5. Relative to the control group, the total hemocyte counts (THC) of shrimp receiving MEL were significantly decreased ($p<0.05$), but no signficant ($p>0.05$) difference was caused by CYA alone in the diet. PO activity in the shrimp received combined doses of MEL and CYA increased from the 5+5 to the 10+10 g kg^{-1} diet, and MEL dosing alone at 10 g kg^{-1} diet had significantly ($p<0.05$) lower PO than the control diet. However, no signficant ($p>0.05$) difference was found in shrimp fed 2.5+2.5 g kg^{-1} diet or with CYA dosing alone at 10 g kg^{-1} diet. Respiratory burst activity in shrimp fed the control diet was found signficantly ($p<0.05$) higher than with the actual treatments. Significantly ($p<0.05$) decreased lysozyme activity was found in the shrimp fed 10+10 g kg^{-1} diet combined dose of MEL and CYA, or MEL dosing alone at 10 g kg^{-1} diet.

4.4.4 Antioxidant enzyme activities

The antioxidant enzyme activities are presented in Table 4-6. Catalase (CAT) activity in both hepatopancreas and gill of shrimp fed the diet with MEL+CYA at 5+5 or 10+10 g kg^{-1} diet, or with MEL alone at 10 g kg^{-1} diet, were significantly ($p<0.05$) lower than with the control diet. Glutathione peroxidase (GPx) activity in hepatopancreas was significantly ($p<0.05$) lower in shrimp fed MEL+CYA at 7.5+7.5 or 10+10 g kg^{-1} diet, or with MEL alone at 10 g kg^{-1} diet compared to those fed the control diet, whereas the GPx activity in gill was significantly ($p<0.05$) affected only in the shrimp fed 10+10 g kg^{-1} diet combined dose of MEL+CYA or with MEL alone at 10 g kg^{-1} diet. Superoxide dismutase (SOD) activities in both hepatopancreas and gill were significantly ($p < 0.05$) lower in shrimp fed the MEL+CYA at 10+10 g kg^{-1} diet, or MEL alone at 10 g kg^{-1} diet.

The levels of lipid peroxidation (LPO) in the hepatopancreas was significantly ($p<0.05$) increased in the shrimp fed the combination MEL+CYA, increasing from 7.5+7.5 to 10+10 g kg^{-1} diet, or MEL dosing alone at 10 g kg^{-1} diet (Table 4-6). The hepato-somatic index (HSI) in shrimp fed combined dose of MEL+CYA increased from the 7.5+7.5 to the 10+10 g kg^{-1} diet, giving significantly ($p<0.05$) lower HSI than the control diet (Table 4-6).

4.4.5 Digestive enzyme activities

Digestive enzyme activities in hepatopancreas are presented in Table 4-6. Total protease and trypsin activities were significantly lower ($p<0.05$) in the shrimp fed combined MEL+CYA doses or the CYA dosing at 10 g kg^{-1} diet compared to those fed the control diet. Furthermore, the shrimp fed combined MEL+CYA doses from 7.5+7.5 to 10+10 g kg^{-1} diet had significantly ($p<0.05$) decreased amylase activity. Total protease, trypsin and amylase were affected by MEL dosing alone at 10 g kg^{-1} diet ($p<0.05$).

4.4.6 Histopathological changes

Histopathological alterations in the hepatopancreas of shrimp after 10-week dietary treatments containing MEL and CYA individually or in combination are presented in Figure 4-1. The normal cells of hepatopancreas, tubular morphology and B-(blister-like) cells, F-(fibrillar) cells, and R-(resorptive/absorptive) cells were found in the control group (Fig.4-1A). Degenerative hepatopancreatic tubules, severe atrophic changes, and lacking B-, F- and R-cells were found in shrimp fed combined MEL+CYA doses from 2.5+2.5 to 10+10 g kg^{-1} diet (Fig.4-1B-D). With MEL or CYA individually, degenerated and shrunken hepatopancreatic tubules were recorded (Fig.4-1E-F).

Table 4-5. Blood parameters of Pacific white shrimp fed experimental diets for 10 weeks

Parameters	Diets (MEL+CYA, g kg^{-1} diet)						
	Diet 1 (Control)	Diet 2 (2.5+2.5)	Diet 3 (5+5)	Diet 4 (7.5+7.5)	Diet 5 (10+10)	Diet 6 (10+0)	Diet 7 (0+10)
Total hemocyte counts (THC, $\times 10^7$ cells mL^{-1})	3.92±0.44	2.83±0.57*	3.11±0.59*	2.37±0.42*	2.30±0.39*	2.92±0.58*	3.58±0.37
Phenoloxidase (PO) activity (Unit min^{-1} mg $protein^{-1}$)	132.69±10.99	129.26±23.58	105.78±23.56*	81.74±14.20*	80.56±29.08*	78.76±13.65*	115.59±26.75
Respiratory burst activity (OD 630 nm)	0.063±0.008	0.055±0.007*	0.054±0.004*	0.055±0.006*	0.055±0.002*	0.054±0.005*	0.054±0.003*
Lysozyme activity (Unit mL^{-1})	16.75±3.44	16.50±3.45	16.59±3.58	13.41±4.05	12.17±2.71*	12.67±2.25*	16.33±4.52

Mean±S.D. from ten individual shrimp. Statistically (*) significant differences ($P<0.05$) between a particular treatment group and control.

Table 4-6. Antioxidant and digestive enzyme activities in hepatopancreas of Pacific white shrimp fed experimental diets for 10 weeks

Parameters	Target organs	Diets (MEL+CYA, g kg^{-1} diet)						
		Diet 1 (Control)	Diet 2 (2.5+2.5)	Diet 3 (5+5)	Diet 4 (7.5+7.5)	Diet 5 (10+10)	Diet 6 (10+0)	Diet 7 (0+10)
Antioxidant enzyme activities (Unit mg $protein^{-1}$)								
CAT	Hepatopancreas	189.17±25.83	159.50±37.82	136.61±26.32*	135.61±34.76*	138.82±35.91*	136.82±44.89*	165.59±28.16
	Gill	72.69±11.49	60.86±20.80	48.65±11.68*	49.30±19.88*	44.43±13.79*	46.60±9.20*	61.23±16.53
GPx	Hepatopancreas	5237.67±421.88	4927.92±615.59	4719.16±416.49*	4656.57±339.32*	4105.59±500.56*	4193.15±538.86*	4864.21±518.49
	Gill	684.63±101.97	564.66±142.52	593.87±103.82	555.10±159.37	512.00±65.45*	544.14±119.54*	589.13±80.61
SOD	Hepatopancreas	5.58±1.14	4.85±0.36	4.13±0.85	4.44±1.33	3.30±0.98*	2.18±0.59*	4.39±0.91
	Gill	6.75±0.35	6.02±0.87	5.89±0.769	5.77±0.86	5.59±0.69*	5.56±0.37*	6.30±0.40
Lipid peroxidation (LPO) in hepatopancreas (nmol g^{-1} wet weight tissue) and hepato-somatic index (HSI)(%)								
LPO	Hepatopancreas	9.86±2.53	11.47±2.08	11.59±2.74	13.03±2.51*	13.10±2.75*	13.04±2.64*	9.95±2.69
HIS	Hepatopancreas	6.14±0.66	6.24±1.40	5.57±0.64	5.21±0.90*	5.20±0.88*	5.41±0.48*	6.00±1.24
Digestive enzyme activities (Unit mg $protein^{-1}$)								
Total protease	Hepatopancreas	0.160±0.026	0.128±0.037*	0.107±0.026*	0.110±0.024*	0.120±0.016*	0.123±0.021*	0.137±0.031
Trypsin	Hepatopancreas	0.176±0.018	0.137±0.026*	0.131±0.043*	0.124±0.021*	0.131±0.020*	0.128±0.023*	0.148±0.034
Amylase	Hepatopancreas	23.25±2.68	22.85±5.00	20.34±4.52	18.35±3.49*	19.11±2.57*	16.53±3.56*	23.49±4.69

Mean±S.D. from ten individual shrimp. Statistically (*) significant differences ($P<0.05$) between a particular treatment group and control.

CAT: catalase, GPx: glutathione peroxidase, SOD: superoxide dismutase

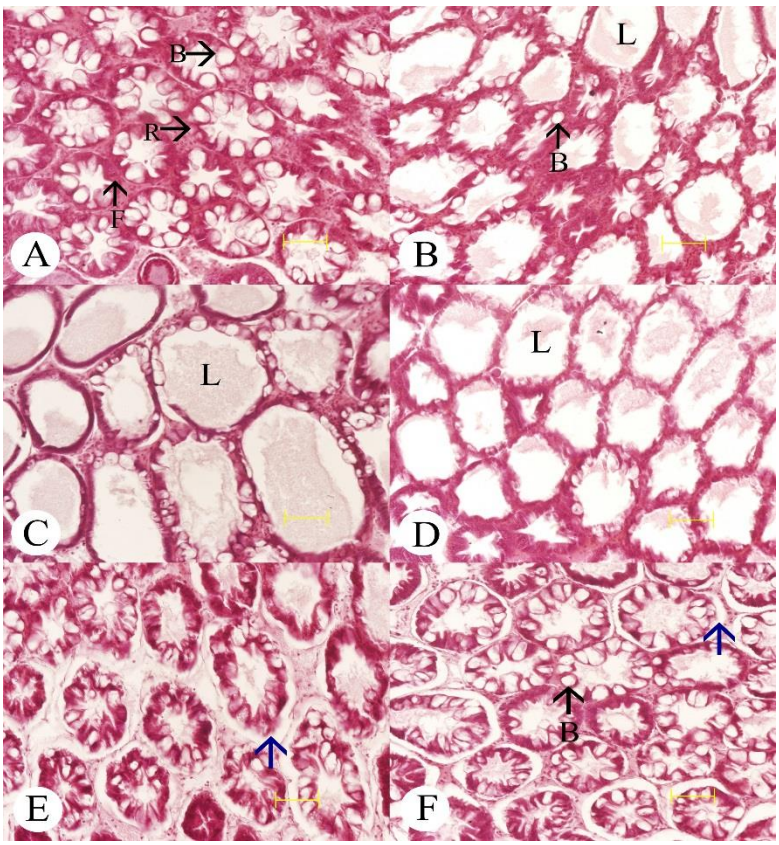

Fig.4-1. Photomicrographs of haematoxylin and eosin (H&E) stained sections of hepatopancreas from Pacific white shrimp fed diets containing various levels of MEL and/or CYA for 10 weeks. (A) The sections of hepatopancreatic tubules of shrimp received the control diet showing normal structure of B (“blasenzenllen”) cells, R (“restzellen”) cells and F (“fibrillenzellen” or fibrous) cells (Magnification ×200; Scale bar = 100 μm); (B) Tubular degeneration and atrophy were observed in shrimp fed combined MEL+CYA doses at 2.5+2.5 g kg^{-1} diet (L=lumen, Magnification ×200; Scale bar = 100 μm); (C) Development of severe atrophic changes of hepatopancreatic tubule were found in shrimp fed combined MEL+CYA doses at 5+5 g kg^{-1} diet (L=lumen, Magnification ×200; Scale bar = 100 μm); (D) shrimp receiving MEL+CYA doses at 10+10 g kg^{-1} diet showed degenerative changes in the hepatopancreas (L=lumen, Magnification ×200; Scale bar = 100 μm); (E-F) Degenerative changes and shrunken hepatopancreatic tubules (blue arrow) were found in shrimp receiving MEL dosing alone (10+0 g kg^{-1} diet) (E) or CYA dosing alone (0+10 g kg^{-1} diet) (f) (Magnification ×200; Scale bar = 100 μm).

4.5 Discussion

Since the pet food crisis in 2007, MEL and its analogues including CYA have been illegally used to elevate the apparent total protein content, thereby adulterating certain protein-rich feed ingredients (Brown et al. 2007; Puschner et al. 2007; Stine et al. 2011). There have been reports of MEL and related analogues in feed ingredients and in animal feeds for swine, poultry, fish, and shrimp (Andersen et al. 2008; Stine et al. 2012). Negative effects from such adulteration in animals have been reported, having as symptoms nephrotoxicity, growth retardation, discoloration, and immune suppression (Baynes et al. 2008; Reimschuessel et al. 2008; 2010; Xue et al. 2011; Phromkunthong et al. 2013; 2015a; 2015b). However, data relating to the toxicity in penaeid shrimp of MEL or CYA exposures is limited. In the present study, the toxicity of MEL and CYA to Pacific white shrimp was carried out under laboratory conditions for 10 weeks. The results from this study demonstrate depressed growth and negative effects by dietary MEL-CYA. In the present study, shrimp fed combined MEL+CYA doses from 2.5+2.5 to 10+10 g kg^{-1} diet had adverse effects on growth and feed conversion ratio. As previously reported, growth retardation is a clinical sign of the toxicity of combined MEL and CYA that has been reported in mice (Lv et al. 2013) and in some farmed fish species, such as Asian seabass (Phromkunthong et al. 2015b) and red tilapia (Phromkunthong et al. 2013; 2015a). Furthermore, the combination of MEL and CYA is highly toxic than exposure to either chemical separately (Karbiwnyk et al. 2010). However, in the present study, the body weight of shrimp receiving MEL alone at 10 g kg^{-1} diet was significantly lower than that of the shrimp fed the control diet. In another experimental study the growth performance and feed utilization of black tiger shrimp (*Peneaus monodon*) were not significantly affected by dietary MEL doses ranging from 0.005 to 0.5 g kg^{-1} diet for 11 weeks (Samranrat et al. 2011). Therefore, the recommended dosage of MEL for Pacific white shrimp should not exceed 0.5 g kg^{-1} diet. Furthermore, the results in this study indicate that shrimp exposure to dietary CYA decreased growth. To date, available data on adulteration by CYA alone in shrimp feed is limited. In our previous study, Phromkunthong et al. (2015b) reported no significant

difference in the growth of Asian seabass between receiving CYA alone at 10 g kg^{-1} diet and control. That the reported results tend to differ qualitatively demonstrates that sensitivity to these contaminants strongly depends on the species. In fish and mammals, the toxic effects of MEL and CYA individually or in combination are directly linked to renal pathology and functions (Dorne et al. 2013). One possible reason for the growth retardation and poor nutrient utilization is directly related to the hepatopancreas dysfunction, which is an important organ for several metabolic pathways of nutrient utilization in crustaceans, including digestion, absorption, and storage (Burgos-Hernández et al. 2005; Zhao et al. 2017). Activities of the digestive enzymes are an important indicator of the diet composition, nutrient quality, feeding habits, digestive functions, and nutrient utilization, which directly relate to shrimp growth (Li et al. 2008a; Zokaeifar et al. 2012; Duan et al. 2017).

The metabolic function of digestive enzymes in shrimp can be influenced by nutrient composition in the diet (Córdova-Murueta and García-Carreño 2002; Song et al. 2017). In the present study, the reductions of digestive enzyme activities (especially of protease) were marked in shrimp receiving MEL alone or in combination with CYA. However, available reports on effects of MEL+CYA on the digestive enzymes in shrimp are limited. In some cases exposure to toxic substances (e.g. pesticides, toxins and heavy metals) can adversely affect the digestive organ and function (Li et al. 2008b; Chiodi Boudet et al. 2015). Moreover, the results in this study found that shrimp fed combined MEL+CYA doses up to 7.5+7.5 g kg^{-1} diet or MEL dosing alone had significantly decreased enzyme activities, indicating poor nutrient utilization. Similar to these results, Seebaugh et al. (2011) reported a significant decrease in digestive enzyme activities of shrimp that was related to reducing food consumption.

Furthermore, hepatopancreas is a vital organ for the detoxification of toxic substances in shrimp and is sensitive to stress responses (Bautista et al. 1994; Yu et al. 2016). Exposure to chronic stress induces reactive oxygen species (ROS) production leading to an imbalance between ROS production and antioxidant defense, which results in cellular oxidative damage (Yang et al. 2010; An et al. 2015; Lee et al.

2016). In this study, the activities of CAT, GPx and SOD were significantly decreased in both gill and hepatopancreas of shrimp fed the highest combined level of MEL-CYA (10:10 g kg^{-1} diet) or MEL alone (10 g kg^{-1} diet). This demonstrates that the main toxic effects of MEL and CYA take place via oxidative stress, resulting in dysfunction of the related detoxification organs and cellular oxidative damage in shrimp, which adversely affects growth performance (Li et al. 2008a). Strong evidence of oxidative stress induced by the combination of MEL and CYA in the diet has been reported by Lv et al. (2013), who found that the SOD activity in mouse kidney was decreased after 13-weeks exposure to a mixture of MEL and CYA. Furthermore, You et al. (2012) found significantly decreased total antioxidant capacity (T-AOC) and SOD activity in mouse testes after oral administration of MEL and CYA mixture for 28 days.

Malondealdehyde (MDA) is generally used as lipid peroxidation indicators in cells and tissues of animals (Zenteno-Savín et al. 2006; Liu et al. 2011; Chiodi Boudet et al. 2015; Liang et al. 2016). The results from this study showed that the MDA content in the hepatopancreas of shrimp fed combined MEL+CYA doses up to 7.5+7.5 g kg^{-1} diet, or MEL dosing alone, was significantly increased from that with the control diet. This indicates oxidative damage induced by these substances. Similarly, Lee et al. (2016) reported an increased of MDA levels in mouse kidney after co-exposure to MEL and CYA, which also caused apoptosis in the renal tubular cells and suppressed antioxidant enzyme activities. In MEL-exposed animals, An et al. (2015) reported increased MDA levels in the hippocampus after oral administration of MEL to male Wistar rats (dosage 300 mg kg^{-1} animal day^{-1} for 28 days). Moreover, there are several reports on significantly increased MDA levels by oxidative stress in many aquatic animals. Liang et al. (2016) reported that MDA levels in the hepatopancreas of Pacific white shrimp significantly increased after exposure to ammonia for 96h.

The hepato-somatic index (HSI) is an important parameter indicative of hepatopancreas conditions, as well as of glycogen reserve. In the present study, HSI was significantly decreased in shrimp fed MEL alone or in combination with CYA at 7.5+7.5 or 10+10 g kg^{-1} diet, relative to the control group. This demonstrates that long-

term dietary supplementation with MEL and CYA to shrimp caused extra energy expenditures in detoxification. Moreover, reduced energy storage may have been induced indirectly through anorexia caused by MEL or CYA (Khalil et al. 2017).

Hemolymph is an important indicator of shrimp performance, welfare, health status, and rearing conditions, and is closely linked to the innate immune system (Li et al. 2008a; Chen et al. 2015; Qiu et al. 2016). In crustaceans, THC and PO system play an important roles in the immune system, especially in shrimp (Sritunyalucksana and Söderhäll, 2000; Li et al. 2008a; Wanlem et al. 2011). In this study, the immune parameters in shrimp fed diets supplemented with MEL alone or in combination with CYA at the highest dose (10+10 g kg^{-1} diet) had decreased THC and PO activities. Moreover, Yin et al. (2016) reported that MEL alone or its combination with CYA appeared to be toxic to the immune response of mice.

The results from this study demonstrate that nitrogen (protein) content in the whole body of shrimp treated with a combination of MEL and CYA, or either of these singly, was significantly increased as compared to those of shrimp fed the control diet. In general, the nitrogen content in the whole body of an animal, including shrimp and fish, is determined by the dietary protein quality, digestibility, and feeding (Halver and Hardy 2002; Kureshy and Davis 2002). The increased nitrogen retention indicates improved protein utilization positively affecting growth performance (Glencross et al. 2007). In present study, in contrast to growth, increased nitrogen content was found in shrimp received with MEL and CYA individually or in combination. These results are similar to previous reports regarding red tilapia (Phromkunthong et al. 2013; 2015a) and Asian seabass (Phromkunthong et al. 2015b), indicating that the protein contents in the whole body and in various parts of fish, such as fillet or viscera, was directly affected by dietary MEL+CYA. Therefore, the increased nitrogen content of shrimp whole body in the MEL+CYA treated group may be attributed to the accumulation of nitrogen from these substances. Karbiwnyk et al. (2010) reported accumulation of CYA in edible tissues of shrimp fed with 1666 or 3333 mg kg^{-1} diet CYA (approximately 55 and 124 mg kg^{-1} body weight) in their diet, respectively. However, the mechanism of these

substances in a shrimp is not well understood. Therefore, the pharmacokinetics/dynamics of MEL and CYA need to be further studied.

Histopathological analysis of hepatopancreas has been used as a practical indicator for assessing the nutrient utilization and toxic substance accumulation in the shrimp (Qiu et al. 2016). According to this study, MEL and CYA directly affect the hepatopancreas. In fish, the formation of melamine-cyanurate crystals in kidney is the main pathological sign of chronic exposure to MEL and CYA contamination, and leads to renal nephropathy (Reimschuessel et al. 2010; Pirarat et al. 2012; Phromkunthong et al. 2015a; 2015b). Base on the previous report in Thailand, “big head syndrome” or “water sac syndrome” have been reported to be pathological signs in shrimp exposed to MEL and CYA contamination (Limsuwan et al. 2009; WHO 2009). Similarly, Lightner et al. (2009) also reported that shrimp received MEL and CYA in their diets could have insoluble crystals in the antennal glands, obstructing and impairing the excretory system and causing swollen cephalothorax. Moreover, the results of present study indicate severe histopathological alterations including severe atrophic changes and cell necrosis in hepatopancreas of shrimp exposed to MEL and CYA.

4.6 Conclusions

This study examined the responses of Pacific white shrimp, *L. vannamei,* to MEL and CYA exposures either individually or in combination in order to understand the mechanisms of toxicity. Results of this study indicate that growth performance and nutrient utilization were adversely affected by MEL and CYA exposure, with reductions in body weight gain, ADG, SGR and an increase in the FCR. Exposure to these substances also damaged the cells of hepatopancreas, leading to the generation of oxidative stress, increased lipid peroxidation and decreased antioxidant and suppress immune responses, which indicare that the hepatopancreas of shrimp is highly sensitive to MEL and CYA. In summary, these results provide some basic knowledge for a toxicological risk assessment of MEL and CYA in the context of shrimp farming. It is noted that further studies should evaluate the accumulation of these

substances in the edible part of shrimp to address public concerns of health risks to consumers.

4.7 References

Amaya E, Davis DA, Rouse DB (2007) Alternative diets for the Pacific white shrimp *Litopenaeus vannamei*. Aquaculture 262:419-425.

An L, Fu J, Zhang T (2015) Reversible effects of vitamins C and E combination on cognitive deficits and oxidative stress in the hippocampus of melamine-exposed rats. Pharmacology Biochemistry & Behavior 132:152-159.

Andersen WC, Turnipseed SB, Karbiwnyk CM, Clark SB, Madson MR, Gieseker CM, Miller RA, Rummel NG, Reimschuessel R (2008) Determination and confirmation of melamine residues in catfish, trout, tilapia, salmon, and shrimp by liquid chromatography with tandem mass spectrometry. Journal of Agricultural and Food Chemistry 56:4340-4347.

AOAC (Association of Official Analytical Chemists) (1995) Official methods of analysis of AOAC International. 16th Edition. Association of Analytical Communities, Arlington, VA.

Ávila-Villa LA, Fimbres-Olivarria D, García-Sánchez G, Gollas-Galván T, Hernández-López J, Martínez-Porchas M (2012) Physiological and immune responses of white shrimp (*Litopenaeus vannamei*) infected with necrotizing hepatopancreatitis bacterium. Aquaculture 324–325:14-19.

Bancroft JD (1967) Histochemical Techniques. Butterworths, London.

Bautista MN, Lavilla-Pitogo CR, Subosa PF, Begino ET (1994) Aflatoxin B1 contamination of shrimp feeds and its effects on growth and hepatopancreas and pre-adult *Penaeus monodon*. Journal of the Science of Food and Agriculture 65:5-11.

Baynes RE, Smith G, Mason SE, Barrett E, Barlow BM, Riviere JE (2008) Pharmacokinetics of melamine in pigs following intravenous administration. Food and Chemical Toxicology 46:1196-1200.

Bradford MM (1976) A rapid and sensitive method for the quantitation of micro-gram

quantities of protein using the principle of protein–dye binding. Analytical Biochemistry 72:248-254.

Brown CA, Jeong KS, Poppenga RH, Puschner B, Miller DM, Ellis AE, Kang KI, Sum S, Cistola AM, Brown SA (2007) Outbreaks of renal failure associated with melamine and cyanuric acid in dogs and cats in 2004 and 2007. Journal of Veterinary Diagnostic Investigation 19:525-531.

Burgos-Hernández A, Farias SI, Torres-Arreola W, Ezquerra-Brauer JM (2005) In vitro studies of the effects of aflatoxin B1 and fumonisin B1 on trypsin-like and collagenase-like activity from the hepatopancreas of white shrimp (*Litopenaeus vannamei*). Aquaculture 250:399-410.

Byrne J (2016) Thai authorities find melamine in imported feed. Available at: https://www.feednavigator.com/Article/2016/10/27/Thai-authorities-find-melamine-in-imported-feed. (Accessed 5 January 2019).

Carvalho RAPLFde, Ota RH, Kadry VO, Tacon AGJ, Lemos D (2016) Apparent digestibility of protein, energy and amino acids of six protein sources included at three levels in diets for juvenile white shrimp *Litopenaeus vannamei* reared in high performance conditions. Aquaculture 465:223-234.

Casu A, Camino G, De Giorgi M, Flath D, Morone V, Zenoni R (1997) Fire-retardant mechanistic aspects of melamine cyanurate in polyamide copolymer. Polymer Degradation and Stability 58:297-302.

CCAC (Canadian Council on Animal Care) (2005) The CCAC guidelines on: the care and use of fish in research, teaching and testing. CCAC, Ottawa, ON.

Chen YY, Chen JC, Lin YC, Yeh ST, Huang CL (2015) White Shrimp *Litopenaeus vannamei* That Have Received *Gracilaria tenuistipitata* Extract Show Early Recovery of Immune Parameters after Ammonia Stressing. Marine Drugs 13:3606-3624.

Chiodi Boudet LN, Polizzi P, Romero MB, Robles A, Marcovecchio JE, Gerpe MS (2015) Histopathological and biochemical evidence of hepatopancreatic toxicity caused by cadmium in white shrimp, *Palaemonetes argentinus*. Ecotoxicology and

Environmental Safety 113:231-240.

Córdova-Murueta JH, García-Carreño FL (2002) Nutritive value of squid and hydrolyzed protein supplement in shrimp feed. Aquaculture 210:371-384.

Dorne JL, Doerge DR, Vandenbroeck M, Fink-Gremmels J, Mennes W, Knutsen HK, Vernazza F, Castle L, Edler L, Benford D (2013) Recent advances in the risk assessment of melamine and cyanuric acid in animal feed. Toxicology and Applied Pharmacology 270:218-229.

Duan Y, Zhang Y, Dong H, Zheng X, Wang Y, Li H, Liu Q, Zhang J (2017) Effect of dietary poly-β-hydroxybutyrate (PHB) on growth performance, intestinal health status and body composition of Pacific white shrimp *Litopenaeus vannamei* (Boone, 1931). Fish and Shellfish Immunology 60:520-528.

Ehling S, Tefera S, Ho IP (2007) High-performance liquid chromatographic method for the simultaneous detection of the adulteration of cereal flours with melamine and related triazine by-products ammeline, ammelide, and cyanuric acid. Food additives and contaminants 24:1319-1325.

Ellis AE (1990) Lysozyme assay. In: Stolen JS, Fletcher TC, Anderson DP, Roberson BS, van Muiswinkel WB (eds) Technique in fish immunology, SOS Publications, Fair Haven, NJ, pp. 101-103.

European Commission (2013) Commission Regulation (EU) No 107/2013 of 5 February 2013 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council as regards maximum levels for melamine in canned pet food. Official Journal of the European Union, Brussels.

FAO (Food and Agriculture Organization of the United Nations) (2016) The State of World Fisheries and Aquaculture 2016. FAO, Rome.

García-Carreño FL, Haard N (1994) Preparation of an exopeptidase enriched fraction from the hepatopancreas of decapods. Process Biochemistry 29:663-670.

Glencross BD, Booth M, Allan GL (2007) A feed is only as good as its ingredients: a review of ingredient evaluation strategies for aquaculture feeds. Aquaculture Nutrition 13:17-34.

Halver JE, Hardy RW (2002) Fish Nutrition. Academic Press, San Diego, CA.

Hsu SW, Chen JC (2007) The immune response of white shrimp *Penaeus vannamei* and its susceptibility to *Vibrio alginolyticus* under sulfide stress. Aquaculture 271:61-69.

Humason G.L. (1979) Animal Tissue Techniques. W.H. Freeman and Company, San Francisco.

JAVMA (2007) Melamine adulterates component of pellet feeds. Journal of the American Veterinary Medical Association 231:17.

Karbiwnyk CM, Williams RR, Andersen WC, Turnipseed SB, Madson MR, Miller KE, Reimschuessel R (2010) Bioaccumulation of cyanuric acid in edible tissues of shrimp following experimental feeding. Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment 27:1658-1664.

Khalil SR, Awad A, Ali SA (2017) Melamine and/or formaldehyde exposures affect steroidogenesis via alteration of StAR protein and testosterone synthetic enzyme expression in male mice. Environmental Toxicology and Pharmacology 50:136-144.

Kim J, Nhut T, Hai T, Ra C (2011) Effect of dietary essential oils on growth, feed utilization and meat yields of white leg shrimp *L. vannamei*. Asian-Australasian Journal of Animal Sciences 24:1136-1141.

Kureshy N, Davis DA (2002) Protein requirement for maintenance and maximum weight gain for the Pacific white shrimp, *Litopenaeus vannamei*. Aquaculture 204:125-143.

Lee IC, Ko JW, Park SH, Shin IS, Moon C, Kim SH, Kim YB, Kim JC (2016) Melamine and cyanuric acid co-exposure causes renal dysfunction and structural damage via MAPKs and mitochondrial signaling. Food and Chemical Toxicology 96:254-262.

Li E, Chen L, Zeng C, Yu N, Xiong Z, Chen X, Qin JG (2008a) Comparison of digestive and antioxidant enzymes activities, haemolymph oxyhemocyanin contents and hepatopancreas histology of white shrimp, *Litopenaeus vannamei*, at various salinities. Aquaculture 274:80-86.

Li N, Zhao Y, Yang J (2008b) Effects of water-borne copper on digestive and metabolic enzymes of the giant freshwater prawn *Macrobrachium rosenbergii*. Archives of Environmental Contamination and Toxicology 55:86-93.

Liang Z, Liu R, Zhao D, Wang L, Sun M, Wang M, Song L (2016) Ammonia exposure induces oxidative stress, endoplasmic reticulum stress and apoptosis in hepatopancreas of pacific white shrimp (*Litopenaeus vannamei*). Fish and Shellfish Immunology 54:523-528.

Lightner DV, Pantoja CR, Redman RM, Hasson KW, Menon JP (2009) Case reports of melamine-induced pathology in penaeid shrimp fed adulterated feeds. Diseases of aquatic organisms 86:107-112.

Limsuwan C, Chuchird N, Prasertsri S, Wongmaneeprateep S, Laisutisan K, Wiriyapattanasub P, Limhang K (2009) *Gross and histopathological changes of Pacific white shrimp (Litopenaeus vannamei) experimentaly induced with contaminated of melamine plus cyanuric acid in pelleted feed*. In: Proceedings of 47th Kasetsart University Annual Conference: Fisheries. 2009 March 17-20; Bangkok: Kasetsart University. (Article in Thai).

Liu XL, Xi QY, Yang L, Li HY, Jiang QY, Shu G, Wang SB, Gao P, Zhu XT, Zhang YL (2011) The effect of dietary *Panax ginseng* polysaccharide extraction the immune responses in white shrimp, *Litopenaeus vannamei*. Fish and Shellfish Immunology 30:495-500.

Liu CH, Yeh ST, Cheng SY, Chen JC (2004) The immune response of the white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio* infection in relation with the moult cycle. Fish and Shellfish Immunology 16:151-161.

Lv Y, Liu Z, Tian Y, Chen H (2013) Effect on morphology, oxidative stress and energy metabolism enzymes in the testes of mice after a 13-week oral administration of melamine and cyanuric acid combination. Regulatory Toxicology and Pharmacology 65:183-188.

Mahardika K, Mastuti I, Zafran Z (2017) Histopathological study on nephropathy caused by oral administration with melamine and cyanuric acid in humpback grouper

(*Cromileptes altivelis*). Aquaculture, Aquarium, Conservation & Legislation - International Journal of the Bioflux Society 10: 328-334.

Maule AG, Gannam AL, Davis JW (2007) Chemical contaminants in fish feeds used in federal salmonid hatcheries in the USA. Chemosphere 67:1308-1315.

O'Keefe T, Campabadal CA (2015) Storage and handling of feeds for fish and shrimp. In: Davis DA (ed), Feed and Feeding Practices in Aquaculture, Woodhead Publishing, Oxford.

Pacini N, Prearo M, Abete MC, Brizio P, Dörr AJ, Reimschuessel R, Andersen W, Gasco L, Righetti M, Elia AC (2013) Antioxidant responses and renal crystal formation in rainbow trout treated with melamine administered individually or in combination with cyanuric acid. Journal of Toxicology and Environmental Health, Part A 76:491-508.

Pettersson H (2012) Mycotoxin contamination of animal feed. In: Gremmels JF (ed), Animal Feed Contamination, Woodhead Publishing, Oxford.

Phromkunthong W, Choochuay P, Kiron V, Nuntapong N, Boonyaratpalin M (2015a) Pathophysiological changes associated with dietary melamine and cyanuric acid toxicity in red tilapia. Journal of Fish Diseases 38:161-173.

Phromkunthong W, Nuntapong N, Boonyaratpalin M, Kiron V (2013) Toxicity of melamine, an adulterant in fish feeds: experimental assessment of its effects on tilapia. Journal of Fish Diseases 36:555-568.

Phromkunthong W, Nuntapong N, Wanlem S, Boonyaratpalin M (2015b) A study on growth, histopathology and oxidative stress in Asian sea bass on diets with various loadings of melamine and cyanuric acid adulterants. Aquaculture 435:336-346.

Pirarat N, Katagiri T, Chansue N, Ponpornpisit A, Endo M, Maita M (2012) The pathological effects of melamine and cyanuric acid in the diet of walking catfish (*Clarius batrachus*). Journal of Comparative Pathology 147:259-266.

Puschner B, Poppenga RH, Lowenstine LJ, Filigenzi MS, Pesavento PA (2007) Assessment of melamine and cyanuric acid toxicity in cats. Journal of Veterinary

Diagnostic Investigation 19:616-624.

Qiu M, Wang Y, Wang X, Sun L, Ye R, Xu D, Dai Z, Liu Y, Bi S, Yao Y, Gooneratne R (2016) Effects of T-2 toxin on growth, immune function and hepatopancreas microstructure of shrimp (*Litopenaeus vannamei*). Aquaculture 462:35-39.

Rick W, Stegbauer HP (1974) Alpha amylase measurement of reducing groups. In: Bergmeyer HV (ed), Methods of enzymetic analysis, Academic Press, New York.

Reimschuessel R, Evans ER, Stine CB, Hasbrouck N, Mayer TD, Nochetto C, Gieseker CM (2010) Renal crystal formation after combined or sequential oral administration of melamine and cyanuric acid. Food and Chemical Toxicology 48:2898-2906.

Reimschuessel R, Gieseker CM, Miller RA, Ward J, Boehmer J, Rummel N, Heller DN, Nochetto C, de Alwis GK, Bataller N, Andersen WC, Turnipseed SB, Karbiwnyk CM, Satzger RD, Crowe JB, Wilber NR, Reinhard MK, Roberts JF, Witkowski MR (2008) Evaluation of the renal effects of experimental feeding of melamine and cyanuric acid to fish and pigs. American Journal of Veterinary Research 69:1217-1228.

Roviello G, Ricciotti L, Ferone C, Ferone C, Colangelo F, Tarallo O (2015) Fire resistant melamine based organic-geopolymer hybrid composites. Cement and Concrete Composites 59:89-99.

Samranrat N, Muangyao P, Thongrod S, Paengmee R, Yaponha L (2011) Effect of Melamine in Feed on Growth, Survival Rate, Feed Conversion Ratio and Pathological Changes in Black Tiger Shrimp (*Peneaus monodon* Fabricius, 1798). Technical Paper No. 8/2011. Coastal Aquatic Feed Research Institute, Department of Fisheries, Bangkok, (Article in Thai).

Seebaugh DR, L'Amoreaux WJ, Wallace WG (2011) Digestive toxicity in grass shrimp collected along an impact gradient. Aquatic Toxicology 105:609-617.

Senphan T, Benjakul S (2012) Compositions and yield of lipids extracted from hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*) as affected by prior autolysis. Food Chemistry 134:829-835.

Shen WY, Fu LL, Li WF, Zhu YR (2010) Effect of dietary supplementation with *Bacillus*

subtilis on the growth, performance, immune response and antioxidant activities of the shrimp (*Litopenaeus vannamei*). Aquaculture Research 41:1691-1698.

Smoker M, Krynitsky AJ (2008) Interim Method for Determination of Melamine and Cyanuric Acid Residues in Foods Using LC-MS/MS. Version 1.0. US FDA/CFSAN Laboratory Information Bulletin LIB No. 4422. US-FDA, Maryland.

Song HL, Tan BP, Chi SY, Liu Y, Chowdhury MAK, Dong XH (2017) The effects of a dietary protease-complex on performance, digestive and immune enzyme activity, and disease resistance of *Litopenaeus vannamei* fed high plant protein diets. Aquaculture Research 48:2550-2560.

Sritunyalucksana K, SÖderhäll K (2000) The proPO and clotting system in crustaceans. Aquaculture 191:53-69.

Stine CB, Nochetto CB, Evans ER, Gieseker CM, Mayer TD, Hasbrouck NR, Reimschuessel R (2012) Depletion of melamine and cyanuric acid in serum from catfish *Ictalurus punctatus* and rainbow trout *Onchorhynchus mykiss*. Food and Chemical Toxicology 50:3426-3432.

Stine CB, Reimschuessel R, Gieseker CM, Evans ER, Mayer TD, Hasbrouck NR, Tall E, Boehmer J, Gamboa da Costa G., Ward JL (2011) A No Observable Adverse Effects Level (NOAEL) for pigs fed melamine and cyanuric acid. Regulatory Toxicology and Pharmacology 60:363-372.

Supamattaya K, Kiriratnikom S, Boonyaratpalin M, Borowitzka L (2005) Effect of a *Dunaliella* extract on growth performance, health condition, immune response and disease resistance in black tiger shrimp (*Penaeus monodon*). Aquaculture 248:207-216.

Thai Agricultural Standards TAS 7401-2009 2009. National Bureau of Agricultural Commodity and Food Standards, Ministry of Agriculture and Cooperatives. “Good Aquaculture Practices for Marine Shrimp Farm” in the Royal Gazette. vol. 126 (Section 187D, Dated 28 December B.E. 2552, 2009).

Trasviña-Arenas CH, Garcia-Triana A, Peregrino-Uriarte AB, Yepiz-Plascencia G (2013) White shrimp Litopenaeus vannamei catalase: Gene structure, expression and

activity under hypoxia and reoxygenation. Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology 164:44-52.

US-FDA (U.S. Food and Drug Administration) (2007) Interim Melamine and Analogues Safety/Risk Assessment Peer Review Report. United States Food and Drug Administration, Washington, DC.

Wanlem S, Supamattaya K, Tantikitti C, Prasertsan P, Graidist P, 2011. Expression and applications of recombinant crustacean hyperglycemic hormone from eyestalks of white shrimp (*Litopenaeus vannamei*) against bacterial infection. Fish and Shellfish Immunology 30:877-885.

WHO (World Health Organization) (2009) Background Paper on Occurrence of Melamine in Foods and Feed. Ottawa Canada. WHO, Geneva.

World Bank (2013) Fish to 2030: Prospects for Fisheries and Aquaculture. Agriculture and Environmental Services Discussion Paper No. 3 World Bank Group, Washington DC.

Xue J, Ai Q, Mai K, Xu W, Yang Y, Liufu Z (2011) Effects of melamine on growth performance and skin color of darkbarbel catfish (*Pelteobagrus vachelli*). Aquaculture 320:142-146.

Yang JJ, Yang Z, Zhang T (2010) Action potential changes associated with impairment of functional properties of sodium channels in hippocampal neurons induced by melamine. Toxicology Letters 198:171-176.

Yin RH, Li XT, Wang X, Li HS, Yin RL, Liu J, Dong Q, Wang WC, Yuan J, Liu BS, Han XH, He JB, Bai WL (2016) The effects of melamine on humoral immunity with or without cyanuric acid in mice. Research in Veterinary Science 105:65-73.

You H, Zhu JF, She RP, Chang LL, Shi RH, Ding Y, Chi LJ, Liu B, Yue Z, Tian JJ, Mao JJ, Su LF (2012) Induction of Apoptosis in the Immature Mouse Testes by a Mixture of Melamine and Cyanuric Acid. Journal of Integrative Agriculture 11:2058-2066.

Yu YY, Chen SJ, Chen M, Tian LX, Niu J, Liu YJ, Xu DH (2016) Effect of cadmium-polluted diet on growth, salinity stress, hepatotoxicity of juvenile Pacific white shrimp (*Litopenaeus vannamei*): Protective effect of Zn(II)-curcumin. Ecotoxicology

and Environmental Safety 125:176-183.

Zenteno-Savín T, Saldierna R, Ahuejote-Sandoval M (2006) Superoxide radical production in response to environmental hypoxia in cultured shrimp. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 142:301-308.

Zhao W, Wang L, Liu M, Jiang K, Wang M, Yang G, Qi C, Wang B (2017) Transcriptome, antioxidant enzyme activity and histopathology analysis of hepatopancreas from the white shrimp *Litopenaeus vannamei* fed with aflatoxin B1(AFB1). Developmental and Comparative Immunology 74:69-81.

Zokaeifar H, Balcázar JL, Saad CR, Kamarudin MS, Sijam K, Arshad A, Nejat N (2012) Effects of *Bacillus subtilis* on the growth performance, digestive enzymes, immune gene expression and disease resistance of white shrimp, *Litopenaeus vannamei*. Fish and Shellfish Immunology 33:683-689.

CHAPTER 5

Effects of Melamine and Cyanuric Acid on Growth Performance, Blood Immune Parameters and Histological Alterations in Hybrid *Clarias* Catfish (*Clarias macrocephalus* (Günther) x *C. gariepinus* (Burchell))

5.1 Abstract

Since the contamination of animal feeds with melamine (MEL) and cyanuric acid (CYA) has been announced in many countries, it resulted in a significant economic impact and consumer confidence. Several studies have been performed to investigate the pathological effects of these substances in various kinds of aquatic animal. Therefore, the objective of this study was to investigate the effects of MEL and CYA singly and in combination on growth performance, blood immune parameters and histological alterations in hybrid *Clarias* catfish (*Clarias macrocephalus* (Günther) x *C. gariepinus* (Burchell)). The experiment comprised of 7 treatments with 3 replicates each. Seven isonitrogenous (350 g kg^{-1}) and isolipidic (70 g kg^{-1}) diets were formulated as follows diet 1 (a control diet without MEL and CYA); diets 2-5 (with MEL+CYA at 2.5+2.5, 5+5, 7.5+7.5, or 10+10 g kg^{-1} diet); diet 6 and diet 7 (with only MEL or CYA at 10 g kg^{-1} diet, respectively). Fingerling catfish with an initial weight of 6.00±0.02 g were randomly assigned into glass aquaria at a stocking density of 15 fish per aquaria. Experimental diets were given twice daily to apparent satiation for 8 weeks. The results from this study indicated that final body weight and specific growth rate (SGR) were significantly ($P<0.05$) lower in fish that exposed with combined MEL+CYA or each alone relative to the control. Compared to control, feed intake was significantly decreased ($P<0.05$) in fish exposed with combined MEL+CYA diets or each alone, while FCR remained unaltered ($P>0.05$) with all treatments. Fish received with combined MEL+CYA diets or each alone developed darkening of the skin, while fish fed control diet did not showed any significant abnormality of skin color. Dietary MEL-CYA did not affect ($P>0.05$) the red blood cell counts, hemoglobin and hematocrit values among fish fed different diets. In contrast, significant decreases were recored in white blood cell counts and NBT

reduction value of MEL-CYA treated group compared to control, except for lysozyme activity which significant differences ($P<0.05$) were found in MEL+CYA at 7.5+7.5, 10+10 and MEL alone 10 g kg^{-1} diet compared to control. Dietary MEL-CYA induced hepato-renal toxicity in fish. The melamine-cyanurate crystals were detected in renal tubules of fish that exposed with combined MEL+CYA diets, whereas fish received only MEL or CYA alone did not have such crystals in the kidneys. In addition, dietary MEL-CYA induced oxidative sress and lipid peroxidation, evidenced by the significant changes of the liver and kidney antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) and heat shock protein (Hsp70) intensity. These findings raise concerns about MEL-CYA contamination in aquatic feed and these substances should be strictly monitored to ensure feed safety, therefore, reducing risk to either animals or consumers.

5.2 Introduction

Melamine (1,3,5-triazine-2,4,6-triamine; abbreviated as MEL) is organic nitrogenous compound which contains up to 67 percent of nitrogen by mass (Bischoff, 2011). For industrial purposes, MEL is extensively used as starting materials for manufacture of thermoplastic materials, kitchenware, fire retardant and coating compound (Onac *et al*., 2018). According to U.S. Food and Drug Administration (US-FDA, 2007), the use of MEL is not approved for supplementation in either foods for human or animal feeds. Unfortunately, due to higher amount of nitrogen content, MEL has been illegally used by unethical manufacturer for commercial purposes to falsely increased protein apparent in requiring samples. In general, the routine analysis of protein content in sample is performed by multiplication amounts of nitrogen with conversion factor and then converting into protein content (US-FDA, 2007). This method has the possible disadvantage of not being recognized the derivation of nitrogen sources. Therefore, high protein feed ingredients are highly susceptible to MEL contamination. Major outbreaks of MEL contamination have been reported between 2004 and 2007 in United States and Canada when wheat gluten, rice protein and corn

gluten imported from China were found contaminated with MEL (Brown *et al*., 2007). In recent years, the toxicity of MEL and its derivatives i.e. cyanuric acid (CYA) has been extensively studied to understand the mechanism of MEL toxicity. Several studies reported that MEL induces a pathological damage in liver and kidney, which are important organs for detoxification and excretion in animal. Also, MEL has been associated with impaired nutrient utilization that related to growth retardation.

Hybrid *Clarias* catfish is a favorite fish for consumers due to their good taste, inexpensive and nutritional value. In 2016, the production of hybrid *Clarias* catfish in Thailand was 102,400 tonnes, accounting for 28% of the total freshwater fish production (Department of Fisheries, 2018). Therefore, this fish make an appropriate representative for examining the effects of MEL and/or CYA on freshwater fish. Recently, oncerns about the contamination of MEL and its derivatives in aquatic feeds are linked to a large variety of products in aquaculture industries. Therefore, the aim of this study was to evaluate the toxicity of MEL and CYA, either singly or in combination in hybrid *Clarias* catfish through the analysis of parameters related to growth performance, feed efficiency, oxidative stress-related enzymes and histopathology of liver and kidney.

5.3 Materials and Methods

5.3.1 Test animals

Hybrid *Clarias* Catfish, *Clarias macrocephalus* (Günther) x *C. gariepinus* (Burchell) were obtained from standard commercial farm located in Songkhla province, Thailand. Prior to the experiment, fish were acclimated under laboratory conditions in 1,000 L fiberglass tank and they were fed with control diet (MEL-CYA-free diet) twice daily (08:30 and 15:30) to apparent satiation. After acclimatization for two weeks, fish were fasted for 24 h, hand sorted and weighed after being anesthetized with eugenol (50 mg L^{-1}). Fish with an average body weight 6.00±0.02 g (mean±SD, n = 315) were randomly allocated in 184 L glass aquarium tanks at a density of 15 fish per tank. Three replicate tanks were assigned to each treatment. Fish were hand-fed to apparent satiation twice daily (08:30 and 15:30) for 8 weeks. The water quality during experimental period were maintained within the acceptable ranges according to the

guidelines for good aquaculture practices for freshwater animal farm (Thai agricultural commodity and food standard TAS 7417(G)-2016), water temperature and pH were measured daily and their values were in the range of 25-32°C and 6.5-8.5, respectively. Dissolved oxygen (DO) was kept at about 7 mg L^{-1}, total ammonia concentration was below 0.5 mg L^{-1}. This experiment was conducted following to the guidelines of the Committee on Care and Use of Experimental Animal for handling and sampling to minimize the stress on fish.

5.3.2 Experimental diets

MEL (purity > 99.5%) was supplied by Chang Chun Petrochemical Co. Ltd., Taiwan and CYA (purity > 98%) was obtained commercially from Sigma-Aldrich (St Louis, MO, USA). Seven experimental diets were formulated m*et al*l nutritional requirement of hybrid *Clarias* catfish according to the recommendations by the Department of Fisheries, Thailand. MEL-CYA (in g kg^{-1} diet) were supplementations as follow: control diet (without supplementation of MEL or CYA, diet1), 2.5+2.5 (diet 2), 5+5 (diet 3), 7.5+7.5 (diet 4), 10+10 (diet 5), 10+0 (diet 6) and 0+10 (diet 7), with a corresponding substitution in cellulose content. Diets were pelleted using Hobart Legacy® Mixer (OH, USA). After processing, pellets were packed in low-density polyethylene bags and stored in a refrigerator until used. Proximate composition of the experimental diets was analyzed according to the standard methods of AOAC (1995). Details of feed ingredients and diet proximate composition are presented in Table 1.

5.3.3 Growth performance

At the end of the experiment, all fishes in each tank were individually weighted using a digital balance after anesthesia with 50 mg L^{-1} clove oil. Growth parameters including weight gain (WG, g $fish^{-1}$), specific growth rate (SGR, % day^{-1}), feed conversion ratio (FCR), were calculated using below equations. Survival rate (SR, %) was recorded by counting daily mortality in each tank and was calculated for each treatment by following the equations

Specific growth rate (SGR, % day^{-1}) = 100 [Ln(Mean final body weight, g) - Ln(Mean initial body weight, g)] / feeding days

Feed conversion ratio (FCR) = dry feed intake (g) / [final biomass (g) - initial biomass (g) + biomass of the dead fish (g)]

Survival (SR, %) = 100× (final amount of fishes) / (initial amount of fishes)

5.3.4 Sampling and chemical analyses

At the beginning of the experiment, 20 fishes were scarified with an overdose of anesthetic and they were stored frozen (-20°C) until analysis of the initial proximate composition. At the end of the feeding trial, 3 fishes from each tank (9 fishes per treatment group) were collected after fasting for 24 h to analyze the whole body composition. Nitrogen, crude lipid, moisture and ash content in the whole body were determined following the procedures of AOAC (1995). Briefly, Dry matter was determined by drying the samples in hot air oven (Memmert UF110, Germany) at 105°C until constant weight. Nitrogen content (N x 6.25) was determined by Kjeldahl method using Kjeltec protein analyzer (Kjeltec™ 8100, FOSS, Tecator, Sweden). Crude lipid was determined by methylene chloride extraction using Soxhlet method (Soxtec™ 8000, China). Ash was measured after combustion in a muffle furnace (Gallenkamp Box Furnace, UK) at 550°C for 6 h.

Table 5-1. Compositions of experimental diets

Ingredients (g kg^{-1} diet)	Diets (MEL+CYA, g kg^{-1} diet)						
	Diet 1 (Control)	Diet 2 (2.5+2.5)	Diet 3 (5+5)	Diet 4 (7.5+7.5)	Diet 5 (10+10)	Diet 6 (10+0)	Diet 7 (0+10)
Fishmeal	250	250	250	250	250	250	250
De-hulled soybean meal	250	250	250	250	250	250	250
Ground pea nut	150	150	150	150	150	150	150
Rice bran	100	100	100	100	100	100	100
Rice flour	147	147	147	147	147	147	147
Fish oil : soybean oil (1:1)	20	20	20	20	20	20	20
Vitamin & mineral premix[1]	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Di-calcium phosphate	10	10	10	10	10	10	10
Choline chloride	1	1	1	1	1	1	1
Inositol	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Alpha-starch	50	50	50	50	50	50	50
Microcrystalline Cellulose	20	15	10	5	0	10	10
Melamine (MEL)[2]	0	2.5	5	7.5	10	10	0
Cyanuric acid (CYA)[3]	0	2.5	5	7.5	10	0	10

[1] Vitamin & Mineral premix deliver the following in unit kg^{-1} diet: Retinal (A) 8,000 IU; Cholecalciferol (D3) 1,500 IU; Tocopherol (E) 100 mg; Menadione sodium bisulfite (K3) 5 mg; Thiamine (B1) 10 mg; Riboflavin (B2) 15 mg; Pyridoxine (B6) 15 mg; Cobalamin (B12) 0.02 mg; Niacin 80 mg; Calcium pantothenate 40 mg; Ascorbic acid (C) 150 mg; Biotin 0.5 mg; Folic acid 4 mg; Cu 5 mg; Fe 30 mg; Zn 40 mg; Mn 25 mg; Co 0.05 mg; I 1 mg; Se 0.25 mg.

[2] Melamine : Chang Chun Petrochemical CO.,LTD, Taipei Taiwan (purity 99.5%).

[3] Cyanuric acid (98%) : Sigma-Aldrich CO., LTD.

Table 5-2. Proximate composition of experimental diets (g kg^{-1} as fed basis, analyses of three batches of diet given as mean±SD)

Proximate composition (g kg^{-1} diet)	Diets (MEL+CYA, g kg^{-1} diet)						
	Diet 1 (Control)	Diet 2 (2.5+2.5)	Diet 3 (5+5)	Diet 4 (7.5+7.5)	Diet 5 (10+10)	Diet 6 (10+0)	Diet 7 (0+10)
Nitrogen content (N×6.25)	355.62±2.55	373.51±9.19	381.69±2.52	416.10±4.03	430.16±1.67	390.68±0.61	373.10±9.68
Crude lipid	69.39±1.89	73.94±0.57	72.03±0.46	72.62±0.25	70.53±1.59	71.63±2.57	67.48±1.61
Crude ash	91.69±1.76	90.89±1.37	86.18±0.73	85.76±1.03	83.68±0.23	89.67±0.28	83.03±0.43
Gross energy (MJ kg^{-1} diet)	17.13	17.13	17.13	17.13	17.13	17.13	17.13
MEL content[1]	0	2.5	4.7	8.0	9.9	10.8	0
CYA content[1]	0	2.6	4.9	7.7	10.5	0	10.6

[1] LC-MS/MS using a modified method of US-FDA LIB No. 4422 with HILIC chromatography (Smoker and Krynitsky, 2008)

5.3.5 Hematological Characteristics

At the end of experiment, 3 fishes from each tank were euthanized by an overdose of clove oil. Blood samples were immediately collected from the puncture of caudal vein using 1 mL syringe with 25 gauge needles and were transferred into 1.7 mL sterile micro centrifuge tubes. Red blood cell count, (RBC) and white blood cell count (WBC) were counted using Bright-Line™ Hemacytometer (Hausser Scientific, USA) after diluted 1:200 with Yokoyama's solution by using diluting pipette (Boeco, Germany) (Blaxhall and Daisley, 1973). Hemoglobin levels (Hb, g dL^{-1}) were analyzed following the Drabkin's cyanmethemoglobin method. A standard curve was performed using two-fold serial dilutions of human hemoglobin at ranging from 13.100 to 0.205 g dL^{-1} and the absorbance was measured with a Multiskan™ GO microplate spectrophotometer (Thermo Fisher Scientific, USA) at 540 nm (Larsen and Snieszko, 1961). The hematocrit (Ht, %) values were analyzed by microcentrifuge technique, using heparinized micro-hematocrit capillary tube (Vitrex, Denmark). Briefly, blood samples were placed in capillary tube and centrifuged by micro-hematocrit centrifuge (model 4203; ALC International, Italy) at 10,000 rpm for 5 min and the values were calculated as percentage of packed red blood cells in a centrifuged column of whole blood (Blaxhall and Daisley, 1973). The nitroblue tetrazolium (NBT) reduction was determined according to the method as described by Anderson and Siwiki (1995). Briefly, 50 µL of the blood was mixed with 30 µL of acid citrate dextrose (ACD) in flat-bottom 96 well plate and incubated at 37°C for 1 h to allow adhesion of cells. After incubation time, the supernatant was discharged and the cells were washed three times with 0.01 M phosphate buffered saline, pH 7.2. Afterward, 50 µL of 0.2% NBT (Sigma, USA) in 0.85% normal saline was added into the wells and incubated at 37°C for 1 h. Then, the supernatant was discharged and the cells were fixed with 50 µL of absolute methanol for 3 minutes and then washed three times with 100 µL 70% methanol. The plates were dried in the laminar flow cabinet for 5 minutes before 120 µL of 2 N potassium hydroxide (KOH) and 140 µL of dimethyl sulphoxide (DMSO) were added into the wells to dissolve

the formazan crystals. The absorbance was measured at 620 nm using 120 µL of 2 N KOH and 140 µL of DMSO as blank. NBT reduction was reported as OD 620 nm.

Remaining part of blood samples were allowed to clot for 15 minutes at room temperature. The clot samples were centrifuged at 5,300×*g* for 15 min at 4°C (Beckman Coulter, Inc., Allegra X-30R, Germany) and supernatant (serum) was collected for biochemical parameters analysis. Lysozyme activity was determined by the turbidimetric methods according to Demers and Bayne (1997) based on lysis of gram positive bacterial cell walls, *Micrococcus lysodeikticus* (Sigma, USA). In brief, 25 µL of serum was mixed with 175 µL of bacterial suspension (0.2 mg/mL in 0.05 M phosphate buffer, pH 6.2). The absorbance (**Δ**OD) at 450 nm was measured at 1 minute intervals for 10 minutes using Multiskan™ GO microplate spectrophotometer (Thermo Fisher Scientific, USA). One unit of lysozyme activity was expressed as the amount of enzyme producing a decrease in absorbance of 0.001 min^{-1} ml^{-1} of serum.

5.3.6 Determination of antioxidant enzyme, Hsp70 and lipid oxidation in liver and kidneys

5.3.6.1 Sample preparation

At the end of the experiment after fasted for 24 h, 3 fishes from each tank were randomly captured and euthanized by an overdose of clove oil. Samples of liver and kidney were collected into cryogenic tubes (Corning, Mexico) and kept in liquid nitrogen until analyzed. Each tissue was weighted and homogenized using a sterile pestle in ice cold acetate buffer, pH 7.5, plus 20% protease inhibitor (Amresco, USA) under an ice bath. Afterward, the homogenates were centrifuged at 12,879 ×*g* at 4°C for 10 min. The supernatant was transferred into 1.7 mL microcentrifuge tube and kept at -80°C until analyzed.

5.3.6.2 Determination of antioxidant enzyme

The activity of superoxide dismutase (SOD, EC1.15.1.1) and glutathione peroxidase (GPx, EC 1.11.1.9) in fish tissues were determined by the assay kit (Cayman Chemical, USA) according to the instructions given by the manufacturer. The activity of catalase (CAT, EC1.11.1.6) was determined following to the method as described by

Trasviña-Arenas *et al*. (2013) using Purpald® (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole, Sigma-Aldrich, USA) as chromogen. Specific activities of the enzymes are expressed in unit mg protein^{-1}.

5.3.6.3 Determination of Hsp70

Western blot analysis was performed according to the modified method of Köhler *et al*. (2009). The homogenates samples were mixed with a sodium dodecyl sulfate (SDS) loading buffer (10 mM Tris, 3% SDS (w/v), 0.3% mercaptoethanol (w/v), 20% glycerol (w/v), 0.005% bromophenol blue (w/v)), and boiled for 5 min. Equal amount of protein samples (100 micrograms) were loaded to a 12% polyacrylamide gel, and electrophoresis was conducted at 80 V for 15 min followed by 120 V for 60 min. Then, separated proteins were transferred to nitrocellulose membrane (Santa Cruz Biotechnology, USA) using semi-dry blotting (Thermo Scientific, USA) at 180 mA for 1 h. The membranes were blocked 50% horse serum in TBS (50 mM Tris pH 7.5, 150 mM NaCl) for 1 h, and then washed three times with TBS. The membranes were incubated overnight at 4°C with a primary antibody against Hsp70 (mouse anti-human IgG, Thermo Scientific, USA). Afterward, the membranes were incubated for 2 h at 4°C with secondary antibody (goat anti-mouse IgG, Dianova, Germany). For detection, 4-chloro-1-naphthol was used as the peroxidase substrate and the relative intensities of the Hsp70 bands were measured by ImageJ software (NIH, Bethesda, MD).

5.3.6.4 Determination of lipid oxidation in liver and kidneys

Lipid peroxidation was determined as thiobarbituric acid reactive substances (TBARS) assay according to the method of Buege and Aust (1978), which measures the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) to produce pink compounds. Briefly, each sample (~0.1 g) was homogenized with TBA reagent (0.375% (w/v) TBA (Sigma-Aldrich, Germany), 15% (w/v) trichloroacetic acid, TCA and 0.25N HCl). The reaction mixtures were heated at 95°C for 15 minutes. After cooling down in cold water, samples were centrifuged at 5,300 ×*g* for 10 minutes at 4 °C, and optical density was measured by a microplate spectrophotometer at 532 nm. A standard curve was prepared using 1,1,3,3-tetramethoxypropane (TEP) and the levels of TBARS were expressed as micromole of MDA equivalents g^{-1} tissue.

5.3.7 Histopathological alteration

At the end of 8 weeks, 3 fish from each tank were collected after euthanasia with an overdose of clove oil. Liver and kidney were immediately dissected for estimation of histopathological alteration. All tissue samples were fixed by immersion in 10% neutral buffer formalin for 48 h and stored in 70% ethanol until processing. Afterward, the samples were processed by standard histological procedures using an automatic tissue processor (Leica TP1020, Germany), and then embedded in paraffin (Leica EG1150C, China). The samples were sectioned at 5 µm thicknesses using a rotary microtome (Leica EG1150H, China) before stained with Haematoxylin & Eosin (H&E) (Bancroft, 1967). The histopathological examination of processed slides was evaluated through Olympus PROVIS AX-70 microscope (Olympus Corporation, Japan) and images were captured with an Olympus DP71 digital camera and software (Olympus Corporation, Japan).

5.3.8 Statistical analysis

Statistical analyses of all collecting data were analyzed by using SPSS statistical software version 11.5 for Windows. Data are presented as mean±standard deviation (SD). Data were tested for normality (Shapiro-Wilks test) and homogeneity of variance (Levene's F test) prior to one-way analysis of variance (ANOVA). The differences between means were compared by the Tukey test at a 95% interval of confidence ($P<0.05$).

5.4 Results

5.4.1 Growth performance

Growth and survival rate of hybrid *Clarias* catfish measured at the end of experiment are presented in Table 5-3. Survival rate of fish in all treatments ranged from 91.11 to 100%, and there were no significant differences ($P>0.05$) among treatments (Table 5-3). Growth performances of fish were significantly affected by dietary MEL and CYA levels. Fish fed control diet obtained significant higher ($P<0.05$) average body weight and SGR compared to those in other groups (Table 5-3). No significant

differences (P>0.05) among treatments were founded for FCR values (Table 5-3). A significantly lower (P<0.05) feed intake (FI) was observed during the first six weeks of the experiment in fish fed with MEL and CYA supplemented diets, either singly or combination compared to fish fed the control diet. During week 6-8, FI of fish fed control diet was significantly higher (P<0.05) than fish fed with MEL-CYA combination, or with MEL alone. However, no significant different (P>0.05) was found from fish fed with CYA alone (Table 5-3).

5.4.2 Assessment of skin discoloration

During this experiment, fish fed control diet did not showed any abnormality of skin color (Figure 5-1A). However, skin discoloration, including darkening of skin of hybrid *Clarias* catfish was markedly affected by dietary MEL and CYA supplementation (Figure 5-1B-G). After receiving experimental diet for 2 weeks, occurrence of skin discoloration was recorded in all treatment groups (Fig 5-1: B-G and Table 5-4). During week 4-6, fish fed MEL+CYA at 10+10 g kg^{-1} diet has the highest percentage of skin discoloration (Figure 5-1: E and Table 5-4), however, there were no significant differences (P>0.05) when compared to those of fish fed MEL+CYA at 5+5, 7.5+7.5 and 10+0 g kg^{-1} diet (Figure 5-1C, D and F, respectively and Table 5-4). At the end of experiment, the percentage of skin discoloration of fish fed MEL+CYA at 2.5+2.5 g kg^{-1} diet was 53.85% (Figure 5-1B), which significantly increased (P<0.05) to 79.52, 81.06, 87.62 and 76.75% for fish fed MEL+CYA at 5+5, 7.5+7.5, 10+10 and 10+0 g kg^{-1} diet, respectively (Figure 5-1C-F and Table 5-4).

Table 5-3. Growth, feed efficiency and survival rate of hybrid *Clarias* catfish fed diets with different levels of MEL and CYA for 8 weeks (values are given as mean±SD; n = 3 replicate tanks)

Parameters	Diets (MEL-CYA, g kg^{-1} diet)						
	Diet 1 (Control)	Diet 2 (2.5+2.5)	Diet 3 (5+5)	Diet 4 (7.5+7.5)	Diet 5 (10+10)	Diet 6 (10+0)	Diet 7 (0+10)
Survival (% cumulative)							
Week 0-8	100.00±0.00^{a}	95.56±7.70^{a}	97.78±3.85^{a}	93.33±6.67^{a}	91.11±10.18^{a}	95.56±7.70^{a}	97.78±3.85^{a}
Average body weight (g $fish^{-1}$)							
Initial	6.00±0.01^{a}	6.00±0.03^{a}	6.00±0.01^{a}	6.00±0.04^{a}	6.00±0.04^{a}	6.00±0.04^{a}	6.01±0.02^{a}
Final	54.29±2.77^{b}	41.16±2.79^{a}	41.68±4.56^{a}	40.95±0.55^{a}	43.84±1.24^{a}	36.32±4.25^{a}	44.52±2.71^{a}
Specific growth rate, SGR (% day^{-1})							
Week 0-8	3.93±0.09^{c}	3.43±0.12^{ab}	3.45±0.20^{ab}	3.43±0.02^{ab}	3.55±0.06^{ab}	3.21±0.20^{a}	3.57±0.10^{b}
Feed conversion ratio, FCR							
Week 0-8	1.26±0.09^{a}	1.38±0.11^{a}	1.28±0.13^{a}	1.39±0.09^{a}	1.28±0.04^{a}	1.47±0.10^{a}	1.42±0.04^{a}
Feed intake, FI (g $fish^{-1}$)							
Week 0-2	7.36±0.68^{b}	4.73±0.96^{a}	5.21±0.26^{a}	5.15±0.13^{a}	5.31±0.35^{a}	4.34±0.20^{a}	5.75±0.62^{a}
Week 2-4	12.33±1.12^{b}	8.41±0.68^{a}	8.35±0.44^{a}	8.40±0.14^{a}	8.42±0.41^{a}	7.39±0.22^{a}	8.25±0.28^{a}
Week 4-6	19.49±0.38^{b}	13.29±1.31^{a}	11.72±2.50^{a}	12.98±0.14^{a}	13.88±0.51^{a}	12.28±0.58^{a}	13.96±0.91^{a}
Week 6-8	19.60±1.85^{b}	15.53±0.88^{a}	14.40±0.64^{a}	14.90±0.76^{a}	15.56±1.67^{a}	14.36±0.40^{a}	16.32±2.03^{ab}

Values within the same row sharing a common superscript are not significantly different by the Tukey test (P>0.05).

Table 5-4. Percentage of skin discoloration of hybrid *Clarias* catfish fed diets with different levels of MEL and CYA for 8 weeks (values are given as mean±SD; n = 3 replicate tanks)

Parameters	Diets (MEL+CYA, g kg^{-1} diet)						
	Diet 1 (Control)	Diet 2 (2.5+2.5)	Diet 3 (5+5)	Diet 4 (7.5+7.5)	Diet 5 (10+10)	Diet 6 (10+0)	Diet 7 (0+10)
Percentage of skin discoloration (%)							
Week 2	0 ± 0^{a}	17.78 ± 3.85^{b}	51.11 ± 3.85^{c}	48.89 ± 3.85^{c}	51.28 ± 4.01^{c}	46.67 ± 6.67^{c}	46.67 ± 6.67^{c}
Week 4	0 ± 0^{a}	35.56 ± 3.85^{b}	71.11 ± 3.85^{c}	71.49 ± 6.27^{c}	73.23 ± 1.79^{c}	65.30 ± 4.76^{c}	58.86 ± 12.16^{c}
Week 6	0 ± 0^{a}	48.89 ± 3.85^{b}	75.56 ± 3.85^{cd}	78.84 ± 5.65^{d}	80.63 ± 2.44^{d}	72.31 ± 5.20^{cd}	61.42 ± 11.36^{bc}
Week 8	0 ± 0^{a}	53.85 ± 7.45^{b}	79.52 ± 0.82^{d}	81.06 ± 3.16^{d}	87.62 ± 4.83^{d}	76.75 ± 3.34^{cd}	61.42 ± 11.36^{bc}

Values within the same row sharing a common superscript are not significantly different by the Tukey test ($P>0.05$).

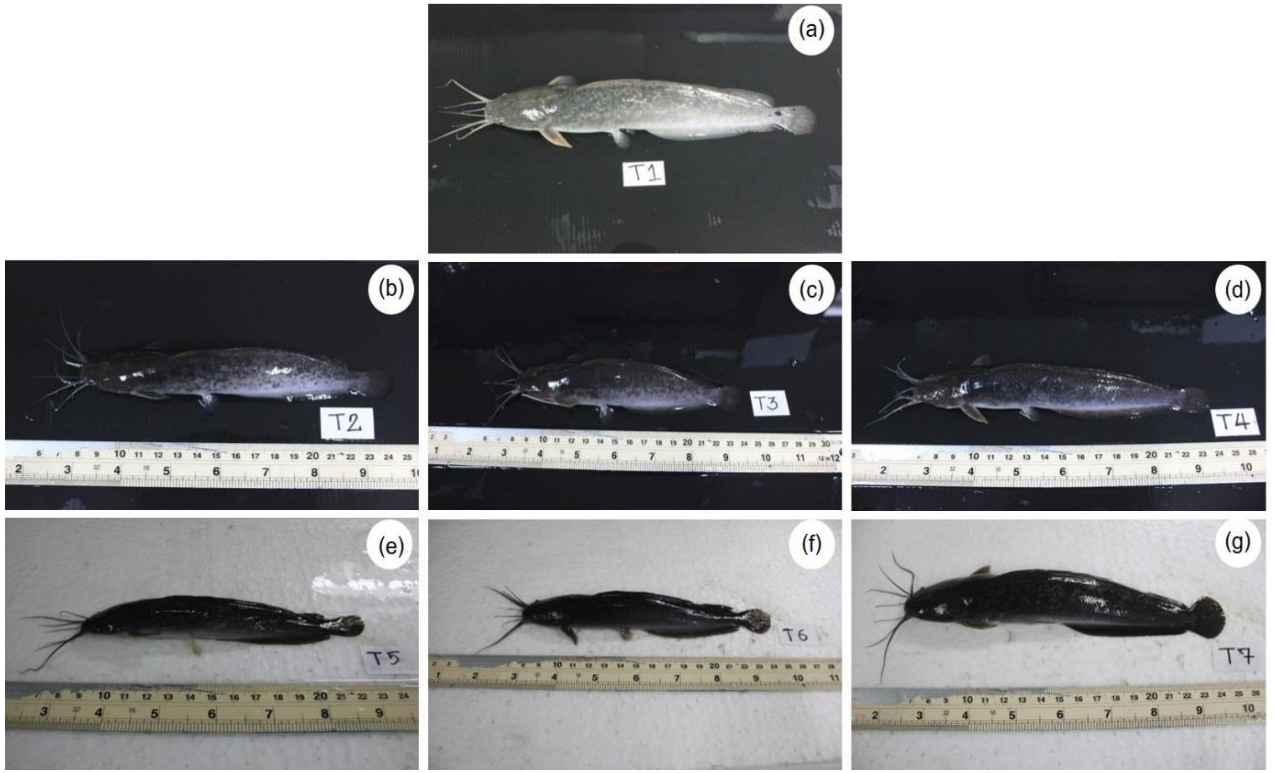

Figure 5-1. Clinical signs and external features of hybrid *Clarias* catfish after fed diets with different levels of MEL and CYA for 8 weeks. (a) Normal appearance of skin color of fish fed on control diet. Darkening of skin was markedly affected by dietary MEL+CYA supplementation as followed (b) 2.5+2.5 g kg^{-1}; (c) 5+5 g kg^{-1}; (d) 7.5+7.5 g kg^{-1}; (e) 10+10 g kg^{-1}; (f) 10+0 g kg^{-1}; (g) 0+10 g kg^{-1}.

5.4.3 Whole body composition

The whole body composition of the fish fed with different dietary MEL and CYA levels are presented in Table 5-5. Dry matter content of fish fed control diet was significantly higher ($P<0.05$) than fish fed with MEL-CYA at 7.5+7.5, 10+10 and 10+0 g kg^{-1} diet (Table 5-5). Higher nitrogen content ($P<0.05$) in whole body was found among fish fed MEL-CYA at 5+5, 7.5+7.5, 10+10 and 10+0 g kg^{-1} diet compared to those fed the control diet. Fish fed the diet with MEL-CYA at 7.5+7.5, 10+10 and 10+0 g kg^{-1} did not differ in lipid content in whole body and were lower ($P<0.05$) than that observed in fish fed control diet (Table 5-5). However, no significant differences ($P>0.05$) of ash content in whole body was observed among all groups (Table 5-5).

5.4.4 Blood parameters

The effects of dietary MEL-CYA on blood parameters are presented in Table 5-6. Dietary MEL-CYA did not affect ($P>0.05$) the hemoglobin and hematocrit values among fish fed different diets. Similarly, RBC counts did not show any significant differences ($P>0.05$) among treatments (Table 5-6). However, fish fed control diet showed the highest ($P<0.05$) WBC counts and NBT reduction compared to the other treatments (Table 5-6). Fish fed with the control and CYA alone at 0+10 g kg^{-1} diets had the highest ($P<0.05$) serum lysozyme activity, whereas the lowest ones were found among fish fed the diet with MEL-CYA at 7.5+7.5, 10+10 and 10+0 g kg^{-1} diet (Table 5-6).

5.4.5 Antioxidant enzyme activities and stress-70 proteins (Hsp70)

The effects of dietary MEL-CYA supplementation on the antioxidant enzyme activities are shown in Figure 5-2 to 5-4. No significant differences ($P>0.05$) of superoxide dismutase (SOD) activities in both liver and kidney were found between control and MEL-CYA supplementation group (Figure 5-2). Catalase (CAT) activity in the liver of fish fed the diet with MEL-CYA at 7.5+7.5 and 10+10 g kg^{-1} diet was significantly higher ($P<0.05$) than that in the control group, while only fish fed the diet with 10+10 g kg^{-1} diet had significantly increased ($P<0.05$) CAT activity in the kidney compared with

control group (Figure 5-3). Glutathione (GPx) activity in the liver was gradually increased with the increasing levels of dietary MEL-CYA up to 5+5 g kg^{-1} diet, whereas the highest GPx activity in the kidney of fish fed the diet with MEL-CYA at 10+10 g kg^{-1} diets and MEL alone (10 g kg^{-1} diets) which were significantly higher ($P<0.05$) than the control group (Figure 5-4).

The relative Hsp70 intensity in the liver was significantly lower ($P<0.05$) in fish fed with control diet than in the fish fed the diet with dietary MEL-CYA at 5+5, 7.5+7.5, 10+10 g kg^{-1} diets and MEL alone (10 g kg^{-1} diets) (Figure 5-5). The relative Hsp70 intensity in the kidney were increased ($P<0.05$) in fish fed the diet with 10+10 g kg^{-1} and MEL alone than the fish fed with CYA alone (Figure 5-5). However, there were no significant differences ($P>0.05$) when compared with control group (Figure 5-5).

5.4.6 Lipid oxidation in liver and kidneys

Thiobarbituric acid reactive substances (TBARS) in the liver of fish fed with MEL-CYA 10+10 g kg^{-1} diet and MEL alone was significantly increased, and it was significantly higher ($P<0.05$) than that of fish fed with control diet (Figure 5-6). TBARS in the kidney of fish fed the diet with 5+5, 7.5+7.5, 10+10 and MEL alone at 10+0 g kg^{-1} significantly increased ($P<0.05$) compared with the control (Figure 5-6).

Table 5-5. Proximate whole body composition (g kg^{-1}) of hybrid *Clarias* catfish fed diets with different levels of MEL and CYA for 8 weeks (values are given as mean±SD; n=3 fish from each of the 3 replicate tanks)

Parameters	Diets (MEL-CYA, g kg^{-1} diet)						
	Diet 1 (Control)	Diet 2 (2.5+2.5)	Diet 3 (5+5)	Diet 4 (7.5+7.5)	Diet 5 (10+10)	Diet 6 (10+0)	Diet 7 (0+10)
Dry matter	249.97±3.63^{c}	236.86±0.74^{bc}	233.27±1.23^{abc}	217.55±9.95^{ab}	211.41±3.39^{a}	222.45±10.36^{ab}	255.97±1.82^{c}
Nitrogen, N×6.25	600.44±1.47^{a}	623.25±6.29^{abc}	637.72±9.28^{bc}	645.88±10.78^{c}	649.57±7.88^{c}	634.03±2.27^{bc}	615.89±2.02^{ab}
Lipid	246.92±0.34^{c}	225.80±6.54^{bc}	224.35±10.28^{bc}	209.63±1.63^{ab}	191.42±2.67^{a}	192.48±4.74^{a}	233.55±7.79^{c}
Ash	133.96±0.17^{a}	135.27±6.73^{a}	138.44±11.52^{a}	134.43±4.50^{a}	138.01±7.61^{a}	136.92±4.83^{a}	134.16±4.70^{a}

Values within the same row sharing a common superscript are not significantly different by the Tukey test (P>0.05).

Table 5-6. Blood parameters of hybrid *Clarias* catfish fed diets with different levels of MEL and CYA for 8 weeks (mean ± SD; n=9 fish from each of the 3 replicate tanks).

Parameters	Diets (MEL-CYA, g kg^{-1} diet)						
	Diet 1 (Control)	Diet 2 (2.5+2.5)	Diet 3 (5+5)	Diet 4 (7.5+7.5)	Diet 5 (10+10)	Diet 6 (10: 0)	Diet 7 (0+10)
RBC (x10^9 cell ml^{-1})	2.79±0.23^{a}	2.46±0.27^{ab}	2.25±0.14^{a}	2.27±0.09^{a}	2.29±0.20^{a}	2.11±0.22^{a}	2.46±0.13^{ab}
WBC (x10^7 cell ml^{-1})	7.25±0.62^{c}	5.06±0.49^{a}	4.76±0.63^{a}	5.01±0.76^{a}	4.27±0.68^{a}	4.42±0.27^{a}	6.15±0.26^{b}
Hemoglobin (g dL^{-1})	14.92±0.57^{a}	13.71±1.62^{a}	13.59±0.73^{a}	13.86±1.82^{a}	14.18±1.18^{a}	13.72±1.54^{a}	14.68±1.59^{a}
Hematocrit (%)	37.30±1.08^{ab}	35.19±4.02^{ab}	33.41±2.23^{a}	33.93±4.96^{ab}	38.36±2.60^{b}	35.24±2.29^{ab}	36.60±5.66^{ab}
NBT reduction (OD 620 nm)	0.095±0.021^{b}	0.051±0.005^{a}	0.054±0.006^{a}	0.050±0.007^{a}	0.062±0.006^{a}	0.059±0.004^{a}	0.061±0.005^{a}
Lysozyme activity (U mL^{-1})	17.10±1.45^{c}	15.28±0.73^{bc}	15.24±1.60^{bc}	13.09±1.18^{ab}	12.09±1.85^{a}	13.14±1.77^{ab}	16.83±1.21^{c}

Values within the same row sharing a common superscript are not significantly different by the Tukey test (P>0.05).

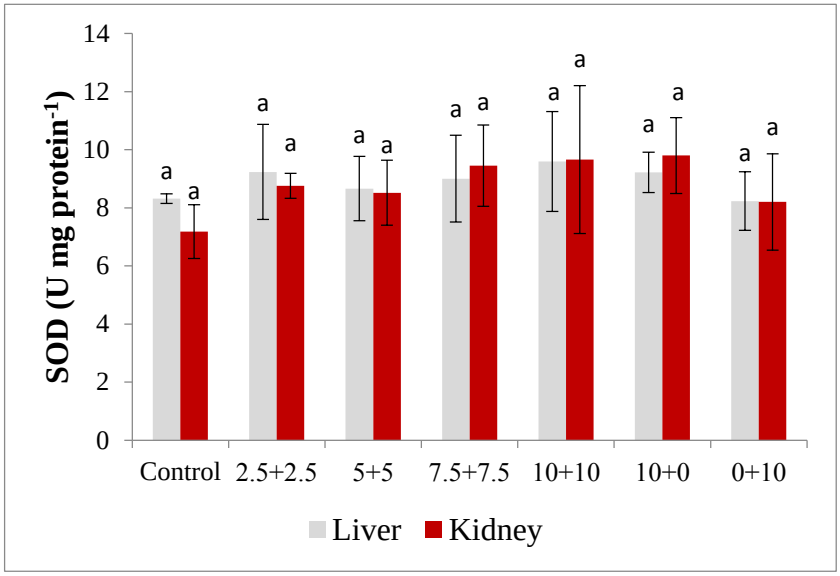

Figure 5-2. Superoxide dismutase (SOD) activity in liver and kidneys of hybrid *Clarias* catfish fed diets with different levels of MEL and CYA for 8 weeks.

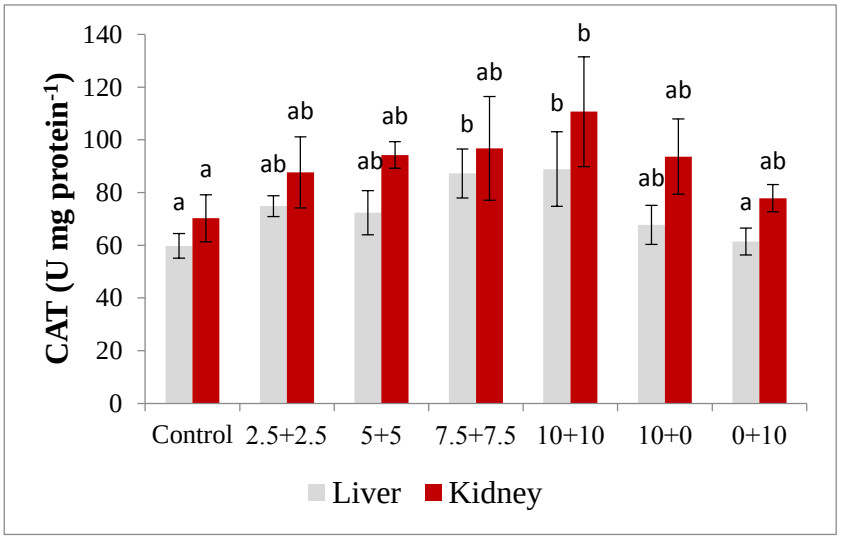

Figure 5-3. Catalase (CAT) activity in liver and kidneys of hybrid *Clarias* catfish fed diets with different levels of MEL and CYA for 8 weeks.

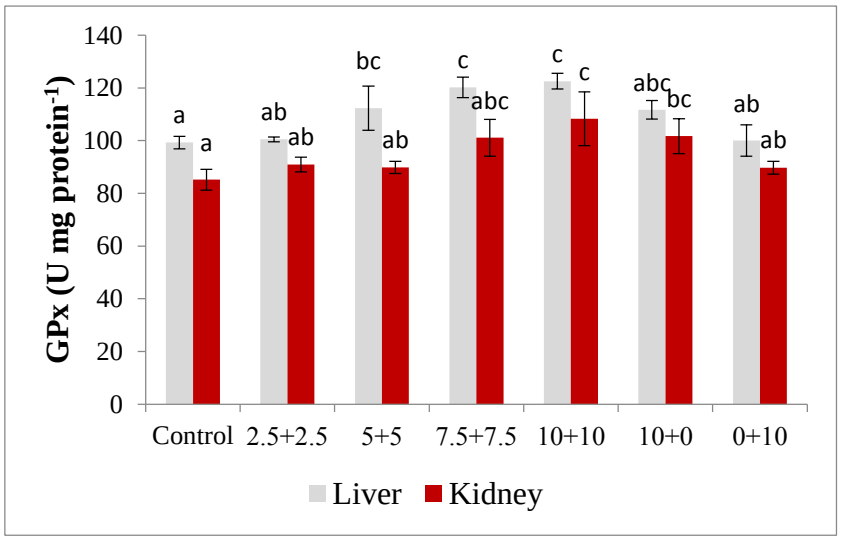

Figure 5-4. Glutathione peroxidase (GPx) activity in liver and kidneys of hybrid *Clarias* catfish fed diets with different levels of MEL and CYA for 8 weeks.

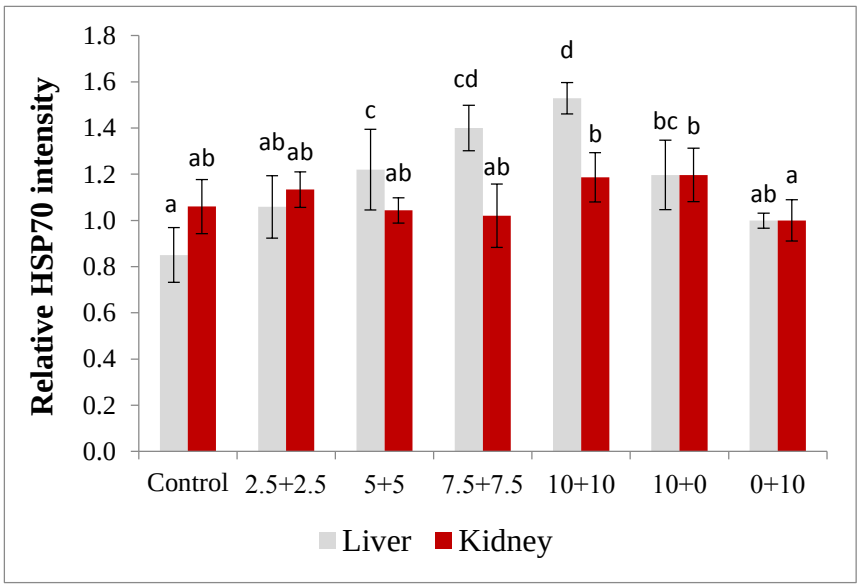

Figure 5-5. Relative Hsp70 intensity in liver and kidneys of hybrid *Clarias* catfish fed diets with different levels of MEL and CYA for 8 weeks.

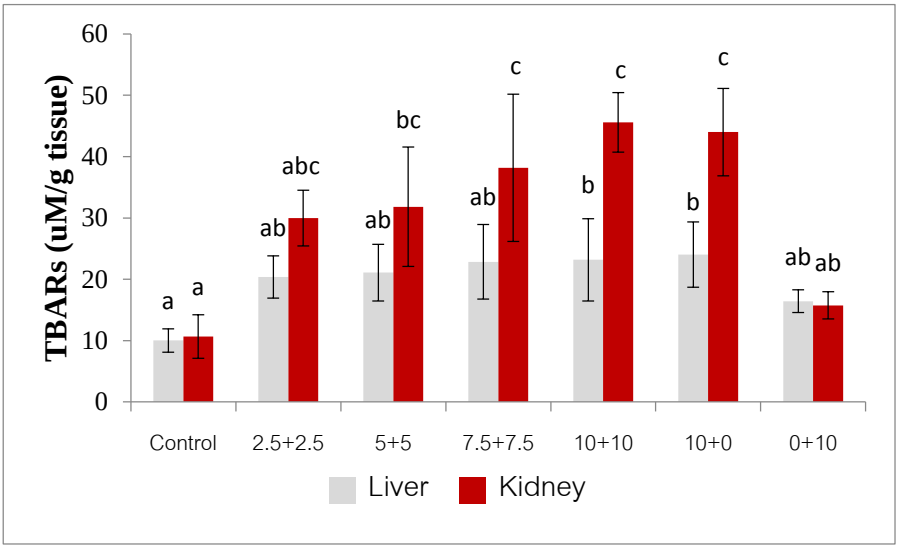

Figure 5-6. Thiobarbituric acid reactive substances (TBARs) in liver and kidneys of hybrid *Clarias* catfish after the various dietary treatments.

5.4.7 Histological alterations

The histological alterations of the fish liver sections are presented in Figure 5-7. The livers of fish fed the control diet exhibited regular morphology of hepatocytes with normal-shape and size (Figure 5-7a). However, abnormal appearance of hepatocytes was observed in fish fed dietary graded levels of MEL-CYA, showing a higher occurrence of cytoplasmic vacuolization with a displacement of cell nuclei (Figure 5-7b-e). However, there were no differences of hepatocytes in fish fed CYA alone at 0+10 g kg^{-1} compared to the control (Figure 5-7f).

The histological alterations of the fish kidney sections are presented in Figure 5-8. Fish fed control diet exhibited normal appearance of kidney structure with well-defined glomerulus and renal tubules (Figure 5-8: a). However, severe histopathological alterations were found in fish fed the MEL-CYA combination diets, showing irregular golden-brown crystals (CYS) in the renal tubules (Figure 5-7: b-d). An enlargement of the renal tubules and the tubular dilation were observed in fish fed high level of MEL and CYA combination (Figure 5-8e-f). Melanomacrophage were found in fish fed MEL or CYA alone (Figure 5-8e-f).

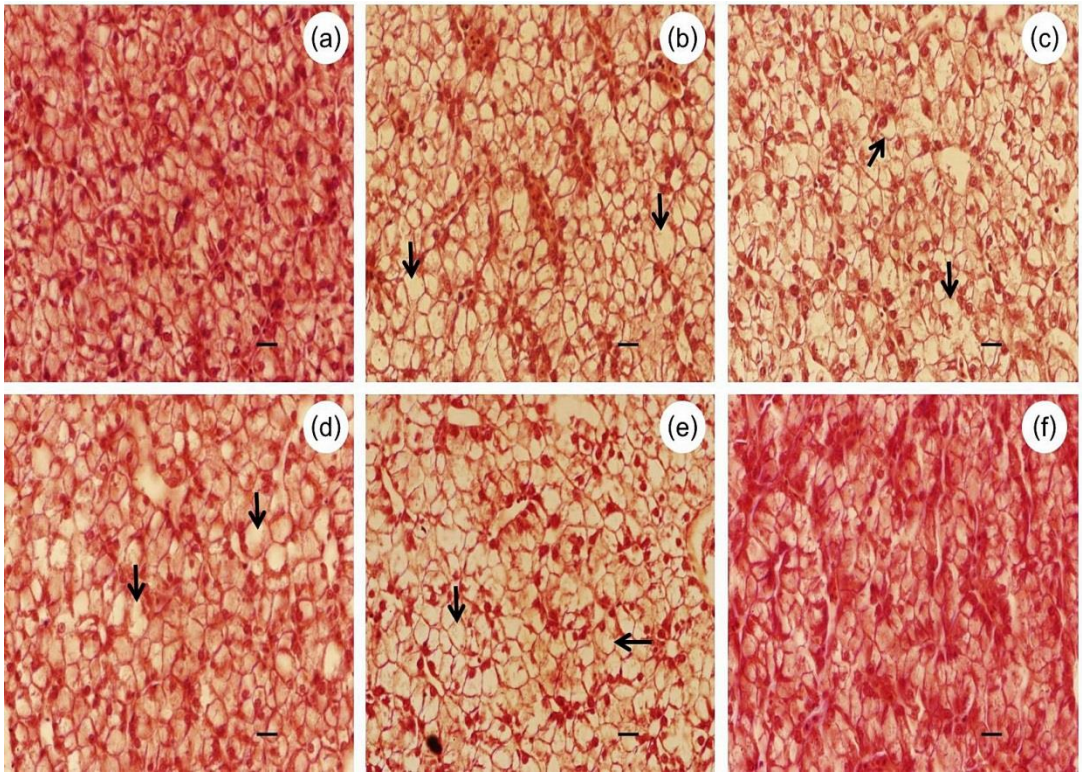

Figure 5-7. Photomicrographs of liver in hybrid *Clarias* catfish fed on diets containing various levels of MEL and/or CYA. (a) Section of liver from fish fed on the control diet exhibited regular morphology of hepatocyte (400×; Scale bar = 20 μm). Enlarged hepatocytes and apparent cytoplasmic vacuolization (black arrow) as the histological changes were found in fish fed MEL-CYA: 2.5+2.5 (b), 5+5 (c) and 10+10 g kg^{-1} diet (d) (400×; Scale bar = 20 μm). (e) Similar symptoms were observed in fish fed on MEL-CYA: 10+0 g kg^{-1} diet (arrow head) (400×; Scale bar = 20 μm). (f) Normal hepatocytes were observed in fish fed on MEL-CYA at 0+10 g kg^{-1} diet (400×; Scale bar = 20 μm).

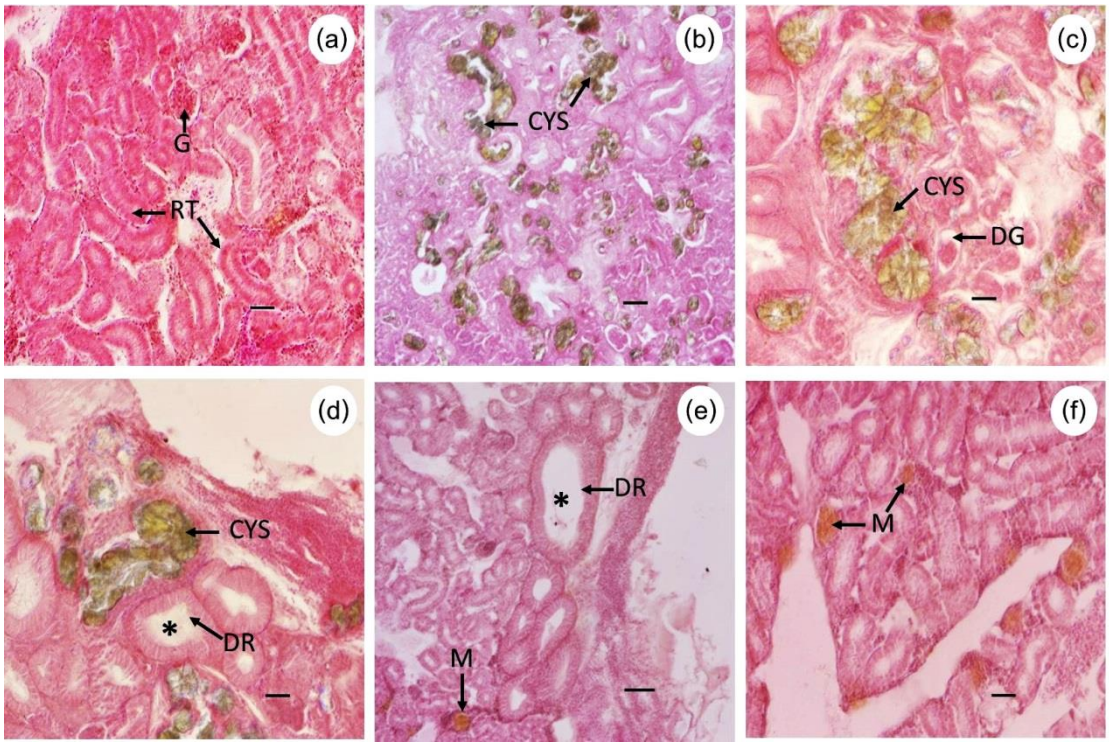

Figure 5-8. Photomicrographs of kidneys in hybrid *Clarias* catfish fed on diets containing various levels of MEL and/or CYA. (a) Normal appearance of renal tubules (RT), glomerulus (G) was found in control group (200×; Scale bar = 50 μm). (b) Trunk kidney from fish fed on a MEL-CYA at 2.5+2.5 g kg^{-1} diet showing irregular golden-brown crystals (CYS) in the renal tubules (100×; Scale bar = 100 μm). (c) Golden-brown crystals and degeneration of glomerulus (DG) were detected in fish fed on combination of MEL-CYA at 7.5+7.5 g kg^{-1} diet. (d) An enlargement (asterisk) and degeneration of renal tubules (DR) were observed in fish fed on combination of MEL-CYA at 10+10 g kg^{-1} diet. (200×; Scale bar = 50 μm). The appearance of the tubular enlargement and melano-macrophage (M) were found in fish fed on MEL alone (10+0 g kg^{-1} diet) (e) (100×; Scale bar = 100 μm) and with CYA alone (0+10 g kg^{-1} diet) (f) (200×; Scale bar = 50 μ)

5.5 Discussion

The contamination of MEL and its derivative compounds becomes a major concern in aqua-feed industry. Recently, the contaminations of these compounds in either food or feed products are restricted by the public health government agency in many countries. In this study, these compounds were not detected in any of the feed ingredients, which were confirmed by non-detection of these compounds in control diet. Therefore, the presence of MEL and/or CYA in the diets was a result of the incorporation.

The results from this study indicated that the toxicity of MEL combined with CYA had an adversely affects growth performance, including average body weight and SGR in hybrid *Clarias* catfish after exposure to 2.5+2.5 g kg^{-1} diet of MEL and CYA for 8 weeks. A similar decrease in average body weight and SGR has been reported in previous study on Asian seabass after exposure to the diet with the identical combination doses (Phromkunthong *et al*., 2015b). In the current study, the SGR was lower in fish that received MEL alone (10 g kg^{-1}diet) relative to control. The similar result in terms of growth retardation after exposure to MEL at a level 10 g kg^{-1} support to the findings of Srisathaporn *et al*. (2014) in fingering hybrid catfish (initial body weight ~9.7 g $fish^{-1}$) exposed to MEL alone at doses 5, 10 and 20 g kg^{-1} diet for 8 weeks, resulting in significant lower final weight in fish exposed to 10 g kg^{-1} of MEL compared to control. Similar results in different species with the same doses were also found in Asian seabass (Phromkunthong *et al*., 2015b) and red tilapia (Phromkunthong *et al*., 2013; 2015a). Whereas in this study, the significant lower on body weight and SGR were noted with CYA alone (10 g kg^{-1}diet) relative to control. This finding is in contrary to previous studies in Asian seabass and red tilapia, which noted that the growth of these fish were not affected by dietary supplementation of CYA at 10 g kg^{-1}diet (Phromkunthong *et al*., 2015a; b), suggesting that hybrid *Clarias* catfish are more sensitive to CYA than those Asian seabass and red tilapia.

In the present study, growth retardation may be caused by lower feeding rate and impairing nutrient utilization due to damage caused by toxic effects of dietary MEL and CYA to the liver, which is an important organ for nutrient metabolism, storage

and detoxification. Therefore, hepatotoxicity can cause negative effects on performance of fish. In this study, the increase lipid vacuolization in the liver point towards a reduction in dietary lipid utilization also directly related to poor feed efficiency due to malfunction of liver (Abdel-Tawwab, 2016; Romano *et al*., 2016; Yin *et al*., 2019). Also, Pirarat *et al*. (2012) reported that alanine transaminase (ALT) and aspartate transaminase (AST), biomarkers for liver health and function, in serum of walking catfish were significantly increased, indicating abnormal liver function.

In the current study, dietary MEL and CYA supplementation significantly affected on protein and lipid content in whole body. In this study, the protein and lipid content in whole body fish showed opposite trend in response to the increase in dietary MEL and CYA supplementation. Generally, protein (as nitrogen) content has positively and significantly contributed to growth performance. However, in contrast to growth, fish fed with a combination of MEL and CYA, or either of these singly, obtained significantly higher nitrogen content in the whole body compared to the control group, indicating that the increased nitrogen content of fish whole body in the MEL and CYA treated group may be directly linked to the accumulation of these substances. Similar effects on higher nitrogen content in whole body of MEL and CYA treated group have also been reported in Asian seabass and red tilapia (Phromkunthong *et al*., 2013; 2015a; 2015b). For lipid, the reduction of lipid content in whole body might be strongly related to the impairing nutrient utilization due to malfunctions of liver. Moreover, fish generally utilize lipid as the energy source in detoxification process, resulting in decrease of lipid content in whole body of fish. The significant decreases of lipid content in whole body of fish after exposure to toxic substances has been reported in previous studies for rainbow trout exposed to deoxynivalenol (Hooft and Bureau, 2017), Nile tilapia exposed to zinc (Abdel-Tawwab, 2016) Nile tilapia exposed to aflatoxin B1 (Hussain *et al*., 2017), Asian seabass exposed to MEL+CYA (Phromkunthong *et al*., 2015b).

In the current study, dietary MEL-CYA induced darkening of the skin due to higher content of melanin pigment. Similar results were also reported by Janlek *et al*. (2009) in hybrid catfish (*Clarias macrocephalus* (Gunther) × *Clarias gariepinus*

(Burchell)) and Pirarat *et al*. (2012) in walking catfish (*Clarius batrachus*). Literature regarding the effect of MEL and CYA on color alterations of skin is scarce. One possible reason may be related to stress and secretion of stress-induced hormone. Two pituitary melanotropic hormones, including alpha-melanocyte-stimulating hormone (α-MSH) and melanin-concentrating hormone (MCH) play an important role for color alterations of fish (Yamanome *et al*., 2007; Takahashi *et al*., 2014). α-MSH can influence the synthesis of melanin, a brown-black pigments, in the skin of fish by an action upon the melanocyte cells. Shishioh-Ikejima *et al*. (2010) reported that over-secretion of α-MSH by melanotrophs in the anterior pituitary was found in animal model under continuous chronic stress, resulting in hyper-pigmentation. However, the contrary finding was reported in darkbarbel catfish by Xue *et al*. (2011) who found that the dorsal skin of darkbarbel catfish fed the diets with 5 and 10 g kg^{-1} MEL supplementation was paler than control. Thus, the skin discoloration, which could be induced by direct brain impairment or high oxidative stress affected by MEL and CYA exposure, could also contribute to the abnormal hormone secretion. This speculation needs to be proven by future studies with high-performance technique i.e. molecular technique or the expression of pigmentation related genes.

Hematological parameters are often used to determine the health condition or effects of exposures to contaminants of fish (Tan *et al*., 2007; Fazio, 2019). In the present study, no significant differences between control and MEL-CYA treated groups were found in RBC, hemoglobin or hematocrit. However, the decrease of WBC was found in fish fed with MEL and/or CYA than those received control diet. The decrease of WBC is critical for fish health because these cells are play an important role for immune responses against infectious agents or pathogens in fish. The decrease of WBC recorded in this study was confirmed by the activities of phagocytes, which quantified using the NBT reduction method. Moreover, we found that a significantly lower lysozyme activity in combination group of MEL and CYA with high dose (up to 7.5+7.5 g kg^{-1} diet) and MEL alone (10 g kg^{-1} diet) compared with control group. In fish, a close relationship between lysozyme and white blood cells including neutrophils and

monocytes has been recognized by Pratheepa *et al*. (2010), who reported that lysozyme production is mainly based on the neutrophils and monocytes present in the blood. The suppression of immune response due to MEL and CYA exposure could be related to cytokines, a small secreted proteins which are key regulators of the immune system (Wang and Secombes, 2013). Cytokines are synthesized and released by innate immune cells under the conditions of cell stress or pathogenic invasion (Lopez-Castejon and Brough, 2011; Zou and Secombes, 2016). Yin *et al*. (2017) noted that co-administration of melamine and cyanuric acid appears to inhibit the secretion of cytokines in serum of ovalbumin-sensitized mice. Thus, the reduction of cytokines observed are likely to cause adverse effects on immune functions. Unfortunately, an anti-inflammatory cytokines were not assayed in this study, although this speculation needs to be proven by future studies.

The exposure to toxic substances has been linked to organ damage and dysfunction. The target organs for MEL and CYA in animal were liver and kidney (EFSA, 2010; Pirarat *et al*., 2012; Phromkunthong *et al*., 2015a; 2015b). The formations of melamine-cyanurate crystals in renal tubules have been reported in animal exposed to MEL and CYA. The obstruction of renal tubular by melamine-cyanurate crystals causes tubular damage and disorder of organ function, which can lead to kidney (renal) failure, as known crystal nephropathy (Reimschuessel *et al*., 2008; 2010; Lee *et al*., 2016). In mammal, Lv *et al*. (2013) reported that crystals were found in the kidney of mice fed on MEL in combination with CYA, resulting in kidney damages due to oxidative stress and hypoxia. In the current study, formations of melamine-cyanurate crystals were detected in renal tubules in the kidneys of fish that received diets with combination of MEL-CYA. Crystal formation within the renal tubules may be dependent on the concentrations of MEL and CYA (Reimschuessel *et al*., 2010)Similar finding are also reported in channel catfish (Reimschuessel *et al*., 2010), walking catfish (Pirarat *et al*., 2012), rainbow trout (Reimschuessel *et al*., 2010; Pacini *et al*., 2014) and red tilapia (Phromkunthong *et al*., 2015a). In present study, exposure of fish to MEL or CYA alone resulted in marked abnormalities which were characterized by enlargement and degeneration of renal

tubules and cell necrosis in the kidney. These pathological findings confirm previous observations in red tilapia exposed to MEL alone (Phromkunthong *et al*., 2013). Moreover, this study showed that the formation of melano-macrophage in kidneys of fish. Melano-macrophage play an important role in the response of fish to infectious agents, including viruses, bacteria, parasites and toxic substances (Agius and Roberts, 2003), or indicating the presence of apoptotic area in the target tissues (Zeng *et al*., 2019). Capps *et al*. (2004) reported that macrophage aggregates were presented in the hemopoietic region of the kidney in zebrafish (*Danio rerio*) after exposed to perchlorate for 8 weeks. However, melamine-cyanurate crystals were absent in the kidneys of fish that received the diet with MEL or CYA alone. Reimschuessel *et al*. (2009) reported that spherulite crystals formed in the kidneys of fish, resulting in intratubular obstruction and renal damage.

The expressions of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in the liver and kidney of the fish after 8 weeks of exposure to dietary MEL and CYA supplementation provide further evidence that these substances induce oxidative stress in these target organs. The oxidative stress in living organisms and cells is indicated by generation of reactive oxygen species (ROS), induction of lipid peroxidation, and altered activity of antioxidant enzymes (Woo *et al*., 2009; Vinagre *et al*., 2012; Birnie-Gauvin *et al*., 2017). Deng *et al*. (2010a, 2010b) reported that a reduction in the hepatic liver contents and hepatic lesions, which could be due to oxidative stress, caused by AFB1 exposure. In the current study, an increased activity of CAT and GPx in liver and kidney tissues were found in liver and kidney of the highest MEL+CYA combination diet suggest that the receiving of these toxic substances induces the oxidative stress of the fish. The results in our study are in agreement with Jin *et al*. (2010) reported significant increased catalase activity in the liver of zebra fish (*Danio rerio*) after exposure to triazine herbicide (atrazine) for 14 days and also similar to the finding in red tilapia (Phromkunthong *et al*., 2015a). The results from the antioxidant enzymes alterations were closely related lipid peroxidation as evaluated by thiobarbituric acid-reactive substances (TBARs). The

significant increases in amount of TBARs in liver and kidney of the highest MEL+CYA combination diet may be a response to oxidative stress caused by MEL-CYA exposure.

Heat shock protein, in particular the 70 kDa (Hsp70) has been widely used for biomarkers of cellular stress in aquatic organisms. Hsp70 play an important roles for protect cells from various conditions of stress. Elevated levels of various HSPs have been measured in tissues of fish exposed to abnormality-producing agents. Under stress condition, HSP could help cells resist deadly conditions and adapt to stress (Iwama *et al*., 1999; Basu *et al*., 2001). In the current study, an increase in relative Hsp70 intensity in liver and kidney of MEL-CYA exposed group is probably an indication of tissue damages. Phromkunthong *et al*. (2015b) reported a significant increase in Hsp70 in the liver of Asian seabass when exposed to MEL and CYA. Similar findings were also reported by same author in red tilapia in response to MEL and CYA exposure (Phromkunthong *et al*., 2014).

5.6 Conclusion

In conclusion, the results of the present study confirmed that MEL and CYA had negative impacts to hybrid *Clarias* catfish, the clinical signs as growth retardation, feed efficiency reduction, skin discoloration, target organs (liver and kidney) dysfunction and histological damage are commonly recorded. The combination of MEL and CYA induces crystal nephropathy. Antioxidant enzymes and Hsp70 play important roles to protect tissues from these substances. To our knowledge, the information will be helpful to understand the toxic effects and clinical signs of target organs induced by MEL and CYA in hybrid *Clarias* catfish.

5.7 References

Abdel-Tawwab, M. 2016. Effect of feed availability on susceptibility of Nile tilapia, *Oreochromis niloticus* (L.) to environmental zinc toxicity: Growth performance biochemical response, and zinc bioaccumulation. Aquaculture 464, 309-315.

Agius, C. and Roberts, R.J. 2003. Melano-macrophage centres and their role in fish pathology. J. Fish Dis. 26, 499–509.

Anderson, D.P. and Siwicki, A.K. 1995. Basic haematology and serology for fish health programmes, *In*: Shhariff, J.R., Subasinghe, R.P. (eds.), Diseases in Asian Aquaculture II. Fish Health Section, Asian Fisheries society, Manila, Philippines, pp. 185-202.

AOAC (Association of Official Analytical Chemists). 1995. Official Methods of Analysis of AOAC International, Sixteenth ed. 2 vols. Association of Analytical Communities, Arlington, VA.

Bancroft, J.D. 1967. Histochemical Techniques. first ed. Butterworths: London.

Basu, N., Nakano, T., Grau, E.G. and Iwama, G.K. 2001. The effects of cortisol on heat shock protein 70 levels in two fish species. Gen. Comp. Endocrinol. 124, 97-105.

Birnie-Gauvin, K., Costantini, D., Cooke, S.J. and Willmore, W.G. 2017. A comparative and evolutionary approach to oxidative stress in fish: A review. Fish Fish. 18, 928-942.

Bischoff, K. 2011. Melamine and cyanuric acid. *In*: Gupta, R.C. (Ed.), Reproductive and Developmental Toxicology. Academic Press/Elsevier, Amsterdam, pp. 367–372.

Blaxhall, P.C. and Daisley, K.W. 1973. Routine haematological methods for use with fish blood. J. Fish Biol. 5, 771–781.

Brown, C.A., Jeong, K.S., Poppenga, R.H., Puschner, B., Miller, D.M., Ellis, A.E., Kang, K.I., Sum, S., Cistola, A.M. and Brown, S.A. 2007. Outbreaks of renal failure associated with melamine and cyanuric acid in dogs and cats in 2004 and 2007. J. Vet. Diagn. Invest. 19, 525-531.

Buege, J.A. and Aust, S.D. 1978. Microssomal lipid peroxidation. Methods Enzymol. 52, 302-309.

Capps, T., Mukhi, S., Rinchard, J.J., Theodorakis, C.W., Blazer, V.S. and Patiño, R. 2004. Exposure to Perchlorate Induces the Formation of Macrophage Aggregates in the Trunk Kidney of Zebrafish and Mosquitofish. J. Aquat. Anim. Health 16, 145-151.

Chang, L., She, R., Ma, L., You, H., Hu, F., Wang, T., Ding, X., Guo, Z. and Soomro, M.H. 2014. Acute testicular toxicity induced by melamine alone or a mixture of melamine and cyanuric acid in mice. Reproductive Toxicol. 46, 1-11.

Demers, N.E. and Bayne, C.J. 1997. The immediate effects of stress on hormones and plasma lysozyme in rainbow trout. Dev. Comp. Immunol. 21, 363-373.

Deng, S.X., Tian, L.X., Liu, F.J., Jin, S.J., Liang, G.Y., Yang, H.J., Du, Z.Y. and Liu, Y.J. 2010. Toxic effects and residue of aflatoxin B1 in tilapia (*Oreochromis niloticus* × *O. aureus*) during long-term dietary exposure. Aquaculture 307, 233-240.

Department of Fisheries. 2018. Fisheries statistics of Thailand for the year 2016. Bangkok: Fisheries Development Policy and Strategy Division, Department of Fisheries.

EFSA (European Food Safety Authority). 2010. Scientific Opinion on Melamine in Food and Feed. EFSA Journal 8, 1-145.

Fazio, F. 2019. Fish hematology analysis as an important tool of aquaculture: A review. Aquaculture 500, 237–242.

Green, J.A. and Baker, B.I. 1991. The influence of repeated stress on the release of melanin-concentrating hormone in the rainbow trout. J. Endocrinol. 128, 261-266.

Hooft, J.M. and Bureau, D.P. 2017. Evaluation of the efficacy of a commercial feed additive against the adverse effects of feed-borne deoxynivalenol (DON) on the performance of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 473, 237–245.

Hussain, A., Mateena, A. and Gatlin III, D.M. 2017. Alleviation of aflatoxin B1 (AFB1) toxicity by calcium bentonite clay: Effects on growth performance, condition indices and bioaccumulation of AFB1 residues in Nile tilapia (*Oreochromis niloticus*). Aquaculture 475, 8-15.

Iwama, G.K., Vijayan, M.M., Forsyth, R.B. and Ackerman, P.A. 1999. Heat shock proteins and physiological stress in fish. Amer. Zool. 39, 901–909.

Janlek, P., Boonyaratpalin, M. and Phromkunthong, W. 2009. Effect of melamine on growth performance, blood components and histological changes in hybrid catfish, *Clarias macrocephalus* (Gunther) × *Clarias gariepinus* (Burchell). Thai Fish. Gaz. 62, 331–340 (in Thai).

Jin, Y., Zhang, X., Shu, L., Chen, L., Sun, L., Qian, H., Liu, W. and Fu, Z. 2010. Oxidative stress response and gene expression with atrazine exposure in adult female zebrafish (*Danio rerio*). Chemosphere 78, 846–852.

Köhler, H.R., Lazzara, R., Dittbrenner, N., Capowiez, Y., Mazzia, C. and Triebskorn, R. 2009. Snail phenotypic variation and stress proteins: do different heat response strategies contribute to Waddington's widget in field populations?. J. Exp. Zool. B Mol. Dev. Evol. 312, 136-147.

Larsen, H.N. and Snieszko, S.F. 1961. Comparison of various methods of determination of haemoglobin in trout blood. Prog. Fish Cult. 23, 8-17.

Lee, I.C., Ko, J.W., Park, S.H., Shin, I.S., Moon, C., Kim, S.H., Kim, Y.B. and Kim, J.C. 2016. Melamine and cyanuric acid co-exposure causes renal dysfunction and structural damage via MAPKs and mitochondrial signaling. Food Chem. Toxicol. 96, 254-262.

Lopez-Castejon, G. and Brough, D. 2011. Understanding the mechanism of IL-1β secretion. Cytokine Growth Factor Rev. 22, 189-195.

Lv, Y., Liu, Z., Tian, Y. and Chen, H. 2013. Effect on morphology, oxidative stress and energy metabolism enzymes in the testes of mice after a 13-week oral administration of melamine and cyanuric acid combination. Regul. Toxicol. Pharmacol. 65, 183-188.

Onac, C., Korkmaz Alpoguz, H., Lutfi Yola, M. and Kaya, A. 2018. Transport of melamine by a new generation of nano-material membranes containing carbon nanotubes and determination with surface plasmon resonance. Innov. Food Sci. Emerg. Technol. 45, 467-470.

Phromkunthong, W., Choochuay, P., Kiron, V., Nuntapong, N. and Boonyaratpalin, M. 2015a. Pathophysiological changes associated with dietary melamine and cyanuric acid toxicity in red tilapia. J. Fish Dis. 38, 161-173.

Phromkunthong, W., Nuntapong, N., Boonyaratpalin, M. and Kiron, V. 2013. Toxicity of melamine, an adulterant in fish feeds: experimental assessment of its effects on tilapia. J. Fish Dis. 36, 555-568.

Phromkunthong, W., Nuntapong, N., Wanlem, S. and Boonyaratpalin, M. 2015b. A study on growth, histopathology and oxidative stress in Asian seabass on diets with various loadings of melamine and cyanuric acid adulterants. Aquaculture 435, 336-346.

Pirarat, N., Katagiri, T., Chansue, N., Ponpornpisit, A., Endo, M. and Maita, M. 2012. The pathological effects of melamine and cyanuric acid in the diet of walking catfish (*Clarius batrachus*). J. Comp. Pathol. 147, 259-266.

Pratheepa, V., Ramesh, S. and Sukumaran, N. 2010. Immunomodulatory effect of *Aegle marmelos* leaf extract on freshwater fish *Cyprinus carpio* infected by bacterial pathogen *Aeromonas hydrophila*. Pharm. Biol. 48, 1224-1239.

Reimschuessel, R., Evans, E., Andersen, W.C., Turnipseed, S.B., Karbiwnyk, C.M., Mayer, T.D., Nochetto, C., Rummel, N.G. and Gieseker, C.M. 2009. Residue depletion of melamine and cyanuric acid in catfish and rainbow trout following oral administration. J. Vet. Pharmacol. Ther. 33, 172–182.

Reimschuessel, R., Evans, E.R., Stine, C.B., Hasbrouck, N., Mayer, T.D., Nochetto, C. and Gieseker, C.M. 2010. Renal crystal formation after combined or sequential oral administration of melamine and cyanuric acid. Food Chem. Toxicol. 48, 2898–2906.

Reimschuessel, R., Gieseker, C.M., Miller, R.A., Ward, J., Boehmer, J., Rummel, N., Heller, D.N., Nochetto, C., De Alwis, G.K., Bataller, N., Andersen, W.C., Turnipseed, S.B., Karbiwnyk, C.M., Satzger, R.D., Crowe, J.B., Reinhard, M.K., Roberts, J.F. and Witkowski, M.R. 2008. Evaluation of the renal effects of

experimental feeding of melamine and cyanuric acid to fish and pigs. Am. J. Vet. Res. 69, 1217–1228.

Romano, N., Simon, W., Ebrahimi, M., Fadel, A.H.I., Chong, C.M., Kamarudin, M.S., 2016. Dietary sodium citrate improved oxidative stability in red hybrid tilapia (*Oreochromis* sp.) but reduced growth, health status, intestinal short chain fatty acids and induced liver damage. Aquaculture 458, 170-176.

Schmitz, N., Kurrer, M. and Kopf, M. 2003. The IL-1 receptor 1 is critical for Th2 cell type airway immune responses in a mild but not in a more severe asthma model. Eur. J. Immunol. 33, 991–1000.

Shishioh-Ikejima, N., Ogawa, T., Yamaguti, K., Watanabe, Y., Kuratsune, H. and Kiyama, H. 2010. The increase of alpha-melanocyte-stimulating hormone in the plasma of chronic fatigue syndrome patients. BMC neurology 10, 73.

Srisathaporn, A., Khajarern, J., Chalorkpunrut, P. and Tengjroenkul, B. 2014. Growth performance and health of hybrid catfish (*Clarias macrocephalus* x *Clarias gariepinus*) fed with supplemental melamine and urea formaldehyde diets. Khon Kaen Agr. J. 42, 81-92 (in Thai).

Takahashi, A., Mizusawa, K. and Amano, M. 2014. Multifunctional roles of melanocyte-stimulating hormone and melanin-concentrating hormone in fish: evolution from classical body color change. Aqua-BioSci. Monogr. 7, 1-46.

Tan, X., Sun, Z., Chen, S., Chen, S., Huang, Z., Zhou, C., Zou, C., Liu, Q., Ye, H., Lin, H., Ye, C. and Wang, A. 2017. Effects of dietary dandelion extracts on growth performance, body composition, plasma biochemical parameters, immune responses and disease resistance of juvenile golden pompano *Trachinotus ovatus*. Fish Shellfish Immun. 66, 198–206.

Thai agricultural commodity and food standard TAS 7417(G)-2016. 2016. National Bureau of Agricultural Commodity and Food Standards, Ministry of Agriculture and Cooperatives. “Good aquaculture practices for freshwater animal farm” in the Royal Gazette. vol. 133 (Dated 18 November B.E. 2559, 2016).

Trasviña-Arenas, C.H., Garcia-Triana, A., Peregrino-Uriarte, A.B. and Yepiz-Plascencia, G. 2013. White shrimp *Litopenaeus vannamei* catalase: Gene structure, expression and activity under hypoxia and reoxygenation. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 164, 44-52.

US-FDA (U.S. Food, Drug Administration). 2007. Interim Melamine and Analogues Safety/Risk Assessment Peer Review Report. United States Food and Drug Administration, Washington, DC.

Vinagre, C., Madeira, D., Narciso, L., Cabral, H. and Diniz, M. 2012. Effect of temperature on oxidative stress in fish: lipid peroxidation and catalase activity in the muscle of juvenile seabass, *Dicentrarchus labrax*. Ecol. Indic. 23, 274-279.

Wang, T. and Secombes, C.J. 2013. The cytokine networks of adaptive immunity in fish. Fish Shellfish Immunol. 35, 1703-1718.

Woo, S., Yum, S., Park, H.S., Lee, T.K. and Ryu, J.C. 2009. Effects of heavy metals on antioxidants and stress-responsive gene expression in Javanese medaka (*Oyzias javanicus*). J. Comp. Biochem. Physiol. C 149, 289-299.

Yamanome, T., Chiba, H. and Takahashi, A. 2007. Melanocyte-stimulating hormone facilitates hypermelanosis on the non-eyed side of the barfin flounder, a pleuronectiform fish. Aquaculture 270, 505–511.

Yin, R.H., Dong, Q., Li, H.S., Yuan, J., Li, X.T., Yin, R.L., Li, W., Wang, W.C., Liu, B.S., Han, X.H., Wang, R.F., Wang, X. and Bai, W.L. 2017. The effects of melamine with or without cyanuric acid on immune function in ovalbumin-sensitized mice. Res. Vet. Sci. 114, 254-261.

Yin, P., Xie, S., Huo, Y., Guo, T., Fang, H., Zhang, Y., Liu, Y., Tian, L. and Niu, J. 2019. Effects of dietary oxidized fish oil on growth performance, antioxidant defense system, apoptosis and mitochondrial function of juvenile largemouth bass (*Micropterus salmoides*). Aquaculture 500, 347-358.

Zeng, Z.Z., Jiang, W.D., Wu, P., Liu, Y., Zeng, Y.Y., Jiang, J., Kuang, S.Y., Tang, L., Zhou, X.Q. and Feng, L. 2019. Dietary aflatoxin B1 decreases growth performance

and damages the structural integrity of immune organs in juvenile grass carp (*Ctenopharyngodon idella*). Aquaculture 500, 1-17.

Zou, J. and Secombes, C.J. 2016. The Function of Fish Cytokines. Biology (Basel) 5, 23.

CHAPTER 6

Conclusions and recommendation

The past decade, the illegal adulteration of melamine and its derivatives had been reported in various animal feed products. The presence of these compounds in feed samples can increase protein content due to a false positive of nitrogen detection in typical protein assays. Melamine has an impact on animal health in numerous ways. Melamine by itself was classified as moderate toxic. However, the combination of melamine with cyanuric acid formed insoluble crystals in kidneys, which can occur more toxic than each substance alone. The melamine-cyanurate crystals induced renal toxicity due to the the accumulation of insolubal crystals in the kidneys. Therefore, MEL should not be present in any feed product. Recently, the contamination of melamine can be monitored by verification of industrial process regulations.

This thesis demonstrates a series of experiments investigating the effects of melamine and cyanuric acid in 3 economic aquatic animal species of Thailand including Asian seabass, Pacific white shrimp and hybrid *Clarias* catfish. Overall, the results of this study can be summarized as follows:

For Asian seabass

• Dietary MEL and CYA or MEL alone had adverse effects on growth and feed utilization in Asian seabass.

• The supplementation of combined MEL and CYA induced gold-brown crystals in the kidney.

• Stress-70 proteins and antioxidant enzyme activities increased in fish ingested diets included with MEL and CYA.

• The highest MEL residue in fillet was detected in fish ingested MEL alone. In contrast, CYA were not detected in all treatment.

For Pacific white shrimp

• Dietary MEL and CYA or MEL alone had adverse effects on growth and feed utilization in Pacific white shrimp.

• Total hemocyte count (THC) and phenoloxidase (PO) activity were significantly decreased in shrimp receiving MEL alone and at high combination dosages.

• The activities of antioxidant and digestive enzyme in shrimp fed the highest combination level of MEL-CYA or MEL alone were significantly decreased in both gill and hepatopancreas.

• Severe histological damages to the hepatopancreas were detected in shrimp ingested diets included with MEL and CYA.

For hybrid *Clarias* catfish

• Growth performance were significantly lower in fish that exposed with combined MEL+CYA or each alone relative to the control.

• Dietary combined malmine and cyanuric acid or each alone decrease feed palatability.

• Abnormalities of skin color were detected in melamine and cyanuric acid exposed group.

• Supression of blood related-immune parameters including white blood cell counts, NBT reduction and lysozyme were noted in melamine and cyanuric acid exposed group.

• The melamine-cyanurate crystals were detected in renal tubules of fish that exposed with combined MEL+CYA diets.

• Dietary MEL-CYA induced oxidative sress and lipid peroxidation in liver and kidney of fish.

VITAE

Name Mr. Nutt Nuntapong

Student ID 6010630019

Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Aquatic Science)	Prince of Songkla University	2007
Master of Science (Aquatic Science)	Prince of Songkla University	2011

Scholarship Awards during Enrolment

The Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (Grant No.: NAT540528S)

List of Publications and Proceedings

Phromkunthong, W., Nuntapong, N., Wanlem, S. and Boonyaratpalin, M. 2015. A study on growth, histopathology and oxidative stress in Asian seabass on diets with various loadings of melamine and cyanuric acid adulterants. Aquaculture 435, 336-346. (Chapter 3)

Nuntapong, N., Wanlem, S., Boonyaratpalin, M. and Phromkunthong, W. 2019. Dietary exposure to melamine and cyanuric acid induced growth reduction, oxidative stress and pathological changes of hepatopancreas in Pacific white shrimp. Int. Aquat. Res. https://doi.org/10.1007/s40071-019-0216-y (Online ISSN 2008-6970) (Chapter 4)

Nuntapong, N., Wanlem, S., Boonyaratpalin, M. and Phromkunthong, W. Effects of melamine and cyanuric acid on growth performance, blood immune parameters and histological alterations in hybrid *Clarias* catfish (*Clarias macrocephalus* (Günther) x *C. gariepinus* (Burchell)). (manuscript, Chapter 5)

นัทท์ นันทพงศ์ สุณีย์ หวันเหล็ม มะลิ บุณยรัตผลิน และวุฒิพร พรหมขุนทอง. 2561. ผลของเมลา-มีนและกรดไซยานูริคต่อการเจริญเติบโต, สีผิว และการเปลี่ยนแปลงของเนื้อเยื่อไตในปลาดุกพันธุ์ผสม. ใน: การประชุมนำเสนอผลงานวิจัยระดับบัณฑิตศึกษาครั้งที่ 13 ปีการศึกษา 2561. มหาวิทยาลัยรังสิต.16 สิงหาคม 2561. หน้า 2995-3002.