EFFECTS OF MICROPLASTICS ON GENE EXPRESSION TO NONSPECIFIC IMMUNE SYSTEM
IN PACIFIC WHITE SHRIMP (*LITOPENAEUS VANNAMEI*).

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A Thesis Submitted in Partial Fulfillment of the Requirements

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Department of Veterinary Medicine

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ผลกระทบของไมโครพลาสติกต่อการแสดงออกของยีนที่เกี่ยวข้องกับระบบภูมิคุ้มกันแบบไม่จำเพาะ
ในกุ้งขาวแวนนาไม (*Litopenaeus vannamei*)

นายทรงศักดิ์ เนี่ยมเจริญ

จุฬาลงกรณ์มหาวิทยาลัย

CHULALONGKORN UNIVERSITY

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ทรงศักดิ์ เนี่ยมเจริญ : ผลกระทบของไมโครพลาสติกต่อการแสดงออกของยีนที่เกี่ยวข้อง
กับระบบภูมิคุ้มกันแบบไม่จำเพาะในกุ้งขาวแวนนาไม (*Litopenaeus vannamei*) . (
EFFECTS OF MICROPLASTICS ON GENE EXPRESSION TO NONSPECIFIC
IMMUNE SYSTEM IN PACIFIC WHITE SHRIMP (*LITOPENAEUS VANNAMEI*).) อ.ที่
ปรึกษาหลัก : รศ. ดร.สพ.ญ.นันทริกา ชันซื่อ, อ.ที่ปรึกษาร่วม : ดร. สพ.ญ.ฐนิดา เห
ตระกูล

การศึกษาถึงผลกระทบของไมโครพลาสติกกำลังได้รับความสนใจ ซึ่งเป็นที่แน่ชัดแล้วว่า
การได้รับไมโครพลาสติกสามารถส่งผลต่อระบบภูมิคุ้มกันแบบไม่จำเพาะในสัตว์จำพวกครัสเตเชียน
(Crustaceans) ได้ ดังนั้นการศึกษาในครั้งนี้มุ่งเน้นการตรวจสอบผลกระทบของไมโครพลาสติกช
นิดพอลิเอทิลีนความหนาแน่นสูง (high-density polyethylene microplastic หรือ HDPE-MP)
ในอาหารกุ้งต่อการแสดงออกของยีนในระบบภูมิคุ้มกันแบบไม่จำเพาะของกุ้งขาว (*Litopenaeus
vannamei*) โดยพบว่า HDPE-MP มีค่า LC_{50} ที่ 28 วันเท่ากับ 3.074% w/w ของ HDPE-MP ใน
อาหาร กุ้งขาวแวนนาไมในกลุ่มที่ได้รับ HDPE-MP ที่ความเข้มข้น 0.1 และ 0.5% w/w ของ
อาหาร มีการแสดงออกของยีนซุปเปอร์ออกไซด์ดิสมิวเตส (superoxide dismutase หรือ SOD)
และกลูตาไธโอนเปอร์ออกซิเดส (glutathione peroxidase หรือ GPx) เพิ่มมากขึ้นอย่างมี
นัยสำคัญ และต่อมาก็มีการแสดงออกของยีนต่ำกว่าปกติอย่างมีนัยสำคัญภายหลังจากการได้รับไม
โครพลาสติกเป็นระยะเวลานาน ทุกกลุ่มการทดลองที่ได้รับ HDPE-MP เป็นระยะเวลามากกว่า 14
วัน มีการแสดงออกของยีนไลโซไซม์ลดลงต่ำกว่าปกติอย่างมีนัยสำคัญ พบการเกิดพยาธิสภาพใน
ตับและตับอ่อนของกุ้งขาวที่ได้รับ HDPE-MP อย่างมีนัยสำคัญ ได้แก่ การแทรกตัวของฮีโมไซต์ใน
เนื้อเยื่อร่างแห (interstitial hemocyte infiltration), การหนาตัวของเนื้อเยื่อบุผิว (epithelium
hyperplasia), การผิดรูปของท่อตับ (tubular deformity), การเกิดโนดูล (nodule formation),
และการสะสมของเมลานินในเยื่อ (melanization) โดยความรุนแรงของรอยโรคที่เพิ่มขึ้นสัมพันธ์
กับความเข้มข้นของ HDPE-MP ที่เพิ่มมากขึ้น ดังนั้นการศึกษาในครั้งนี้ได้เสนอว่ากุ้งขาวที่ได้รับ
อาหารที่ปนเปื้อน HDPE-MP สามารถรบกวนการแสดงออกของยีนของเอนไซม์ต่อต้านอนุมูลอิสระ
(antioxidant) และต่อต้านแบคทีเรีย (anti-bacterial) ที่สำคัญ และสามารถก่อความเสียหายต่อ
เนื้อเยื่อตับและตับอ่อนของกุ้งขาวแวนนาไม

สาขาวิชา	อายุรศาสตร์สัตวแพทย์	ลายมือชื่อนิสิต ....
ปีการศึกษา	2565	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....
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Microplastics pollution effects are increasingly observed as new studies emerge in different ecosystem and organismal levels. It is becoming clear that exposure to different types of microplastic can impair the nonspecific immune system of crustaceans. Therefore, this study investigated the impact of high-density polyethylene microplastic particles (HDPE-MP) in feed on nonspecific immune system gene expression of Pacific white shrimp (*Litopenaeus vannamei*). The LC_{50} at day 28 of HDPE-MP ingestion was 3.074% w/w of food. Superoxide dismutase (SOD) and the Glutathione peroxidase (GPx) gene were upregulated significantly in groups supplemented with 0.1 and 0.5% of HDPE-MP and then down-regulated significantly after ingested for extended period of time. Lysozyme (LYZ) gene was down-regulated significantly in all treatment groups after 14 days of exposure. Significant histopathological changes in the hepatopancreas such as interstitial hemocytic infiltration, epithelium hyperplasia, tubular deformity, nodule formation, and melanization were observed in the HDPE-MP treatment groups. The severity of each lesion increased, corresponding to the increasing concentration of HDPE-MP. Ingestion of HDPE-MP contaminated feed can interfere with expression of major antioxidant and anti-bacterial genes and damage the hepatopancreas in Pacific white shrimp.

Field of Study:	Veterinary Medicine	Student's Signature	....
Academic Year:	2022	Advisor's Signature	....
		Co-advisor's Signature	....

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Songsak Niemcharoen

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Chapter 1

Introduction

1.1 Background and significance of the problem

At present, the problem of plastic waste in the ocean is a major issue affecting the environment and marine ecosystems, including marine life. In 2019, it is estimated that there were 1.13 million tons of plastic waste on the surface of the ocean (Lebreton et al., 2019). One of the major types of plastic waste is polyethylene which accounted for 38% of all plastic waste in the oceans in 2007. Polyethylene may be divided into low-density polyethylene (LDPE), which is 21% of plastic waste in the sea, and high-density polyethylene (High-density polyethylene; HDPE), 17% of plastic waste in the sea (Andrady, 2011). Such plastic waste is generally highly durable in the environment and can affect marine life both directly and indirectly. The United Nations estimates that more than 800 species of marine and coastal creatures are being affected by plastic waste (Dias, 2016). Besides the macro-plastic, small plastic particles called microplastics are also a major concern in the global marine environment. Microplastics, or synthetic plastic particles between 1 μm and 5 mm in diameter, are often caused by direct production or by the breakdown of large plastic waste in the environment (Frias and Nash, 2019). Today, many microplastics are reported to accumulate in the global marine environment (Desforges et al., 2014; Zhang et al., 2015; Cincinelli et al., 2019). In addition, microplastic contamination has been found in the gastrointestinal tract of many marine species (Foekema et al., 2013; Cannon et al., 2016; Naidoo et al., 2016). The problem of microplastic contamination is one of the threats to the environment and important ecosystems of the world in the 21st century.

The Pacific white shrimp (*Litopenaeus vannamei*) is one of the most important marine species in Thailand. Pacific white shrimp, an important food source for humans worldwide and a top priority in the world's aquaculture industry, which 4.4 million tons of Pacific white shrimp were cultivated in 2017, out of a total of 9.1 million tons of global shrimp production (FAO, 2019). Even though microplastic contamination from shrimp raised in aquaculture systems has never been reported,

shrimp raised in such systems are still at risk of getting microplastics from the food, water, and pond environment. Microplastic contamination has now been reported in the fish meal (Hanachi et al., 2019), which is an important component of shrimp feed (Tantikitti et al., 2016). The practice of using of HDPE-pond lining have been widely adopted by shrimp farm to increase survivability of shrimp (Prawitwilaikul et al., 2006). However, without proper maintenance, these HDPE-pond lining could degrade and become a source of HDPE microplastic in shrimp ponds (Kershaw, 2016; Schoof and DeNike, 2017). This contamination could affect the health of shrimp in the aquaculture system as well.

Microplastics can affect living creatures in many ways whether it is a direct physical effect of plastic particles or the chemical effects of polymers and their contaminants (Jovanoví et al., 2018). Microplastics can interfere with nonspecific immune function in crustaceans. From studies in Chinese mitten crabs (*Eriocheir sinensis*) (Yu et al., 2018; Liu et al., 2019) and Penaeid shrimp (Wang et al., 2021), microplastics were found to alter the expression of enzymes and genes related to immunity. And microplastics can also cause histopathological lesions of the Hepatopancreas of Pacific white shrimp (Hsieh et al., 2021). The results of those studies indicate that microplastics can have a significant impact on the health of crustaceans since crustaceans rely on a nonspecific immune system as the only primary immune system to defend themselves against pathogens (Aguirre-Guzman et al., 2009). Current studies on the effects of microplastics on these groups of animals, especially Pacific white shrimp are still very rare. Further studies of its effects on health and the immune system are needed to help better understand the toxicity of microplastics in this group of animals, especially Pacific white shrimp.

Microplastic research is becoming a major topic for researchers around the world. Although there have been many reports of microplastic contamination in aquatic animals, knowledge of the apparent effects of microplastics on animal and human health is still limited. In 2017, the Food and Agriculture Organization of the United Nations (FAO) assessed that microplastics have a very low risk of introducing contaminants into the human body (Lusher et al., 2017; Everaert et al., 2018). However, microplastics will have a greater impact on the health of animals and

humans in the future (Lusher et al., 2017). Therefore, a precise study of its effects on humans and animals, particularly marine animals as human's source of food is very critical for the future.

This study examined the effects of HDPE microplastics on the immune system of Pacific white shrimp. The toxicology of microplastics through ingestion was studied as well as gene expression associated with the nonspecific immune system of shrimp and histopathological occurring lesions in shrimp. The data obtained from this study can be used to further understand the potential impact of microplastics on the shrimp farming industry which is one of the important industries with high value in the country. This will establish guidelines for farm management and shrimp health management to cope with an increasing trend of microplastic contamination in the environment and to strengthen food security in the future. It will also increase the awareness of the effects of microplastics on shrimp and other creatures in sub-phylum crustaceans that may ingest microplastics in nature. Further study on the impact of microplastics on the health of humans, shrimp, and other marines may lead to a wider awareness of the dangers of microplastics to farmers, veterinarians, related agencies, and the general public. This information can also help in formulating environmental policies in the future.

1.2 Objectives

1) To study the toxicity of microplastics on Pacific white shrimp.

2) To study the effects of microplastics on the expression of nonspecific immune system genes of Pacific white shrimp: *Superoxide dismutase* (*SOD*), *Glutathione peroxidase* (*GPx*), and *Lysozyme* (*LYZ*).

3) To study histopathological lesions at the hepatopancreas of Pacific white shrimp.

1.3 Scope and limitations of the research

This study was divided into two parts namely; Part I examined the toxicity of HDPE 50 μm in diameter and the LC_{50} of this microplastic in Pacific white shrimp. The second part of the study examined the response and alteration of gene

expression in nonspecific immune systems and the histopathology of the hepatopancreas of Pacific white shrimp.

1.4 Expected Outcomes

This study will provide basic information for further studies of the toxicity of microplastics to humans and animals as well as raise awareness of the effect of microplastics on both the environment and public health. The study will also create understanding and information that will lead to policy formulation regarding plastic waste in the future. In addition, the data obtained from the study could raise awareness about the seriousness of microplastic contamination in the shrimp farming industry and suggest a new approach to shrimp farm management in the future.

Chapter 2

Review literature

2.1 Pacific white Shrimp

2.1.1 Taxonomy

The Pacific white shrimp or Whiteleg shrimp is classified in the Kingdom Animalia, Phylum Arthropoda, Class Crustacean, Subclass Malacostraca, Super order Eucarid Ecarida, Order Decapoda, Suborder Natantia, Section Penacidea, Family Penaeidae, Genus *Peneus Litopenaeus*, Subgenus *Penacus Litopenaeus*, Species *Litopenaeus Vannamei*.

2.1.2 Importance of Pacific white shrimp

Pacific white shrimp are one of the most important aquatic species in the global aquaculture industry. Pacific white shrimp is the most cultivated species of marine shrimp in the world. In 2017, global shrimp production was 9.1 million tons, of which 4.4 million tons or up to 48% of global shrimp production is Pacific white shrimp (FAO, 2019). In 2017, Thailand can produce 327 thousand tons of marine shrimp, of which 314 thousand tons is white shrimp (Rubel et al., 2019). Thailand is rated as the 6th largest producer of marine shrimp in the world with an 8% share of the global marine shrimp market (Rubel et al., 2019).

2.1.3 Immune system of Pacific white shrimp

All animals need an immune system to cope with infections and foreign substances that can harm the animal's body upon ingestion. The immune system of animals can be divided into two types: specific immunity system, which is the immune response to repeated exposure to the same specific pathogen or foreign body (Immunological memory) (Iriti and Faoro, 2007). The main defense of the specific immunity system is antibodies, which are produced by B lymphocytes and circulating in the blood, and T lymphocytes, which could destroy infected cells and kill pathogens (Dempsey et al., 2003). However, the specific immunity is restricted to

vertebrate animals only (Iriti and Faoro, 2007). Despite invertebrate animals do not possess a true specific immune system, The recent studies in shrimp suggested an immune mechanism that could exhibit immune specificity and immune memory called alternative specific immunity (Amatul-Samahah et al., 2020; Kulkarni et al., 2021). However, the mechanism and function of alternative-specific immunity have a lot to be discovered. Secondly, the nonspecific immunity system is the main line of defense against foreign bodies and pathogens (Menezes and Jared, 2002; Iriti and Faoro, 2007). Although the true specific immune system is lacking in shrimp, an efficient innate immune system renders protection against invading pathogens (Aguirre-Guzman et al., 2009; Kulkarni et al., 2021).

Pacific white shrimp are invertebrates that rely heavily on the non-specific immune system to protect the body from infection with the assistance of novel alternative-specific immune systems and protection by physical defenses, in which these components must work in conjunction with each other to protect the body from pathogens (Aguirre-Guzman et al., 2009; Kulkarni et al., 2021).

2.1.3.1 Physical defenses

Shrimp's physical defenses are the first line of defense against pathogens and foreign matter which consists of a rigid exoskeleton or cuticle that acts to prevent dangerous injuries and the invasion of microorganisms into the body (Aguirre-Guzman et al., 2009). The main constituent of the exoskeleton of animals in the subphylum Crustacean is chitin, a polysaccharide net, protein, and calcium carbonate, which makes the cuticle hard. There are also other minor components such as proteoglycan, lipids, and other minerals (Nagasawa, 2012). The distribution of hemocyanin (HC) and phenol (which are in the Prophenoloxidase system.) on the outer shell (Exocuticle) and the inner shell (Endocuticle) of the crustacean have also been reported (Adachi et al., 2005), which act as the line of defense against injury and infection during the process of molting and cuticle hardening (Moret and Moreau, 2012).

2.1.3.2 Nonspecific immunity

The non-specific immunity of shrimp in the Penaeidae family consists of cell-mediated immunity and Humoral immunity (Aguirre-Guzman et al., 2009; Kulkarni et al., 2021).

2.1.3.2.1 Cellular immune system

Crustacean has an open circulatory system where hemolymph circulates through the hemocele to nourish the body's tissues. Blood cells, or hemocytes, and fluid in the immune system are transported through the hemolymph to eliminate foreign matter and pathogens that enter the body (Rendón and Balcázar, 2016). Hemocytes play an important role in cellular immunity. These cells have functions and biological properties similar to those in the vertebrate immune system, including macrophages, granulocytes, and natural killer cells (Braak, 2002). Hemocytes are responsible for phagocytosis, the process of encapsulating foreign bodies, nodule formation, wound repair, clotting, and prophenoloxidase (Kannan and Jain, 2000; Jiravanichpaisal et al., 2006; Kulkarni et al., 2021).

Phagocytosis

Phagocytosis is the primary defense mechanism in invertebrates (Aguirre-Guzman et al., 2009), which consists of chemotaxis, adherence, ingestion, pathogen destruction, and, exocytosis (Kondo et al., 1998; Vargas-Albores et al., 1998). The Granulocyte cells and Semi-granulocytes cells have the ability to remove foreign matter by phagocytosis (Zhang et al., 2006).

After ingesting the microorganisms, cells have two methods of eliminating ingested pollutants. The first is aerobic, which uses Nicotinamide adenine dinucleotide phosphate (NADPH) in the reduction reaction with electrons of oxygen (Aguirre-Guzman et al., 2009) to generate Superoxide ion. It is one of the reactive oxygen species (ROS) that can destroy microorganisms (Dupré‑Crochet et al., 2013). The second method is an anaerobic one, which uses active enzymes in eliminating microorganisms by producing lysozyme and antimicrobial peptides (AMP).

Encapsulating foreign and nodules formation

Semigranulocyte is responsible for identifying foreign bodies and encapsulating them with 76kD protein, which acts as an opsonin involved in the prophenoloxidase system. Moreover, these proteins are responsible for the degranulation process and adhesion of semigranulocyte. They also act as a catalyst for the encapsulation process of the granulocyte as well (Vargas-Albores et al., 1998; Yeh et al., 1999; Wang et al., 2001; Braak, 2002).

Nodule formation takes place after the action of the prophenoloxidase system, melanization, and microorganisms destruction (Wang et al., 2001; Braak, 2002). Nodules can be found in the gills and hepatopancreas of shrimp which is caused by the systematic merger of many hemocytes in order to encapsulate microorganisms or large antigens that cannot be eliminated by phagocytosis processes (Aguirre-Guzman et al., 2009).

Prophenoloxidase system

The prophenoloxidase system is an important foreign body removal mechanism in invertebrates (Mak and Saunders, 2005). The cascade reaction of the prophenoloxidase system is shown in **Figure 2.1**. Granulocyte may synthesize, collect and release phenol oxidase by molecules of fungal β-glucan, peptidoglycan. and lipopolysaccharide, which is recognized by a pattern recognition protein (PRP), namely peptidoglycan binding protein (PGBP), LPS and β-1,3-glucan binding protein (LGBP) and β-1,3-glucan binding protein (βGBP), which leads to the activation of the serine proteinase cascade reaction. Serine proteinase enzyme will activate the pro-prophenoloxidase activating enzyme (ProPPAE) to become prophenoloxidase activating enzyme (PPAE), which is in an active form. As a result, prophenoloxidase (ProPO), an enzyme that is in an inactive form is converted to Phenoloxidase (PO), which is an active enzyme. PO will activate the phenoloxidase which converts phenols into quinones that have antimicrobial properties (Lee et al., 2004; Hellio et al., 2007; Söderhäll et al., 2013). Quinone can induce oxidative stress (Bolton and Dunlap, 2017), and later on, melanin is synthesized. Both melanin and substances

undergoing melanin synthesis are also capable of eliminating pathogens (Mak and Saunders, 2005; Söderhäll et al., 2013).
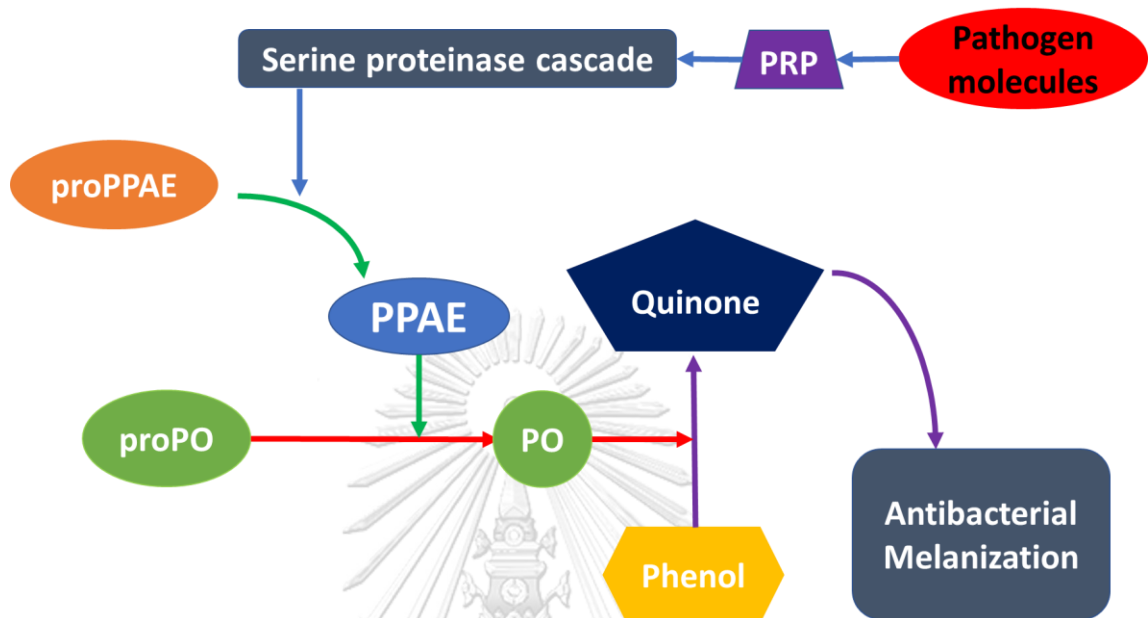
Figure 2.1: The activation process of the prophenoloxidase system (Adapted from Söderhäll et al. (2013))

Melnization

Melanization is a biochemical process of several protease enzymes regulated by the prophenoloxidase system (Robalino et al., 2007; Pais et al., 2008). Melanin, the dark brown pigment is a product of the prophenoloxidase system. Melanin is produced around foreign bodies and has antimicrobial properties (Holmblad and Söderhäll, 1999; Mak and Saunders, 2005; Barillas-Mury, 2007).

Cytokine

Hemocytes produce cytokines, which regulate the antimicrobial processes in invertebrates. Cytokines in invertebrates are similar to those in vertebrates, i.e. interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α). These cytokines have the same function as cytokines in vertebrates (Nappi and Ottaviani, 2000). Invertebrates also have heat shock protein (HSP) which is

a cytokine that can protect and repair proteins damaged by stress factors such as heat (Frankenberg et al., 2000; Lo et al., 2004).

Clotting protein cascade

Blood coagulation is the process of preventing the loss of hemolymph through the wound and inhibiting the spread of pathogens (Chen et al., 2005). Crustaceans have a total of three hemolymph coagulation systems. Type 1 is an agglutination of hemocytes without hemolymph coagulation. Type 2 consists of agglutination of hemocytes and coagulation of hemolymph. And Type 3 involves agglutination of hemocytes, then disintegration followed by hemolymph coagulation (Yeh et al., 1999; Van de Braak et al., 2002).

Blood coagulation in crustaceans is regulated by coagulation proteins such as coagulogen and compartmentalized cellular factors protein within the hemocyte that circulate in the blood system. Blood coagulation proteins are converted to polymers linked by covalent bonds by calcium-dependent transglutaminase secreted by hemocytes (Wang et al., 2001). Blood coagulation is induced by lipopolysaccharide or β-1,3 glucan) and correlate with the activity of the prophenoloxidase system (Roux et al., 2002).

Antioxidant enzyme

Antioxidant enzymes were created to protect the body from cytotoxicity caused by metabolic processes at the cellular level and oxidative stress (Downs et al., 2001). Oxidative stress occurs when the oxidative substance, or ROS, is produced in quantities greater than the Antioxidant enzymes. ROS plays a major role as a secondary messenger in intracellular signaling in process of maintaining cell homeostasis (Burton and Jauniaux, 2011). However, excess ROS can cause damage to biomolecules which resulted in the loss of functions and apoptosis of cells and organs (Kannan and Jain, 2000). Damages from the excess oxidative reaction can lead to a loss of strength in organ structures (Grezzana et al., 2004). The hepatopancreas of Pacific white shrimp is susceptible to ROS caused by stress factors (Han et al., 2018a; Han et al., 2018b).

Antioxidant enzymes found in Pacific white shrimp include SOD, catalase (CAT), glutathione peroxidase (GPx), and thioredoxin (TRx). These enzymes are responsible for protecting cells from oxidative stress (Wang et al., 2009). SOD is one of the main mechanisms of defense against oxidative stress caused by pollution, infection, hypoxia, hyperoxia, hypothermia, and immunomodulators (Neves et al., 2000). Moreover, there is an antioxidant mechanism without using enzymes, including Ascorbate, β-carotene), Flavonoids, α-tocopherol, and vitamin E, which can eliminate active free oxygen derivatives and repair cell damage (Nathan and Shiloh, 2000).

The SODs are responsible for catalyzing the dismutation reaction of Superoxide anion (O^{2-}) into hydrogen peroxide, which is less toxic, as shown in **Figure 2.2** (Matés and Sánchez-Jiménez, 1999; Halliwell and Gutteridge, 2015). SODs are classified as manganese superoxide dismutase (MnSOD), which is found in mitochondria and in prokaryotic cells. Secondly, iron superoxide dismutase (FeSOD) is found in bacteria and plants. And the copper superoxide dismutase (CuSOD), which is found in eukaryotic cells. Extracellular superoxide dismutase (EC-SOD) has been reported in lobsters. This enzyme is involved in phagocytosis, opsonization, foreign body encapsulation, and the production of antimicrobials (Holmblad and Söderhäll, 1999).

$$O_2^- + O_2^- + 2H^+ \xrightarrow{\text{SOD}} O_2 + H_2O_2$$

Figure 2.2: The reduction of superoxide anion with superoxide dismutase (Adapted from Nimse and Pal (2015))

Although hydrogen peroxide has lower toxicity than superoxide anions, it is still cytotoxic and can damage the genetic material (Cantoni et al., 1989; Mahaseth and Kuzminov, 2017). Therefore, cells rely on CAT and GPx to neutralize the toxicity of hydrogen peroxide by which CAT converts hydrogen peroxide into water and oxygen as shown in **Figure 2.3** (Alfonso-Prieto et al., 2009). GPx catalyzes the

reduction reaction between glutathione (GSH) and hydrogen peroxide to become water and glutathione disulfide (GSSG). Then GSSG is converted back to glutathione through a reduction reaction with glutathione reductase (GR) as the catalyst. As shown in **Figure 2.4** (Mattmiller et al., 2013).

$$2H_2O_2 \xrightarrow{\text{CAT}} O_2 + 2H_2O$$
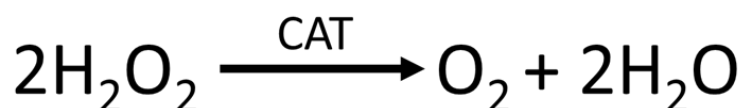
Figure 2.3: The reduction of hydrogen peroxide with catalase (Adapted from Nimse and Pal (2015))

Figure 2.4: The reduction of hydrogen peroxide with glutathione peroxidase (Adapted from Higuchi (2014))

Hydrogen peroxide also creates disulfide bonds in proteins, which cause the deformation of proteins and inhibit the enzymes from functioning properly. Therefore, reduced thioredoxin (TRx-2SH) is required to catalyze the reduction reaction of disulfide bonds. It is formed into proteins with sulfhydryl groups (Sulfhydryl; SH) and the oxidized thioredoxin (TRx-S2). And subsequently reverted to reduction form, which is an active form through the reduction reaction with thioredoxin reductase (TRxR) as the catalyst (Holmgren, 1995), as shown in **Figure 2.5.**
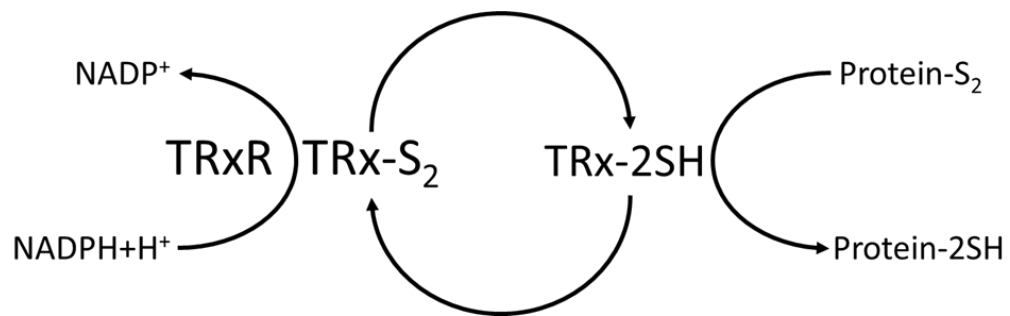
Figure 2.5: Deformation of disulfide bond of protein with thioredoxin (Adapted from Matsuo and Yodoi (2013))

Ubiquitination

Ubiquitination is an essential type of post-translation modification, whereby ubiquitin protein units are attached to a target protein and allows these proteins to be recognized by other enzyme complexes or organelles in the cell, thereby executing their functions such as cell cycle, proliferation, differentiation, DNA repair, energy metabolism, etc. (Nakayama and Nakayama, 2006; Reinstein and Ciechanover, 2006; Naujokat and Šarić, 2007; Kwon and Ciechanover, 2017; Lavie et al., 2018; Zhang et al., 2021). Ubiquitination consists of three steps catalyzed by the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-ligase (E3) (Ciechanover, 2015). During this cascade of reaction, E1 activates and transfers the ubiquitin to E2, which uses ATP. Then, the interaction between E2 and E3 allows the transfer of ubiquitin to the target protein. The seven lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63) on ubiquitin or its N-terminal methionine are the residues through which other ubiquitin units bind to increase the number of ubiquitin molecules on the target protein (Kwon and Ciechanover, 2017).

Besides the primary functions of ubiquitination, recent studies have suggested ubiquitination as a mechanism of immune response (Zhang et al., 2021). Penaeid shrimp have been to adopted ubiquitination as an antiviral mechanism. Since viruses need to enter host cells and be followed by exploiting host nutrients for their replication, proliferation, etc. Hence viruses have to hijack the various host's proteins such as polymeric immunoglobulin receptor (pIgR), ferritin, etc., and immune signaling pathways and proteins such as Toll/IMD-nuclear factor-**K**B (NF-**K**B), JAK-

STAT, Wnt/β-catenin signaling pathways, TRIM50-like, etc. (Wen et al., 2014; Lin et al., 2015a; Sun et al., 2017; Niu et al., 2019; Zhu et al., 2019; Zhao et al., 2021) Ubiquitination intercepted these proteins by the binding of ubiquitin, which lead to inhibition or degradation of these proteins and result in the inhibition of virus proliferation within the host cell (Sun et al., 2017; Sun et al., 2019; Zhao et al., 2021).

2.1.3.2.2 Humoral immunity system

Antimicrobial peptides

Antimicrobial peptides are one of the key mechanisms against pathogens in invertebrates (Marshall and Arenas, 2003). Antimicrobial peptides have a broad-spectrum antimicrobial property, low specificity, and low toxicity to animal cells. These peptides cause holes in the cell membrane of bacteria, fungi, parasites, viral envelop, and cancer cells. This leads to an imbalance of cellular energy and ions (Hancock, 1998; Bulet et al., 1999; Lehrer and Ganz, 1999).

Penaeidins are one of the antimicrobial peptides whose genetic sequences have been described in Pacific white shrimp and other shrimp in the Penaeid family (Destoumieux et al., 1997; Gross et al., 2001; Rojtinnakorn et al., 2002; Supungul et al., 2002). Penaeidins are synthesized and stored in granulocytes and are active against gram-positive bacteria and fungi (Destoumieux et al., 1997; Bachère et al., 2000; Destoumieux et al., 2000).

Hemocyanin is a protein in the hemolymph and is responsible for transporting oxygen to nourish the body. Hemocyanin can be found in the phylum of Arthropods and Mollusca (Markl and Decker, 1992; Lieb et al., 2001; Lippitz et al., 2002). Hemocyanin can convert its peptides at the C-terminal to have anti-fungal activity (Destoumieux-Garzón et al., 2001). Histone proteins found in the hemocytes of Pacific white shrimp have properties against gram-positive bacteria, similar to the function of histone proteins in vertebrates (Patat et al., 2004).

Lysozyme

Lysozyme is an enzyme that plays an important role in the nonspecific immune system. The lysozyme has the ability to degrade the cell wall of gram-

negative bacteria. It can modify the arrangement of molecules on the cell membrane to facilitate the cells that work in the process of phagocytosis (De-La-Re-Vega et al., 2004; Callewaert and Michiels, 2010; Van Herreweghe and Michiels, 2012).

The lysozyme found in animals can be divided into three types: c-type lysozyme or chicken-type lysozyme, g-type lysozyme or goose-type lysozyme, and i-type lysozyme or invertebrate-type lysozyme. Animals in the phylum of arthropods have only c-type and i-type lysozyme (Callewaert and Michiels, 2010). Shrimp in the Penaeid family studied for c-type lysozyme were Kuruma shrimp (*Marsupenaeus japonicas*) (Hikima et al., 2003), Pacific white shrimp (Sotelo-Mundo et al., 2003), Black tiger shrimp (*Penaeus monodon*) (Tyagi et al., 2007; Xing et al., 2009), Banana shrimp (*Fenneropenaeus merguiensis*) (Mai and Hu, 2009), and Chinese white shrimp. (*Fenneropenaeus chinensis*) (Bu et al., 2008). The i-type lysozyme in Penaeid shrimp has been reported in black tiger shrimp and Pacific white shrimp. It can be further divided into two forms: i-type lysozyme 1 and i-type lysozyme 2 (Supungul et al., 2010; Chen et al., 2016). Expression of the *c-type lysozyme* gene can be detected in the gills, antennal glands, epipodite, heart, hemocytes, hepatopancreas, eye stalk, lymphoid organs, and intestines. Most gene expression is found in hemocytes. Expression of the *i-type lysozyme 1* gene can only be detected in the hepatopancreas. Whereas the expression of the *i-type lysozyme 2* gene is detectable in all tissues, with the exception of the gills, lymphatic organs, and intestines, while the heart showed the most gene expression (Supungul et al., 2010; Chen et al., 2016). The c-type and i-type lysozymes 2 play a role in inhibiting and suppressing bacteria and viruses, whereas i-type lysozyme 1 acts as an enzyme in the digestive system (Supungul et al., 2010; Chen et al., 2016; Liu et al., 2016).

2.1.3.3 Alternative specific immunity

Specific immunity, which has antigen specificity and immunological memory, was thought to exist only in vertebrate animals. However, the evidence has suggested that invertebrate animals are also capable of exhibiting specific immune responses through antibody-independent mechanisms (Chambers and Schneider, 2012). Shrimp that were previously injected with antigen (Vaccine-like treatments) increased

tolerance to the pathogen from which the antigen was originally derived (Johnson et al., 2008; Powell et al., 2011; Amatul-Samahah et al., 2020). However, the mechanism that underlines these phenomena is not fully understood.

Down syndrome cell adhesion molecule

Down syndrome cell adhesion molecule (Dscam) is a pathogen recognition protein, which exhibits extremely high variability generated from a single copy of a gene through alternative splicing (Schmucker and Chen, 2009). Dscam was purposed to be the crucial mediator in specific immunity in insects and crustaceans. Since hemocytes of crustaceans and arthropods were already known to express pattern recognition proteins that identify self and antigen molecules, it was suggested that Dscam might be involved in phagocytosis. It was proposed that the hypervariability of Dscam might account for its ability to recognize diverse ligands, epitopes, and pathogens (Ng et al., 2014).

2.1.4 Hepatopancreas

The hepatopancreas is an important organ of animals belonging to the crustacean phylum. They are the organs responsible for the digestive and metabolic processes of the body. The functions of the hepatopancreas can be equivalent to the functions of the intestines, liver, and pancreas of vertebrates. The main functions of the hepatopancreas in shrimp include the production of digestive enzymes, the absorption of nutrients, the storage of nutrients and energy, and the metabolism of fats and carbohydrates. They also play important role in the growth and reproduction process of shrimp (Vogt, 2019).

2.1.4.1 Overall structure of the hepatopancreas

The hepatopancreas is an organ located in the cephalothorax. They consist of a network of close-ended tubules and are wrapped in a network of longitudinal muscle fibers and circular fibers. The duct network of the hepatopancreas connects to the pyloric stomach through a filter structure called the Pyloric filter (**Figure 2.6**).

It is responsible for filtering digested food into the hepatopancreatic ducts. Then, nutrients are further absorbed within the hepatopancreatic ducts (Vogt, 2019).
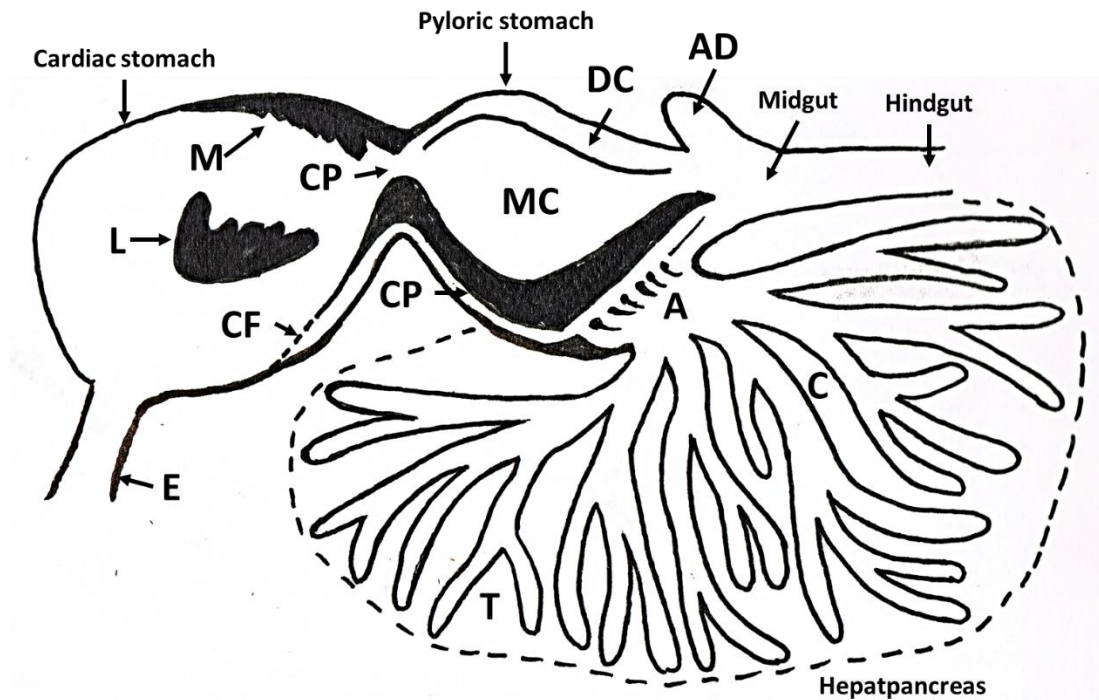
Figure 2.6: Structure of the gastrointestinal tract and hepatopancreas of decapod (Adapted from Vogt (2019))

Abbreviation: T = Terminal end of hepatopancreatic duct, C = Collecting duct, A = Atrium, PF = Pyloric filter, E = Esophagus, M = Medial tooth, L = Lateral tooth, CF = Cardiac filter, CP = Cardiac filter channel, MC = Medial chamber, DC = Dorsal chamber and AD = Anterior dorsal cecum

2.1.4.2 Histological structure and functions of the hepatopancreas

2.1.4.2.1 E-cell

E- cells, or embryonic cells, are stem cells and progenitor cells of the hepatopancreas. The E-cells are located at the distal end of the hepatopancreatic ducts which is known as the embryonic zone. E-cells are cuboidal cells with a high nucleus-to-cytoplasm ratio (Cervellione et al., 2017), microvillus border faces the lumen, and the basal lamina faces the hemolymph sinus. The E-cells in the mitotic stage are mostly located at the distal ends of the hepatopancreatic ducts. The E-

cells are generated by dividing and differentiating as cells are further pushed away from the embryonic zone (Vogt, 2019).

2.1.4.2.2 R-cell

R-cell or reabsorption cells are the most abundant cells in the hepatopancreas and are responsible for the absorption of nutrients. R-cells are columnar cells (Cervellione et al., 2017) with an apical part comprising the microvillus. Its cytoplasm consists of numerous nuclei and lipid vacuoles (**Figure 2.7**). The basal parts are attached to the hemolymph sinus. R-cells can be found in the proximal and central parts of the hepatopancreas. R-cell are the storage of fat and glycogen, which are the energy of crustaceans. The nutrients and energy stored in R-cell is used in cases of molting, malnutrition, or Vitellogenesis. R-cell contains granules that can accumulate minerals such as calcium, iron, mercury, and plutonium. R-cell is also responsible for the synthesis of lipoprotein and vitellogenin (Cervellione et al., 2017; Vogt, 2019).

2.1.4.2.3 F-cell

F- cells, or fibrillar cells, are columnar cells that can be found in the anterior and central part of the hepatopancreatic ducts (Cervellione et al., 2017) with a rough endoplasmic reticulum (RER) and a large number of Golgi bodies (**Figure 2.7**). F- cells are responsible for the synthesis and secretion of digestive enzymes such as astacin, trypsin, carboxypeptidase, chymotrypsin, cathepsin L, amylase, cellulase and. lipase. These digestive enzymes are produced are released into the lumen of the hepatopancreatic ducts and are stored in the cardiac part of the stomach, waiting for further digestion. These enzymes are synthesized in the form of pro-enzymes that are not active. These pro-enzymes are activated when they move into the stomach. It was also found that the F- cells of the Penaeid shrimp were able to synthesize hemocyanin (Vogt, 2019).

2.1.4.2.4 B-cell

B-cell or Blister cell is a cell with a large vacuole (**Figure 2.7**). It is found only in the proximal part of the hepatopancreatic duct (Cervellione et al., 2017). Mature B-cell will be detached from the lamina basal and enter the lumen of the hepatopancreas. The exact function of the B-cell is not yet known. It is assumed that B cells are primarily responsible for secretion, production of digestive enzymes and intracellular digestive enzymes, intracellular digestion, absorption and, accumulation of nutrients within the vacuoles before excreting them for R-cell for to uptake and storage after the intracellular digestion process is complete (Cervellione et al., 2017). It has also been suggested that B-cells are involved in the lipid digestion process by the synthesis of emulsifiers such as acyltaurine and acylsacrosiltaurine (Vogt, 2019).
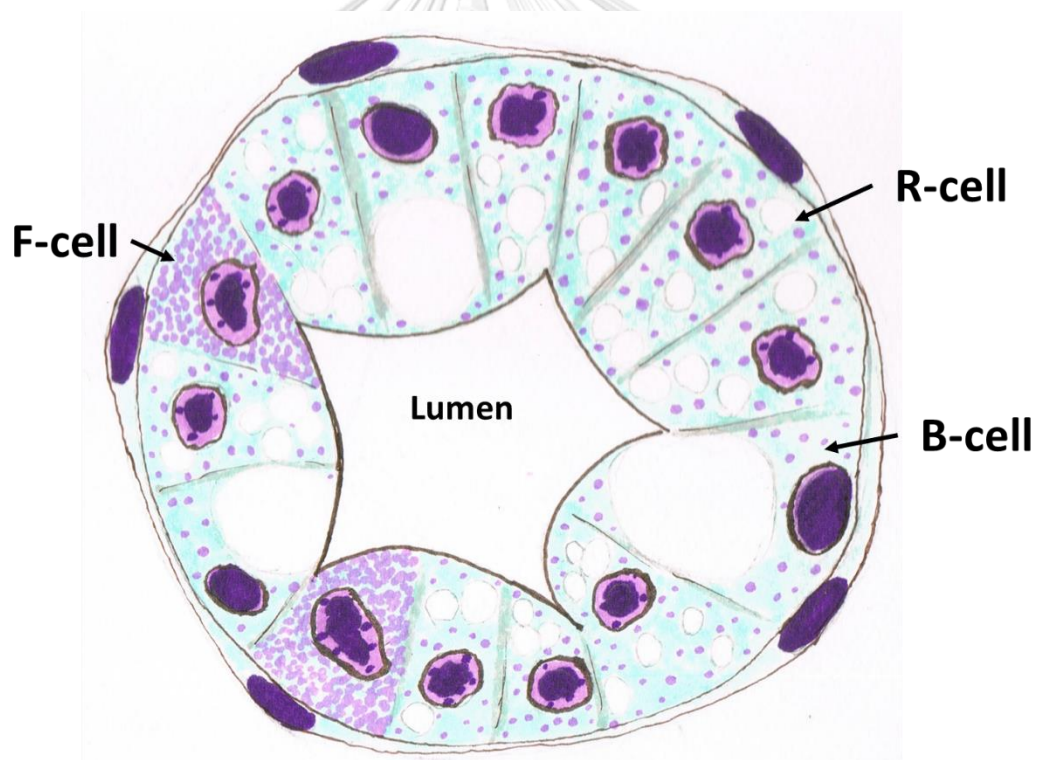
Figure 2.7: A cross-sectional view of the hepatopancreatic ducts and the characteristic structures of the R-cells, F-cells and B-cells (Adapted from Cervellione et al. (2017))

2.2 Microplastics

2.2.1 Definitions and Meanings

Nowadays, there is concern about the effects of microplastics on living organisms. Microplastics are plastic particles that are less than 5 mm in diameter but more than 1 µm (Frias and Nash, 2019). Microplastics can be divided into two categories according to their origin. The first type is primary microplastics which are microplastics that are directly discarded into the environment through wastewater from households or industries. Primary microplastics found in wastewater can be classified according to their shape into spherical, irregular, fibers, and film (Kang et al., 2015; Lusher et al., 2015; Nobre et al., 2015; Rummel et al., 2016; Su et al., 2016). The second type is secondary microplastics which are caused by the degradation or breaking down of plastic material larger than 5 mm in diameter caused by photo-oxidative from ultraviolet, mechanical transformation such as wave abrasion, wind erosion, tire friction, and biodegradation by microorganisms (Browne et al., 2007; Andrady and Neal, 2009; Cole et al., 2011; Löhr et al., 2017). In addition, microplastics present in the environment can also be degraded into nano-plastics with diameters less than 1000 nm (Frias and Nash, 2019), whose effects and toxicity are unknown (Koelmans et al., 2015; da Costa et al., 2016).

2.2.2 Microplastic contamination in the environment

There is evidence of microplastic contamination and accumulation in the environment. Microplastic contamination in water and sediment has been reported in the natural environment including rivers, lakes, estuarine, coastal areas, and oceans (Desforges et al., 2014; Lahens et al., 2018; Tibbetts et al., 2018; Cincinelli et al., 2019; Hitchcock and Mitrovic, 2019). All of the studies indicated a direct correlation between microplastic content to community size, water waste volume and, human activities. Microplastics can also be found in man-made bodies of water, including reservoirs, dams, and water supply systems (Zhang et al., 2015; Danopoulos et al., 2020). It is now widely accepted that the major source of microplastics in the environment is land-based plastic waste that is washed into rivers before continuing

into seas or lakes, and only a small fraction is released directly into the sea by human activities, including maritime, oil rigs, fisheries and, aquaculture (Kershaw, 2016).

2.2.3 Microplastic contamination in aquatic animals

To date, more than 690 species of aquatic animals have been reported to have ingested microplastics (Gall and Thompson, 2015). Aquatic animals confuse microplastics for food or get microplastic by eating smaller animals contaminated with microplastics. Examples of marine species reported for microplastic contamination are Herring (*Clupea harengus*), Sandfish (*Merlangius merlangus*), Mackerel (*Trachurus trachurus*), Haddock (*Melanogrammus aeglefinus*), Cod (*Gadus morhua*) (Foekema et al., 2013), Snow fish (*Dissotichus mawsoni*) (Cannon et al., 2016), Brackish mullet (*Mugil cephalus*) (Naidoo et al., 2016). , Humpback whale (*Megaptera novaeangliae*) (Besseling et al., 2015), Mussel (*Mytilus edulis*) (Van Cauwenberghe et al., 2015; Li et al., 2018b), Oysters (*Saccostrea cucullate*, *Crassostrea gigas*, *Crassostrea angulate*, *Crassostrea hongkongensis* and *Crassostrea sikamea*) (Li et al., 2018a; Teng et al., 2019), Brown shrimp (*Crangon crangon*) (Devriese et al., 2015), Norwegian lobster (*Nephrops norvegicus*) (Welden and Cowie, 2016a), Black tiger shrimp (*Penaeus monodon*) and Pink Shrimp (*Metapenaeus monoceros*) (Hossain et al., 2020), etc.

2.2.4 Microplastics and the aquaculture industry

Although each species of aquaculture has different methods of raising, common equipment that is used in aquaculture are buoyancy, rope, fish cages, plastic pond lining, water turbines, feeders, and fish tanks are all made of or contain plastic materials. The fact that plastic is a cheap and durable material, makes plastics widely used in the global aquaculture industry (Lusher et al., 2017). Plastic materials used in aquaculture can break down into microplastics and enter the aquaculture system (Jang et al., 2020).

The microplastics in the environment are at risk of leaking into the aquaculture system and building up in the water and sediment as well as the aquatic

products. As a result, aquaculture patterns affect the number of microplastics in the environment and microplastics contaminating aquatic animals. It was found that aquaculture in recirculating water systems generates less accumulation of microplastics in the environment and in aquatic animals than aquaculture without recirculating systems such as concrete ponds (Ibrahim et al., 2017; Lv et al., 2020).

Since microplastics contaminate a wide variety of marine life, fish, that are processed into a fish meal, are also contaminated with microplastics (Hanachi et al., 2019; Thiele et al., 2021). Microplastic contamination in fish meal increases the potential for microplastics to enter aquatic products from the aquaculture system. As the fish meal is an important source of protein for aquatic animals and is widely used (Tacon and Metian, 2008). Thus, fish and shellfish consumers are at risk of ingesting microplastics.

Currently, the cultivation of Pacific white shrimp requires plastic material in every step of cultivation, from raising in the hatchery to the incubator. The essential equipment and materials for raising Pacific white shrimp include plastic pond lining, plastic water pipes, water turbines, feeders, and bird netting (Lusher et al., 2017). When these materials deteriorate during use, they can become a source of microplastics in shrimp farms. Together with the use of fishmeal as an ingredient for shrimp feed (Tacon et al., 2013), Pacific white shrimp are more likely to obtain microplastics from fish meal contaminated with microplastics from the sea (Hanachi et al., 2019; Thiele et al., 2021).

2.2.5 Effects of microplastics on aquatic animals

Although plastic is an inert material, plastic particles can be toxic to living things including physical hazards, translocation of microplastics into the body, oxidative stress, inflammation, apoptosis, necrosis, genotoxicity, and immunotoxicity (Volkheimer, 1975; Wright et al., 2013; Wright and Kelly, 2017; Hirt and Body-Malapel, 2020).

2.2.5.1 Toxicity of microplastics

2.2.5.1.1 Physical hazard

There are currently many studies indicating the effects of Macro-plastic ingestion in vertebrates. The reported impacts include ulcers and erosion of tissues of internal and external organs and obstruction of the gastrointestinal tract leading to false satiation, starvation, and deterioration of the body (Laist, 1997; Denuncio et al., 2011; Lazar and GraČan, 2011; Van Franeker et al., 2011; Yamashita et al., 2011). Plastic ingestion led to decreased fertility, drowning, deterred agility to evade predators, and ability to eat as well as increased the chance of toxins from seawater being passed into the body and leading to death (Gregory, 2009). The impact of macroplastics on the aforementioned vertebrates is also similar to the effects of microplastics on small organisms, including invertebrates and small vertebrates.

Microplastics have the potential to clog and attach to the appendages that invertebrates use for finding food or are even embedded in the tissue (Moore et al., 2001; Derraik, 2002). Microplastics can induce false saturation. There is evidence of gastrointestinal obstruction caused by clumps of microplastic fibers entangled within the stomach of Norwegian lobsters. The microplastics that are retained in the stomach can lead to false saturation followed by decreasing consumption and absorption of nutrients. Due to these factors, the growth rate of the Norwegian lobster population is declining. It may impair health and fertility in the population of Norwegian lobster (Welden and Cowie, 2016b; Welden and Cowie, 2016a).

2.2.5.1.2 The translocation of microplastics

The study of the transportability of microplastics into the circulatory system of mammals and poultry revealed that microplastic particles between 10 and 60 µm in diameter were found to be absorbed through the enterocyte of rats, guinea pigs, rabbits, chickens, dogs, and pigs. The previous study demonstrated that polyvinyl chloride particles (PVC) could accumulate in the internal organs and cause an embolism (Volkheimer, 1975). Despite the lack of an enzyme capable of digesting plastic which makes plastic unable to be digested or absorbed, particles of microplastics could pass through the cell membrane. A study in mussels has found

evidence of displacement of spherical PS particles with a diameter of 3 and 9. 6 μm into the hemolymph and hemocytes three days after exposure at a concentration of 15,000 particles per 350 ml of water. This study indicates the ability of spherical microplastics to move through the gastrointestinal lining into the circulatory system. But the mechanism is still unknown (Browne et al., 2008). A study in crustaceans showed that PS particles with a diameter between 180 and 250 nm were able to move from the gastrointestinal tract into the hepatopancreas of fiddler crab (*Uca rapax*) (Brennecke et al., 2015).

2.2.5.1.3 Inflammatory response

Microplastics can induce inflammation in the aquatic digestive system. The secretion of cytokines namely TNF-α, interleukin-1 alpha (IL-1α), interleukin-1beta (IL-1β), Interferon-gamma (INF-γ), IL-6, and interferon (IFN) (Jin et al., 2018; Qiao et al., 2019a; Huang et al., 2020) were found. An increase in D-lactate levels and diamine oxidase (DAO) enzymes were found in gastrointestinal tissue (Qiao et al., 2019b; Kang et al., 2021). D-lactate and DAO are used as indicators of the intestinal inflammatory response (Schmidt et al., 1990; Pucino et al., 2019). There was also a report of increasing expression of the *nuclear factor erythroid 2-related factor 2 (Nrf2)* gene in intestinal tissue (Espinosa et al., 2019), whereas Nrf2 is a protein in the Nrf2 signaling pathway that responds to inflammation (Saha et al., 2020). Evidence of gills inflammation was found after exposure to microplastics from an increased expression of the *TNF-α* and *Prostaglandin-endoperoxide synthase 2a (PTGS2a)* gene (Umamaheswari et al., 2021).

2.2.5.1.4 Histopathological lesions of internal organs

Although plastic is an inert material, there is evidence that suggested internal organs damaging by microplastic ingestion. Initial studies indicated that joint damage was caused by plastic particles from eroded joint endoprostheses. Small plastic fragments that make up the prosthesis were found in the joint capsule, joint cavity, and articular tissue of patients who received a plastic prosthesis. The plastic that slips into joint spaces and tissues was classified as a foreign body and causes

granuloma inflammation, which leads to necrosis, fibrosis, and scar formation of the joint (Willert et al., 1996). This study showed the possibility of internal organ damage after exposure to microplastics.

Microplastics cause histopathological damage to the gastrointestinal tract. A study in Gilthead seabream (*Sparus aurata*) fed with PVC microplastics at a concentration of 500 mg/kg showed an increase in goblet cell proliferation and villous hyperplasia (Espinosa et al., 2019). Microplastics can cause wounds on the intestinal mucosa. In this study, villus rupture, enterocyte dissociation, and intestinal membrane damage of Zebrafish (*Danio rerio*) have been identified (Lei et al., 2018b; Qiao et al., 2019a). The study in European sea bass (*Dicentrarchus labrax*) found that PVC microplastic can damage the intestines and induce goblet cells hyperplasia, villous hyperplasia, mucosal epithelium detachment, and vacuolization of enterocyte (Peda et al., 2016). There was evidence of inflammation in *Girella laevifrons* that ingested carboxylate polystyrene microplastics (PS-COOH) such as leucocyte infiltration in intestinal tissue, intestinal hyperemia, and damaging of villus and Crypt cells (Ahrendt et al., 2020). A study in goldfish (*Carassius auratus*) showed that PS microplastics, Ethylene-vinyl acetate (EVA), and PE could cause intestinal inflammation, villi, and mucous membranes damaging and detachment of the lamina proria (Jabeen et al., 2018). In invertebrates, studies have shown gastrointestinal damage in *Artemia Salina* exposed to PS microplastic. The mucous membranes of the central and peripheral gastrointestinal tract of *Artemia Salina* that were exposed to microplastic were thinned and replaced by basal lamina (Suman et al., 2020). Pacific white shrimp treated with PE microplastics revealed atrophy and damage to intestinal villi and detachment of intestinal wall cells (Hsieh et al., 2021).

Microplastics can also cause inflammation and histopathology lesions in the liver of fish. Histopathological lesions such as hepatic necrosis, and hepatocytes vacuole formation were observed as well as hepatic leukocyte infiltration, hepatocyte lipid droplet and, hepatic lipid deposition observed in Zebrafish that were treated with PS microplastics (Lu et al., 2016). In goldfish treated with microplastic fibers, evidence of inflammation and liver damage was found including micro-granulation, sinusoid dilation, hepatic venous congestion, and hepatic vacuole

formation (Jabeen et al., 2018). A study on European sea bass found congestion in the sinusoid blood vessels and the formation of vacuoles of liver cells after receiving PVC or PE microplastics (Espinosa et al., 2019). The histopathological lesions were identified in the hepatopancreas of Pacific white shrimp that were treated with PE microplastics and nano-plastics, including lumen enlargement, epithelium detachment, B-cell detachment, deformation and rupture of the lumen of the hepatopancreas (Hsieh et al., 2021).

In addition, the microplastic particle could harm the structure and integrity of gills. The study of Zebrafish treated with PS microplastics showed damage to the gills lamellar, capillary dilatation, and necrosis of the gills observed (Umamaheswari et al., 2021). The study on Pacific white shrimp treated with both PE microplastics and nano-plastics showed gill stalk deformation (Hsieh et al., 2021).

2.2.5.1.5 Oxidative stress

All plastics have some initial level of ROS due to the polymerization process and other related production protocols. However, ROS can increase significantly when plastic is exposed to light or in contact with transition metals. The degradation of plastics also leads to the generation of ROS from the cleavage of carbon-hydrogen bonds (White and Turnbull, 1994; Gewert et al., 2015).

Currently, there are many studies that indicate the development of oxidative stress from microplastic exposure in marine animals. The *in vitro* effects of PVC and PE microplastics on head-kidney leucocytes were studied in two fish species: Gilthead Sea Bream and European Snapper, which found an increase in a respiratory burst in cells (Espinosa et al., 2018). Increased antioxidant enzyme activity such as SOD and CAT in the liver and gastrointestinal tract has been reported in Zebrafish that treat with microplastic (Lu et al., 2016; Qiao et al., 2019a; Qiao et al., 2019b). A study in medaka showed an increase in ROS, and decreased expression of *SOD* and *CAT* gene after exposure to PE microplastics with 45 µm in diameter, whereas the group treated with PE microplastic with a diameter of 50 µm showed decreased levels of ROS but increased expression of *SOD*, *CAT* and *GST* gene (Kang et al., 2021).

Besides evidence indicating the occurrence of oxidative stress from microplastic exposure in vertebrates, there is also evidence that suggests oxidative stress in invertebrates. In a study on *Caenorhabditis elegans*, accumulation of ROS and lipofuscin was found after ingestion of PS microplastic (Yu et al., 2020). Evidence of oxidative stress response was also found from the increase in expression of *Glutathione-s-transferase 4* (*GST4*) and *SOD* gene in the gut tissue after ingestion of microplastics (Lei et al., 2018b; Yu et al., 2020). The study in *Paracyclopina nana* treated with PS microplastics (0.5 and 6 μm) and nano-plastics (0. 05 nm) showed the elevation of ROS level, along with an increase in the activity of antioxidative enzymes such as SOD, GPx, GR, and GST, in response to the oxidative stress that generated by microplastics (Jeong et al., 2017). The study on Chinese mitten crab also indicated increasing activity of antioxidative enzymes, including SOD, GSH, and GPx (Yu et al., 2018). The study on three species of shrimps in the Penaeid family namely; Black tiger shrimp, Kuruma shrimp (*Marsupenaeus japonicus*), and Pacific white shrimp, found that after 48 hours of aqueous infusion of PE microplastics, there was a significant increase in the expression of *CAT* gene (Wang et al., 2021). The study by Hsieh et al. (2021) found an increase in thiobarbituric acid (TBARS) in the hepatopancreas which indicates that lipid peroxidation has occurred in the hepatopancreas from microplastic exposure.

2.2.5.1.6 Apoptosis

Apoptosis is an important physiological process in the development and maturation of tissues. Abnormality in apoptosis regulation resulted in diseases and disorders of the body. Inadequate apoptosis led to the accumulation of cells which may cause diseases such as cancer, coronary artery stenosis (Restenosis), etc. On the other hand, Excessive apoptosis caused the deterioration of the organs which corresponds to the occurrence of diseases such as stroke, congestive heart failure, neurodegenerative disease, AIDS, etc. (Reed, 2000; Favaloro et al., 2012). ROS can induce cellular apoptosis (Kannan and Jain, 2000). Thus, acquiring microplastics can induce apoptosis from microplastic ROS generation (White and Turnbull, 1994; Gewert et al., 2015).

Studies in mammals have shown that microplastics can induce apoptosis of ovarian granulosa cells and cardiomyocytes through the signaling pathway of nucleotide-binding oligomerization domain, Leucine-rich Repeat, and Pyrin domain containing 3 / cysteine-aspartic proteases 1 (NLRP3/Caspase-1) and Wnt/β-catenin (Li et al., 2020b; An et al., 2021; Hou et al., 2021). Store-operated calcium channels (SOC) played a role in cellular apoptosis from microplastic exposure. There was evidence of increasing calcium ions in hepatocytes with alteration of gene expression that involved calcium ion signaling. The expression of *calcium release-activated calcium channel protein 1* (*Orai1*) and *stromal interaction molecule 1* (*Stim1*) gene were increased together with decreased expression of *sarcoplasmic reticulum Ca2+ ATPase; Calcium pump* (*SERCA*) gene. This pattern of gene expression was the cause of calcium ion overload in cells. Moreover, in the group receiving PS microplastics in combination with BTP2, which is an Orai1 inhibitor, the amount of intracellular calcium was alleviated. A decrease in intracellular calcium levels was also found to be associated with a decrease in the apoptosis of hepatocytes (Li et al., 2021). A study in Zebrafish found that ROS caused by microplastic exposure can induce apoptosis by increased expression of the apoptosis-related gene including *p53 protein*, *growth arrest, and DNA-damage-inducible beta a* (*gadd45ba*) and *cysteine-aspartic proteases 3b* (*Caspase 3b*) (Umamaheswari et al., 2021). This study suggests that apoptosis can occur through the p53 signaling pathway.

ROS can result in the phosphorylation of proteins in the Mitogen-activated protein kinases (MAPK) signaling pathway, p38, and Extracellular signal-regulated kinase (ERK) signaling via nuclear factor erythroid 2-related factor 2 (Nrf2) as shown in **Figure 2.8** (Jeong et al., 2017), which has a crucial role in the regulation of antioxidative gene expression such as *GPx*, *GR* and *SOD* in response to oxidative stress (Itoh et al., 1997; Chan and Kan, 1999; Li et al., 2004). ERK signaling is associated with cell survival and cell proliferation in response to oxidative stress. While the p38 protein can induce cellular apoptosis, which results in a decrease in the growth rate and fertility (Wada and Penninger, 2004; McCubrey et al., 2007; Matsuzawa and Ichijo, 2008).

A study in crustaceans found that long-term exposure to microplastics showed a substantial increase in the expression of the *cysteine-aspartic proteases 3* (*Caspase 3*) gene (Liu et al., 2019), Whereas Caspase 3 plays a key role in cellular apoptosis, which activated via the p38 signaling pathway (Bian et al., 2011; Choi et al., 2018). This study supports the mechanism of cellular apoptosis through the p38 signaling pathway. The decreasing expression of the *Inhibitor of apoptosis protein* (*IAP*) gene was observed in penaeid shrimp, including Black tiger shrimp, Kuruma shrimp, and Pacific white shrimp that received microplastics, which indicated apoptosis in organs of shrimp treated with microplastics (Wang et al., 2021).
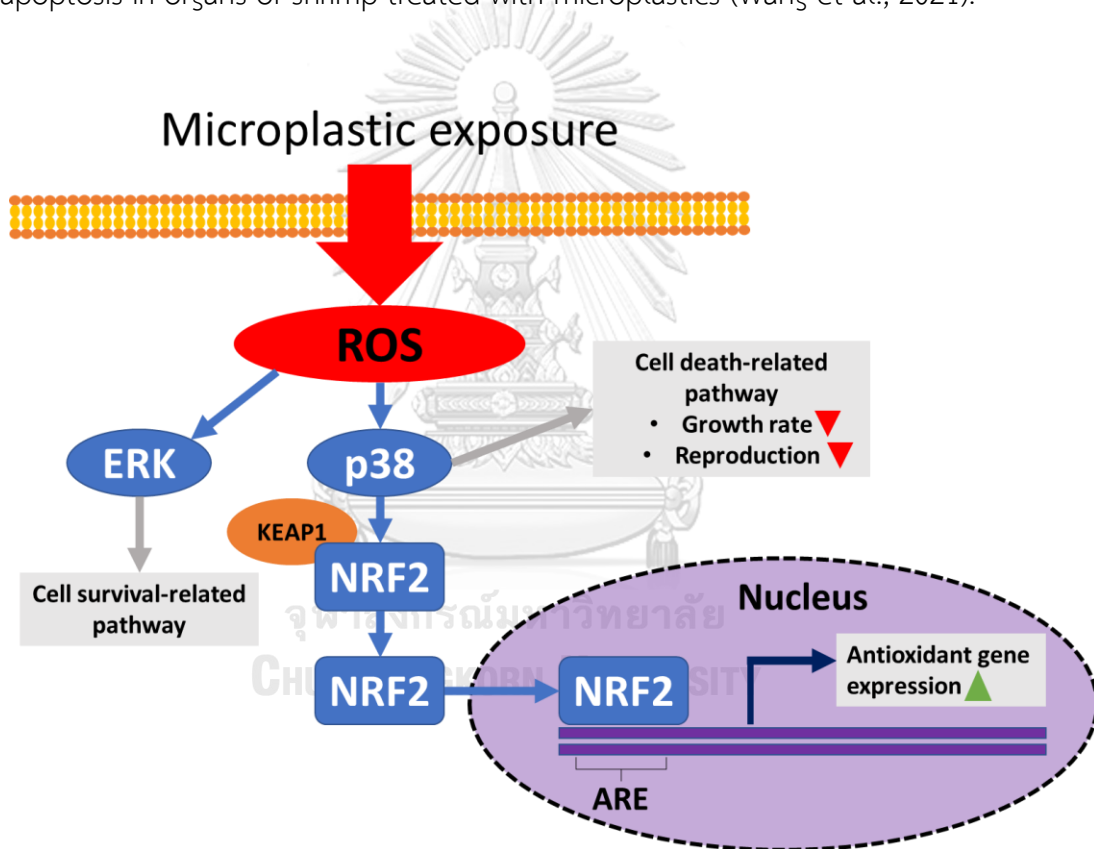
Figure 2.8: Mechanism of ROS responding via cellular signaling, p38 and ERK proteins, which are transmitted via the Nrf2 pathway (Adapted from Jeong et al. (2017))

2.2.5.1.7 Immune system toxicity

A study of the in vitro effects of nano-plastics and microplastics on cellular function in immunity systems of fish by Greven et al. (2016) showed that neutrophils

of fathead minnow (*Pimephales promelas*) were increased in myeloperoxidase and neutrophil extracellular trap (NET) activity, which directly proportional to the exposing quantity of PS nano-plastics and polycarbonate (PC) microplastic. Myeloperoxidase is an enzyme that produces by leukocytes, responsible for the elimination of microorganisms (Winterbourn et al., 2000). NET is a mechanism of neutrophils that is responsible for trapping and neutralizing microorganisms and foreign bodies (Brinkmann et al., 2004). Microplastic could interfere with phagocytosis of the lymphocytes in the anterior part of the kidney of gilthead seabream and European seabass (Espinosa et al., 2017; Espinosa et al., 2018; Espinosa et al., 2019).

The *In vivo* study indicated the immunotoxicity of microplastics. The study in rats treated with PE microplastics showed a sign of immune responses such as neutrophilia and increasing of immunoglobulin A (IgA) in blood, increasing serum interleukin 1 alpha (IL1α) levels, and decreasing level of serum granulocyte-colony stimulating agents (G-CSF), decreasing amount of regulatory T cell (Treg cell) in the spleen, and the increasing amount of T helper 17 cells (Th17 cell) in the spleen (Li et al., 2020a; Park et al., 2020). This study suggested that the immunity system of mammals can respond to microplastic exposure. A study in Common carp (*Cyprinus carpio*) found that microplastics could interfere with the complement system, decreased total plasma immunoglobulin levels, and impaired enzyme activity including lysozyme, Acetylcholinesterase (AChE), γ-glutamyl-transferase (GGT), Lactate dehydrogenase (LDH) and Alkaline phosphatase (AKP) (Banaee et al., 2019). A study in the liver of zebrafish showed that HDPE and PS microplastics were able to reduce the expression of an immune-mediated gene such as *leukotriene B4 receptor* (*ltb4r*) and *interferon induced transmembrane protein* (*iftm1*) (Limonta et al., 2019). There was also the study in small spotted sharks (*Scyliorhinus canicula*) from the Mediterranean, which found that the number of macro-plastic pieces in the digestive tract was associated with increased expression of *T-cell receptors beta* (*TCRβ*) and *delta* (*TCRδ*) and *immunoglobulin M* (*IgM*) gene in the spleen (Mancia et al., 2020).

Several studies found that microplastics or nano-plastics can interfere with the immunity system of invertebrates. A study in *Daphnia magna* that was treated with PS nano-plastics for a period of 1 year was associated with an increased number

of hemocytes (Sadler et al., 2019). A study in Mediterranean mussel (*Mytilus galloprovincialis*) found that microplastic and nano-plastic could cause alterations in hemocytes structure, hemocyte mortality, abnormalities of hemocyte organelle such as mitochondria and lysosomes, and abnormalities of immune-related proteins (Paul-Pont et al., 2016; Green et al., 2019; Auguste et al., 2020a). The study in mussels also demonstrated the increasing bactericidal activity and upregulation of immune-related genes after exposure to PS nano-plastic for 24 hours (Auguste et al., 2020a). On the other hand, mussels that exposing to PS nano-plastic for 96 hours found a decrease in hemolymph phagocytic activity and an increase in oxidative stress levels (Auguste et al., 2020b). The study in Mediterranean urchin (*Paracentrotus lividus*) treated with PS microplastics showed an increasing number of coelomocytes along with the levels of ROS and reactive nitrogen species (RNS) within the coelomocytes, which indicated the effects of oxidative stress on immunity cells (Murano et al., 2020). Two studies in blood cockle (*Tegillarca granosa*) found that microplastics and nano-plastics can cause abnormalities in hemocytes as well as a decrease in the number of hemocytes, phagocytic activity, immune system parameters related to oxidative stress, apoptosis, and the inflammatory response. In both studies, PS nano-plastics could cause more damage to the immunity system than microplastics (Shi et al., 2020; Tang et al., 2020).

There were studies in decapod that indicated the immunotoxicity of microplastic. The studies of PS microplastics toxicity in Chinese mitten carbs showed an increase in the antioxidant enzyme activities and gene expression, including *SOD*, *GSH*, and *GPx*, in the carb that was exposed to low doses of microplastics (40 and 400 µg/l), while carb that exposed with the high doses of microplastics (4000 and 40000 µg/l) showed decreasing is an antioxidant enzyme activity and gene expression (Yu et al., 2018). Long-term exposure to PS microplastics could also result in a decline of nonspecific immunity enzyme activities and gene expression. The study in Chinese mitten crab also found that the expression of immune parameters genes such as *hemocyanin, AKP, PO,* and *lysozyme* was increased when exposed to microplastic for a short period (<21 days) and/or have received low concentration of microplastics (≤0.04 mg/l). On the other hand, a carb that was exposed to

microplastics for a long period (21 days) and/or a high concentration of microplastics ($\geq$0.04 mg/l) showed less activity and expression of the immunity gene (Liu et al., 2019).

The study of Liu et al. (2019) also found an upregulation of the *Caspase3* gene when exposed to a low concentration of microplastics ($\leq$0.4 mg/l) (Liu et al., 2019). Caspase 3 plays an important role in cellular apoptosis, where it can be activated via the p38 signaling pathway (Bian et al., 2011; Choi et al., 2018). Thus, this was evidence of apoptosis induced by microplastic.

The study in Chinese mitten carb by Liu et al. (2019) also suggested a possible mechanism of the alteration of immunity gene expression by microplastic. The upregulation of *MyD88* gene expression in the group receiving microplastic (0. 4 to 4 mg/l) was observed. Which consequence in upregulated immunity gene expression such as hemocyanin and lysozyme (Liu et al., 2019). Since MyD88 plays a role in cellular signaling in response to the nonspecific immune system (Ren et al., 2017), it is possible that microplastics can induce an immune response through such signaling pathways. However, the expression of the *MyD88* gene in the carb exposed to the high concentration of microplastics (40 mg/l) also resulted in a decrease in *MyD88* gene expression (Liu et al., 2019). This phenomenon might suggest that s high concentration of microplastics can result in decreased cellular signaling and result in immunosuppression.

The study in penaeid shrimp supported the evidence of immunosuppression by microplastic. The study in tiger shrimp and Japanese tiger shrimp treated with PE microplastics (100 mg/l) for 48 hours showed a down-regulation of *lysozyme* gene expression (Wang et al., 2021). The study in Pacific white shrimp by Hsieh et al. (2021) also showed that PE microplastics ingestion at doses of 0.5 and 1 µg/g body weight for 7 days were able to suppress the activity of SOD and CAT enzyme and PE microplastics ingestion at a dose of 1 mcg/g body weight for 7 days were able to suppress GPx enzyme, which was associated with decreased survival of Pacific white shrimp.

2.2.5.1.8 Effects on the microbiome in the gastrointestinal tract

Plastic material is highly suitable for microbial growth. The surface of plastic material in water or sediment can form biofilms within at least 7 days (Lobelle and Cunliffe, 2011; Harrison et al., 2014). However, the population of bacteria in biofilms on plastic material is clearly different from the surrounding environment. The bacteria such as Vibrio spp., which is a pathogen can grow on such biofilms (Zettler et al., 2013; Kirstein et al., 2016).

An analysis of the microbiome composition in the gastrointestinal tract of fish treated with PS microplastics for 28 days revealed that the microplastics could induce dysbiosis related to the proportions of different bacterial populations (Gu et al., 2020; Huang et al., 2020; Kang et al., 2021). Microplastics can impair the biodiversity of the gastrointestinal microbiome (Qiao et al., 2019a; Wan et al., 2019). The same is true in crustaceans, where the microbiome was changed and there was a decrease in the biodiversity of the microflora after being exposed to microplastic (Zhu et al., 2018; Ju et al., 2019; Liu et al., 2019).

2.2.5.2 Factors promoting the toxicity of microplastics

2.2.5.2.1 Physical factors of microplastics

Size of microplastics

Microplastics are particles smaller than 5 mm in diameter but larger than 1 µm. This allows microplastic particles to disperse in the environment and contaminate a wide range of aquatic species, especially non-selective forager species (Baldwin, 1995). The size of the microplastics is a factor affecting the microplastic consumption rate because aquatic animals have the behaviors to choose food of different sizes. This resulted in the different sizes of microplastics ingested by different aquatic species (Fernández, 2001; Ory et al., 2017; Kokalj et al., 2018). The size of microplastics is also related to the translocation rate into the body. In the study of mussels, microplastics with a diameter of 3 µm were absorbed into the hemolymph easier than 9.6 mm of microplastics (Browne et al., 2008).

The size of the received microplastics also affects the toxicity of microplastics. However, the size of the microplastic that induced high toxicity was

specific to each aquatic species (Jeong et al., 2016; Lei et al., 2018a). The relationship of microplastic size to microplastic toxicity in each species was unclear due to the wide variety of anatomical and physiological features in each animal species.

The shape of microplastics

The shape of microplastics can be divided into two types: regular shapes; and irregular shapes or divided into specific types of shapes namely spherical, fragment, fibrous, and film (Albanese et al., 2012). The shape of microplastics affects the dispersibility of microplastics in the environment, absorbability, and toxicity of microplastic (Khatmullina and Isachenko, 2017). The shape of the microplastics affects the buoyancy and sedimentation of the plastic particles. These factors affect the likelihood of microplastics ingesting and absorbed by the organism (Filella, 2015). The shape of microplastics affects the retention time of microplastics in the body. Irregular-shaped microplastics had a lower excretion capacity and persisted in the gastrointestinal tract longer than regular-shaped microplastics (Frydkjær et al., 2017). Fibrous-shaped microplastics are more capable of persisting in the gastrointestinal tract than spherical microplastic. As a result, the fibers of microplastics were the most toxic shape of microplastic (Au et al., 2015). Rough-surfaced microplastics such as irregular shapes can be more toxic than microplastics with less concavity or smooth surfaces, for example, spherical microplastics (Choi et al., 2021).

2.2.5.2.2 Chemical factors of microplastics

Endogenous Chemical additives

The plastic items were added with chemical additives during production (Deanin, 1975) to give the plastics the desired properties such as corrosion resistance, thermal and electrical conductivity, durability, etc. The additives in plastics were leaked into the environment during decomposition into microplastics. The organisms exposed to microplastics were also exposed to plastic additives which cause toxicity as well (Zarfl and Matthies, 2010; Velzeboer et al., 2014). Most additives in plastics have a low molecular weight and no chemical bonds to plastics. This allows the chemicals to easily leaked into the body of organisms (Tickner et al., 1999).

Chemicals added to plastics were reported to be toxic to humans, such as chemicals toxic to the reproductive system include Bis(2-ethylhexyl), phthalate, DEHP, and Bisphenol A (BPA); carcinogens, including vinyl chloride and butadiene; mutagenic substances, including benzene and phenol, as well as toxins such as Brominated flame retardants, phthalates, and lead heat stabilizers (Lithner et al., 2011).

Adsorbed Chemical Pollutants

Microplastics have a high surface area-to-volume ratio and are hydrophobic. So, hydrophobic organic contaminants (HOCs), including PCBs (polychlorinated biphenyl), DDT dichlorodiphenyltrichloroethane, PAHs (polycyclic aromatic hydrocarbons), FOSA (perfluorooctane sulfonamide) and PFAS (perfluoroalkyl substance) were likely to bind to microplastic surfaces (Mato et al., 2001; Browne et al., 2007; Rios et al., 2007; Endo et al., 2013; Wang et al., 2015; Llorca et al., 2018). HOCs on microplastic surfaces can be released into tissues when ingested by marine organisms (Tickner et al., 1999; Besseling et al., 2013; Browne et al., 2013; Rochman et al., 2013; Bakir et al., 2014). The study by Rainieri et al., (2018) also found that microplastics can increase toxicity in an environment.

Microplastics can absorb antibiotics, including sulfadiazine (SDZ), Amoxicillin (AMX), Tetracycline (TC), Ciprofloxacin (CIP), Triclosan (TCS), and Trimethoprim (TMP). Thus, microplastics could increase the toxicity of some antibiotic drugs (Li et al., 2018b; Sheng et al., 2021).

In addition, heavy metals can also adhere to the surface of microplastics. The heavy metals that have been reported to contaminate with microplastics include Silver, Aluminum, Arsenic, Barium, Cadmium, and Cobalt. , Iron, Mercury, Manganese, Molybdenum, Stibium, Tin, Titanium, Uranium, Zinc, Nickel, and Lead (Ashton et al., 2010; Holmes et al., 2012; Rochman et al., 2014; Torres et al., 2020).

Chapter 3

Materials and methods

The research was divided into two phases as follows:

1. A study on the toxicity of microplastics in Pacific white shrimp.

2. A study of the response and alteration of nonspecific immune system gene expression and histopathology of the hepatopancreas in Pacific white shrimp.

3.1 Toxicity of microplastics

3.1.1 Animal preparation

Pacific white shrimp (*Litopenaeus vannamei*) with approximately 4 g of weight and 7 cm of total body length was used in this study. Pacific white shrimp were obtained from shrimp farms in Phetchaburi province. The quality of shrimp health and external parasites was examined. Shrimp were reared in a 500 L tank with aerated seawater of 10 ppt salinity for two weeks. The stock density in the quarantine tank was 70 shrimps per square meter. The water was changed twice a week and the water parameters such as ammonia, nitrite, nitrate, water alkalinity, pH, dissolved oxygen, and salinity were monitored daily. Commercial shrimp pellet feed was given at 4% of total body weight (Tacon et al., 2013).

3.1.2 Preparation of treatment feeding

The microplastic-contaminated feed was prepared from powdered commercial shrimp feed mixed with 3% wheat gluten flour and microplastics at various concentrations. Shrimp feed mixed with high-density polyethylene (HDPE) spherical 50 μm diameter in 5 different concentrations as follows: 0, 0.5, 5, 10, and 20% of HDPE-MP in shrimp feed. The mixture was extruded into pellets through an extruder equipped with a 2 mm sieve. The pellets are then dried in the hot air oven at 60°C for 6 hours. The feed pellets were divided into vacuum-sealed bags and stored in a dry place with a temperature of 4 °C.

3.1.3 Toxicity test

Pacific white shrimp were divided into 5 groups of 10 shrimp and this study was done in triplicate. Each group of shrimp received different concentrations of HDPE microplastic for 28 days, as follows: 0, 0.5, 5, 10, and 20% of HDPE-MP. Ten shrimp were reared in a glass tank filled with 45 L of seawater at a salinity of 10 ppt. Tanks were installed with a filtration system and aerated for 24 hours. The water was changed twice a week and water quality was monitored every day. Pacific white shrimp were fed with different concentrations of HDPE in shrimp feed. Shrimp was fed at 4% of the total body weight. The feeding was divided into three meals, the first meal at 9:00 a.m., the second meal at 1:00 p.m., and the third meal at 5:00 p.m. Shrimp behavior, physical changed, and mortality rate were observed. The lethality concentration of 50% (LC_{50}) was calculated at 28 days of microplastic ingestion.

3.1.4 Calculation of LC_{50}

The cumulative mortality rate at day 28 of Pacific white shrimp in each treatment group was assessed for the calculation of LC_{50} of HDPE in Pacific white shrimp by Probit analysis done in SPSS version 22.

3.2 Study on the effects of microplastics on the expression of nonspecific immune system gene

3.2.1 Animals and treatment feeding preparation

A total number of 150 Pacific white shrimp with average weight and total length of 4 g and 7 cm went through the same quarantine protocol as in the phase1 of this study. During the quarantine, external parasites were detected. Thus, shrimp were treated with formalin bathing at a concentration of 20 ppm for 24 hours. External parasite testing was repeated after treatment and quarantine time was extended for 7 days. Shrimp feeds were prepared using the same raw materials and methods as in phase 1 of this study. However, the amounts of HDPE plastics added to the experimental diets were 0, 0.1, 0.5, 1, and 3% of HDPE-MP.

3.2.2 Experimental design and microplastic exposing scheme

Pacific white shrimp were divided into 5 groups of 10 shrimp with 3 replications each, comprising 1 control group, which was not exposed to plastics throughout the experiment. Four experimental groups received HDPE microplastic with a diameter of 50 µm in different concentrations: 0.1, 0.5, 1 and, 3% of HDPE-MP.

Ten shrimp were reared in a glass tank with 45 L of aerated seawater and equipped with a filtration system. The water was changed twice a week and the water quality was monitored daily. Each group of shrimp was fed a different concentration of HDPE in shrimp feed. Shrimp were fed at 4% of total body weight daily. The feeding was divided into three meals at 9:00 a.m., 1:00 p.m. and, 5:00 p.m. Physical changes, behavior, and mortality rate were observed during the experiment.

3.2.3 Sampling

One shrimp was randomly collected from each tank on days 7, 14, 21, and 28 of the experiment to study the expression of nonspecific immunity system gene by Reverse Transcriptase Real-time Polymerase Chain Reaction (RT-qPCR). On day 28 of the experiment, two shrimp were collected from each tank to study the histopathological changes of hepatopancreas.

Shrimp were euthanized according to the guideline of the American Veterinary Medical Association (AVMA) (Underwood and Anthony, 2013). Shrimp were sedated with clove oil (Eugenol 99%) at a concentration of 0.125 ml/L as the first step of euthanasia. Then the shrimp were kept on ice as the final euthanasia. Finally, the hepatopancreas was then collected for further study.

Hepatopancreas samples for the study of gene expression were collected in 1.5 mL sterile centrifuge tubes containing 1 mL of QIAzol (Qiagen®) solution and stored at -80°C prior to administration. Hepatopancreas samples for the study of histopathological lesions were kept in glass jars containing Davidson's solution at room temperature before being used.

3.2.4 Molecular biology

3.2.4.1 Ribonucleic acid (RNA) extraction

RNA was extracted from the hepatopancreas with the RNeasy Mini kit (Qiagen®) extraction. The sample was homogenized with the vortex mixer set at high velocity for 2 minutes. The solution was transferred into a new centrifuge tube and left at room temperature for 5 minutes. The 200 µl of chloroform was added and mixed well with a vortex mixer and left solution at room temperature for 2 to 3 minutes. The solution was centrifuged at a velocity of 12000xg for 15 minutes. Then, transferred approximately 600 µl of the supernatant into the new centrifuge tube and avoided contamination of the bottom sediment. The 70% ethanol was added equal to the amount of solution obtained in step 5 and mixed well with a vortex mixer. The 700 µl of the solution was transferred to the RNeasy Mini kit (Qiagen®) extraction column placed on the 2 ml collection tube and centrifuged the solution at a speed of 8000xg for 15 seconds, then discarded the solution in the collection tube. The 700 µL of buffer RW1 was added to the extraction column placed on the 2 mL collection tube and centrifuged the solution at a speed of 8000xg for 15 seconds, then discarded the solution contained in the collection tube. The 500 µL of buffer RPE was added to the extraction column placed on the 2 mL collection tube and centrifuged at a speed of 8000xg for 15 seconds, then discarded the solution contained in the collection tube. Then added another 500 µL of buffer RPE to the extraction column placed on the 2 mL collection tube, centrifuged the solution at a speed of 15000xg for 2 minutes, and discarded the solution in the collection tube. Finally, the extraction column was transferred into a 1.5 mL sterile centrifuge tube, and add 50 µl of RNase-free water, was then centrifuged the solution at a speed of 8000xg for 1 minute and resulting in the RNA extracted solution inside the 1.5 mL centrifuge tube and then synthesized into cDNA immediately after RNA extraction.

3.2.4.2 Synthesis of deoxyribonucleic acid (Complementary deoxyribonucleic acid, cDNA)

cDNA was synthesized from extracted RNA using the QuantiNova reverse transcription kit (Qiagen®) following the procedure below. The extracted RNA was

quantified by a nanodrop spectrophotometer. The initial RNA was diluted with RNase-free water, calculated to contain 5 µg of RNA in 12 µl of solution. Then the following solutions and chemical reagents were added into diluted RNA solution; 2 µl of gDNA Removal mix, 1 µl of internal control RNA, 1 µl of reverse transcription enzyme, and 4 µl of reverse transcription mix. Then cDNA was synthesized with the Rotor-Gene Q (Qiagen)® thermocycling machine with the following steps; Annealing period at 25 degrees Celsius for 3 minutes, Reverse-transcription at 45 degrees Celsius for 10 minutes, and Inactivation of reaction at 85 °C for 5 minutes. The cDNA was measured for the amount of DNA and RNA using a nanodrop spectrophotometer. Finally, the cDNA was diluted with RNase-free water, and calculated to contain 50 ng cDNA in 1 mL solution.

3.2.4.3 DNA proliferation measurement

The QuantiNova SYBR green PCR kit (Qiagen®) was used for real-time polymerase chain reaction (qPCR). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control or housekeeping gene in this study. The details of the gene and their primers used in this study are shown in **Table 3.1**. This study performed qPCR reaction per sample per 1 gene expression and was done in triplicate. A no-template control (NTC) was used to monitor the contamination during preparation. A master mix of qPCR was prepared for each gene as shown in **Table 3.2**. The quantity of PCR product during the process of thermocycling was measured with a Rotor-Gene Q (Qiagen®) thermocycling machine, with a thermocycling profile setting shown in **Table 3.3**.

Table 3.1: Gene and primer used in the study (Wang et al., 2010)

Gene	Primer's Sequence(5'-3')	Tm (°C)	Product Size (BP)	NCBI ref.
LYZ	F-GAAGCGACTACGGCAAGAAC	56.3	213	XM_027352840.1
	R-AACCGTGAGACCAGCACTCT	56.0		

SOD	F-ATCCACCACACAAAGCATCA	55.9	229	XM_027376216.1
	R-AGCTCTCGTCAATGGCTTGT	56.2		
GPx	F-TTTTTCCGTGCAAAAAGGAC	56.5	239	XM_027372127.1
	R-TAATACGCGATGCCCCTAAC	56.4		
GAPDH	F- AAAGGTAGGAATTGCCCCCG	60.9	169	XM_027372388.1
	R- GAAAGGGATGAGACTAGCACGAC	58		

Table 3.2: Preparation of master mix for qPCR reaction

Reaction Component	Final concentration	Volume/reaction	The volume of 240 reactions (for 1 gene and including the excess)
SYBR Green PCR Master Mix	1x	10 µl	2400 µl
10 µM Forward primer	0.2 µM	0.4 µl	96 µl
10 µM Reverse primer	0.2 µM	0.4 µl	96 µl
cDNA template	≤100 ng	2.0 µl	Add to PCR tube individually
RNase-Free water	Variable	7.2 µl	1728 µl
Total reaction volume		20 µl	4800 µl

Table 3.3: Details of temperature recirculation settings for qPCR

Step	Time	Temperature	Number of cycles
PCR initial	2 minutes	95℃	1

activation			
3 step cycling			
Denaturation	10 seconds	95℃	
Annealing	30 seconds	60℃	45
Extension	30 seconds	72℃	

3.2.4.4 Gene expression analysis

The relative gene expression in this study was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

3.2.5 The study of histopathological changes in the Hepatopancreas

Two Pacific white shrimp were randomly selected from each tank for euthanasia on 28 days of the experiment. The hepatopancreas was collected and preserved in glass jars containing Davidson's solution, after which the preserved samples were immersed in a 70% ethanol solution, 95%, and 100% respectively to dehydrate the sample. The sample was then immersed in xylene for further paraffin embedding. The sample embedded in paraffin was cut into a thin section with a thickness of 5 µm by microtome. The paraffin was then dissolved by immersing it in xylene. The sectioned sample was rehydrated by immersion in 100%, 95%, and 70% ethanol respectively. Finally, the sample was stained with hematoxylin and eosin staining (H&E) (Gonçalves et al., 2018; Jovanović et al., 2018; Hsieh et al., 2021).

Histopathological lesions of the hepatopancreas were observed through a light microscope. Histopathological scores were assessed base on the distribution of each histopathological lesion that could be visible on all fields of a histopathological slide. Histopathological lesions were observed on all high-power fields (100X) in the histopathological section. Each field was determined as present or absence of lesion

on the field. The scoring was calculated as the percentage of the field that presented with a histopathological lesion on the total number of fields that could be observed in the histopathological section. The scoring criteria are shown in **Table 3.4**.

Table 3.4: Histopathological Scoring Criteria (Littik, 2003)

Score	Indication
0	No histopathological lesion in any field on the slides
1	Histopathological lesions present in <25% of the fields on the slides
2	Histopathological lesions present between 25% to 50% of the fields on the slides
3	Histopathological lesions present between >50% to 70% of the fields on the slides
4	Histopathological lesions present in >75% of the fields on the slides

3.2.6 Statistical analysis

Gene expressions were considered as dependent variables and the difference between groups was assessed using a one-way analysis of variance (one-way ANOVA) combined with a posthoc test. The difference in histopathological scores between the group was assessed using the Kruskal-Wallis test with a posthoc test (Meyerholz et al., 2019). Histopathological lesions were assessed by descriptive analysis The statistical significance was set at a p-value less than 0.05 and the statistical analysis was calculated using SPSS version 22.

Chapter 4

Results

4.1 LC_{50} and mortality rate

LC_{50} at day 10 of HDPE microplastic ingestion was 3.074% of HDPE-MP. Shrimp started dying on day 6 of HDPE microplastic ingestion. The mortality rate was correlated with increases in HDPE microplastic concentration as shown in **Figure 4.1**. Shrimp that were fed with 20% of HDPE-MP had the highest mortality rate on day 14.
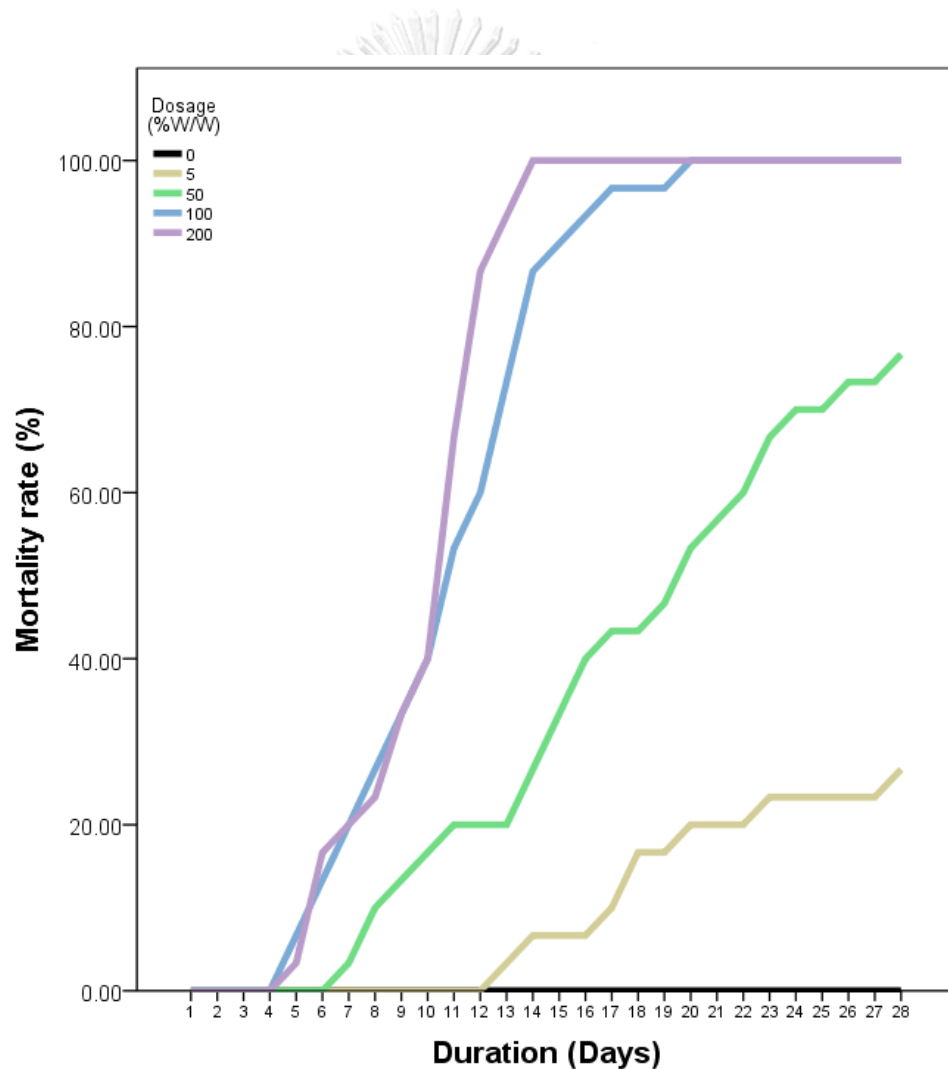
Figure 4.1: Mortality rate of shrimp fed with different concentrations of HDPE microplastic and at different times.

4.2 Fecal examination

The feces of the shrimp were collected during the toxicity test and observed under a light microscope. HDPE-MP was rediscovered in the feces of shrimp that fed with HDPE-MP (**Figure 4.2**)
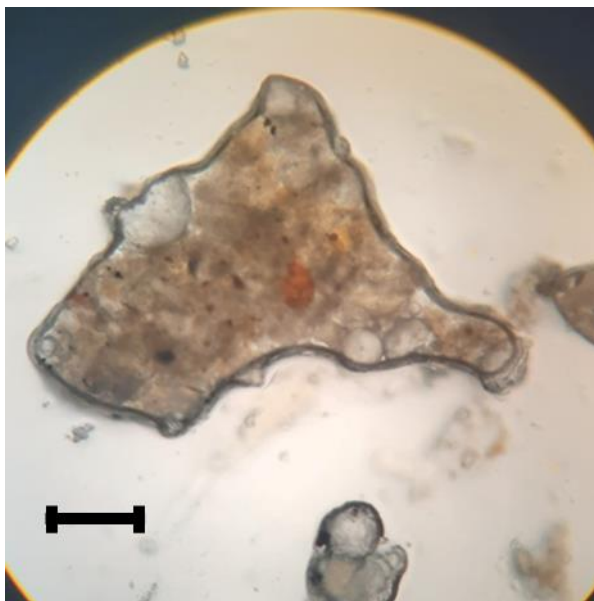
Figure 4.2: HDPE-MP was rediscovered in the feces of shrimp that fed with HDPE-MP (Bar = 55 μm).

4.3 Nonspecific immunity gene expression

4.3.1 Lysozyme

LYZ gene expression of shrimp fed with 0.5, 1, and 3% of HDPE-MP for 7 days was upregulated significantly (P<0.05) (**Figures 4.3 and 4.4**). *LYZ* gene expression was down-regulated significantly (P<0.05) in all groups that were fed with HDPE microplastic for at least 14 days (**Figures 4.3 and 4.4**).
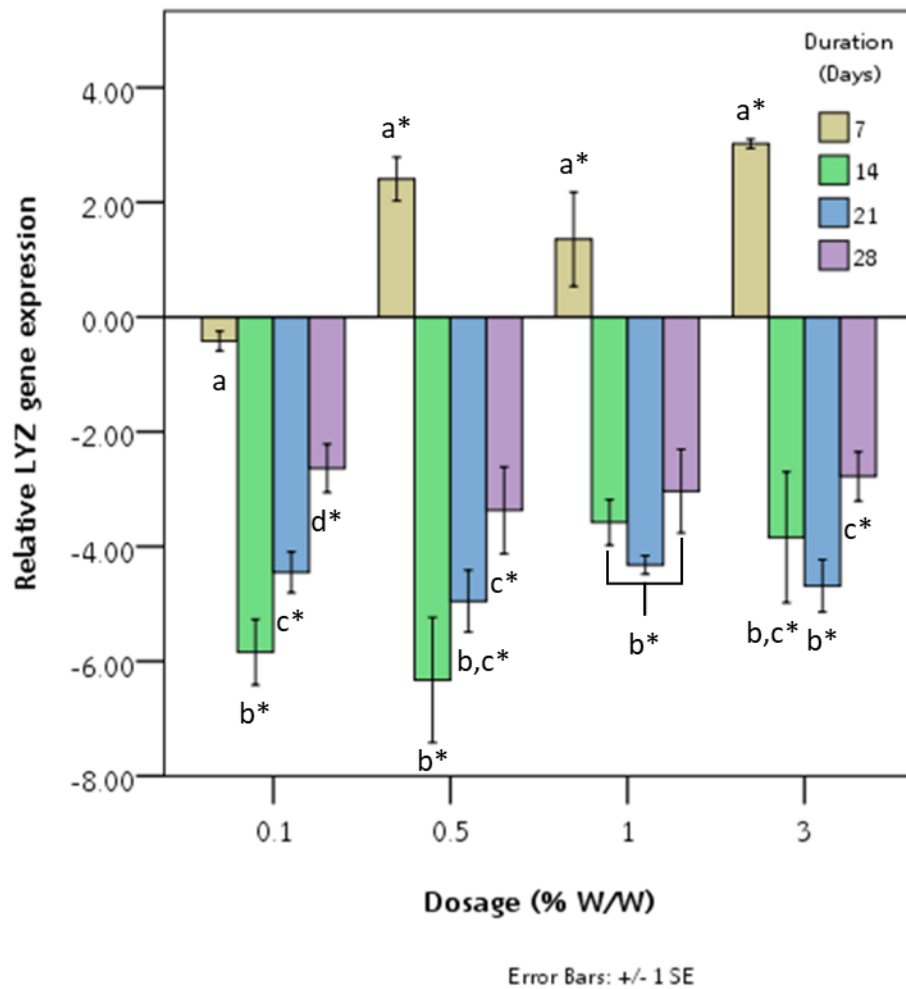
Figure 4.3: The fold change of *LYZ* gene expression normalized to the negative control ($2^{-\Delta\Delta Ct}$). Different letters above bars of the same series indicate significant differences among treatments and "*" indicates significant differences from the negative control.
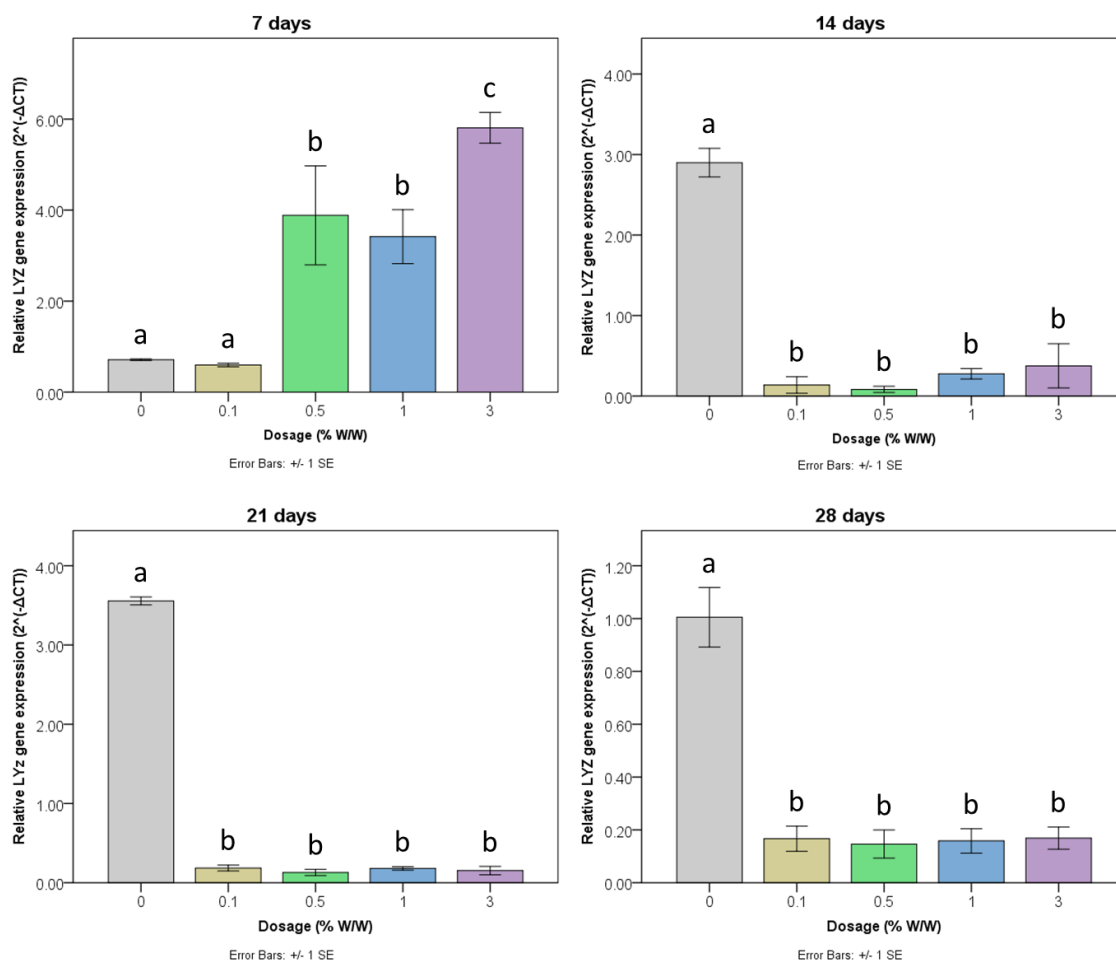
Figure 4.4: The relative *LYZ* gene expression normalized to the *GAPDH* gene and negative control ($2^{-\Delta Ct}$). Different letters above bars of the same series indicate significant differences among treatments.

4.3.2 Superoxide dismutase

SOD gene expression of shrimp fed with 0.1% of HDPE-MP was upregulated significantly (P<0.05) at days 14 and 21, while shrimp fed with 0.5 of HDPE-MP food was upregulated significantly (P<0.05) at day 21 (**Figures 4.5 and 4.6**). There was no significant change (P>0.05) in *SOD* gene expression of shrimp that fed with 1 and 3% of HDPE-MP for not more than 21 days (**Figures 4.5 and 4.6**). *SOD* gene expression of shrimp fed with 0.5, 1, and 3% of HDPE-MP for 28 days was down-regulated significantly (P<0.05) (**Figures 4.5 and 4.6**).
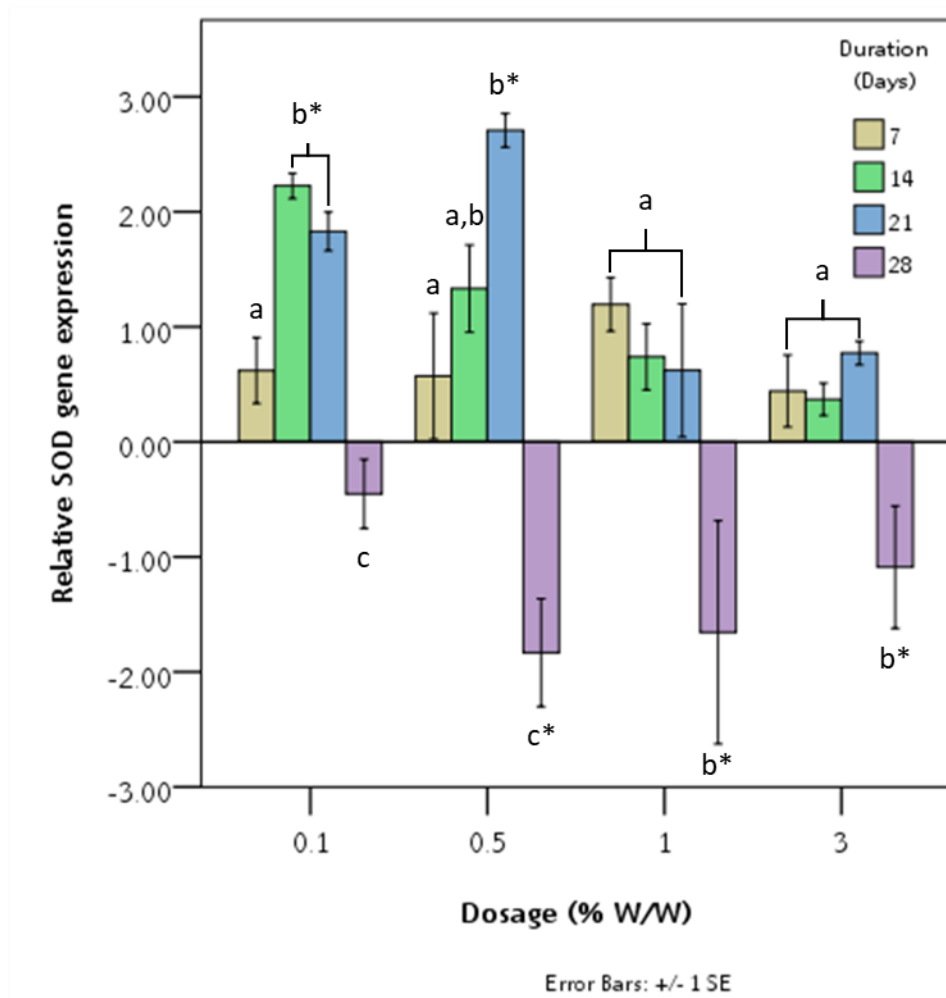
Figure 4.5: The fold change of SOD gene expression normalized to the negative control ($2^{-\Delta\Delta Ct}$). Different letters above bars of the same series indicate significant differences among treatments and "*" indicates significant differences from the negative control.
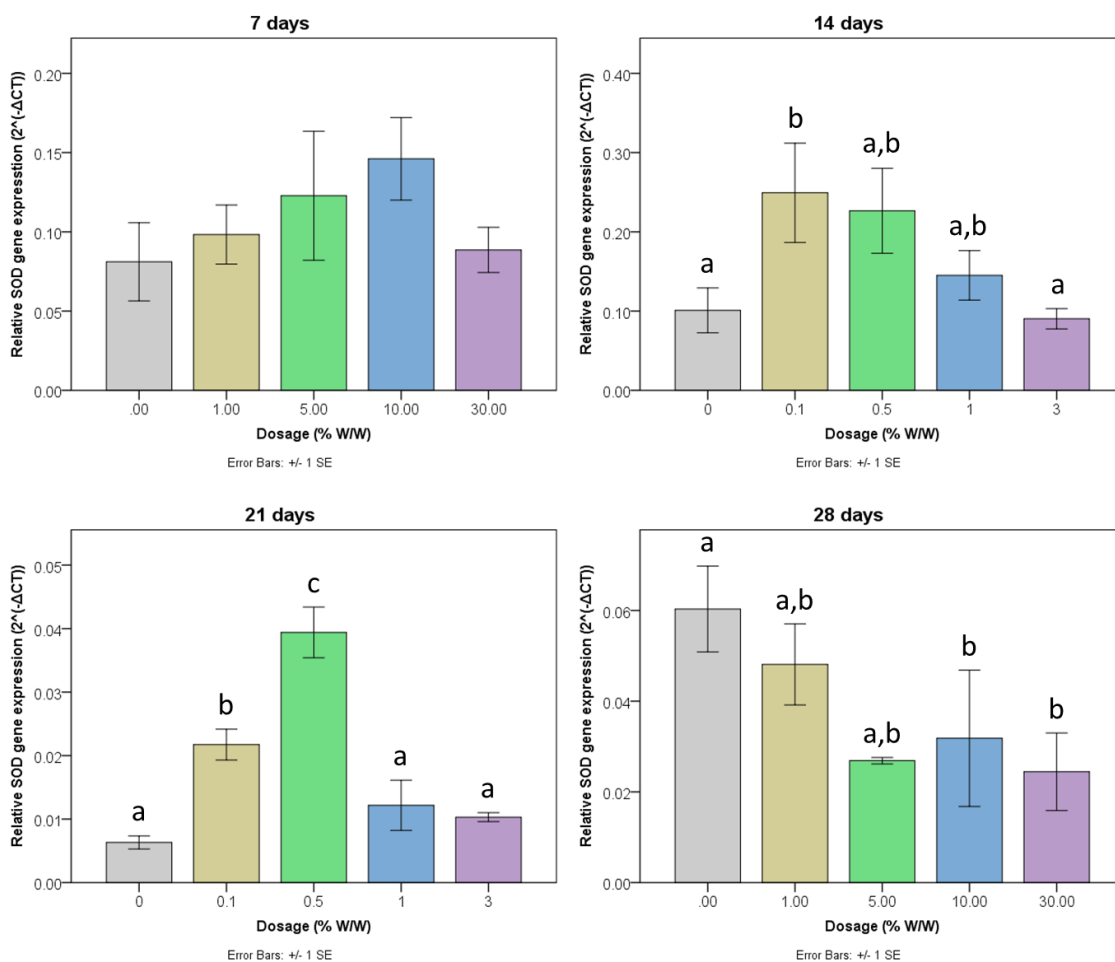
Figure 4.6: The relative SOD gene expression normalized to the *GAPDH* gene and negative control ($2^{-\Delta Ct}$). Different letters above bars of the same series indicate significant differences among treatments.

4.3.3 Glutathione peroxidase

GPx gene expression of shrimp that fed with 0.5% of HDPE-MP was upregulated significantly (P<0.05) at day 14, while shrimp that fed with 0.1% of HDPE-MP was upregulated significantly (P<0.05) at day 21 (**Figures 4.7 and 4.8**). There was no significant change (P>0.05) in *GPx* gene expression of shrimp that fed with 1 and 3% of HDPE-MP for not more than 21 days (**Figures 4.7 and 4.8**). *GPx* gene expression of shrimp that fed with HDPE microplastic not more than 1% of HDPE-MP was down-regulated (P<0.05) at day 28, while shrimp that fed with 3% of HDPE-MP was down-regulated significantly (P<0.05) at day 14 (**Figures 4.7 and 4.8**).
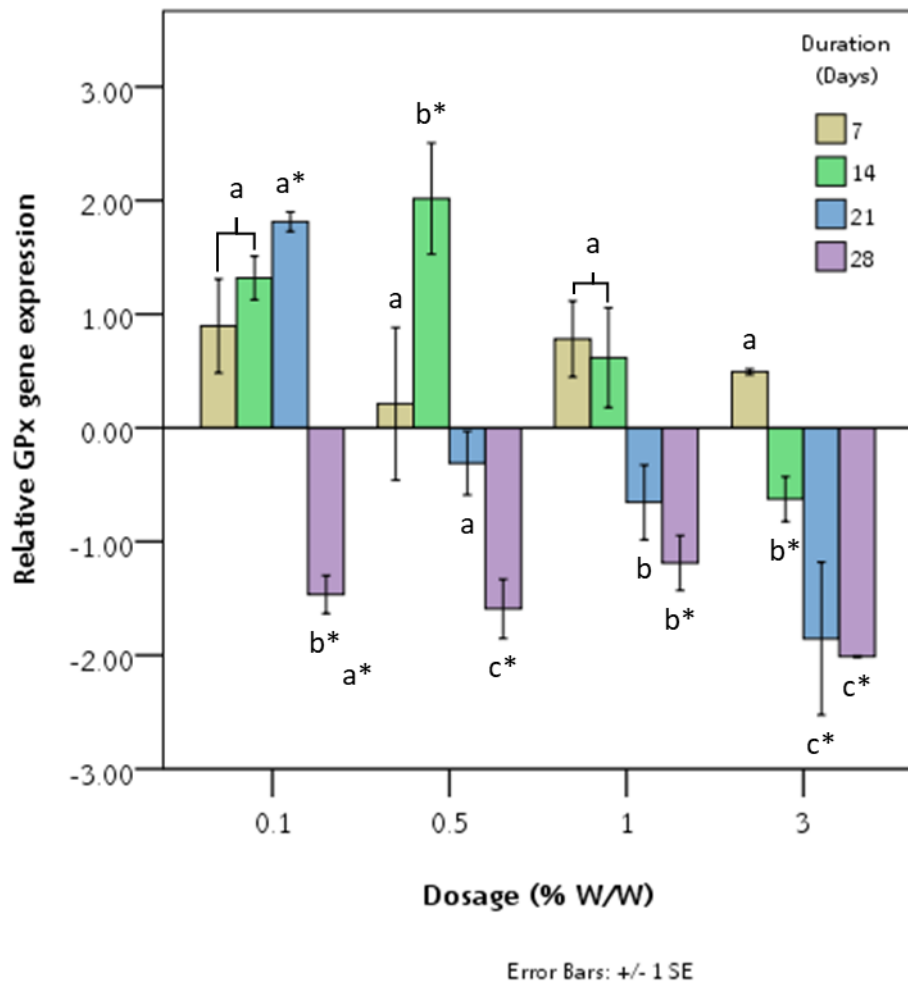
Figure 4.7: The fold change of *GPx* gene expression normalized to the negative control ($2^{-\Delta\Delta Ct}$). Different letters above bars of the same series indicate significant differences among treatments and "*" indicates significant differences from the negative control.
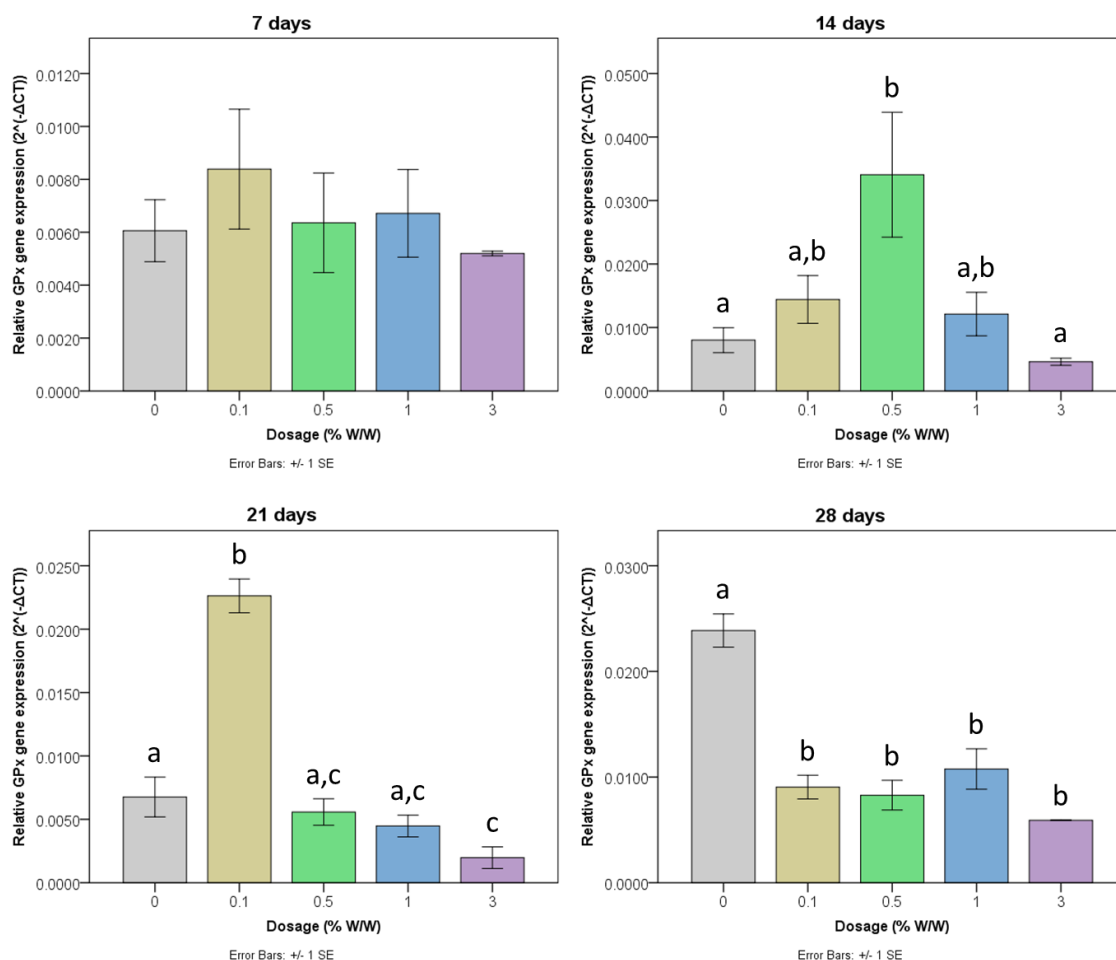
Figure 4.8: The relative *GPx* gene expression normalized to the *GAPDH* gene and negative control ($2^{-\Delta Ct}$). Different letters above bars of the same series indicate significant differences among treatments.

4.4 Histopathology lesion

During the toxicity test, one of the deceased shrimp fed with 20% of HDPE-MP had intestine obstruction by a clump of HDPE-MP when observed under the light microscope (**Figure 4.9**). There was no histopathological lesion in the hepatopancreas of shrimp in the negative control group (**Figures 4.10, 4.11, and 4.12**). On the other hand, histopathological damage was found in the hepatopancreas of shrimp that fed with HDPE microplastic (**Figure 4.10**) The histopathological lesions such as interstitial hemocyte infiltration (**Figures 4.13 and 4.15**), epithelium hyperplasia (**Figures 4.16 and 4.17**), hepatopancreatic tubular

deformity (**Figures 4.13 to 4.17**), nodule formation (**Figures 4.18 to 4.21**), and melanization (**Figures 4.18 to 4.21**) were found in this study. The severity depended on the concentration of HDPE microplastic. Interstitial hemocyte infiltration score was increased significantly (P<0.05) with a rising concentration of HDPE microplastic ingestion (**Figure 4.22**). Shrimp fed with 0.1% of HDPE-MP had the most epithelium hyperplasia score, while shrimp fed with a higher concentration of HDPE microplastic had a lower epithelium hyperplasia score. Epithelium hyperplasia score in shrimp that fed with 3% of HDPE-MP was not a significant difference (P>0.05) from the negative control group (**Figure 4.23**). Tubular deformity and nodule formation score of shrimp that fed with HDPE microplastic was a significant difference (P<0.05) from negative control, but not a significant difference (P>0.05) among the groups of shrimp that fed with different concentrations of HDPE microplastic (**Figure 4.24 and 4.25**). Melanization score in shrimp that fed with 3% of HDPE-MP was a significant difference (P<0.05) from the negative control group, while shrimp that fed with 0.1, 0.5, and 1% of HDPE-MP did not have a significant difference (P>0.05) from the negative control group (**Figure 4.26**). The histopathological score of each lesion was showen in **Table 4.1**.
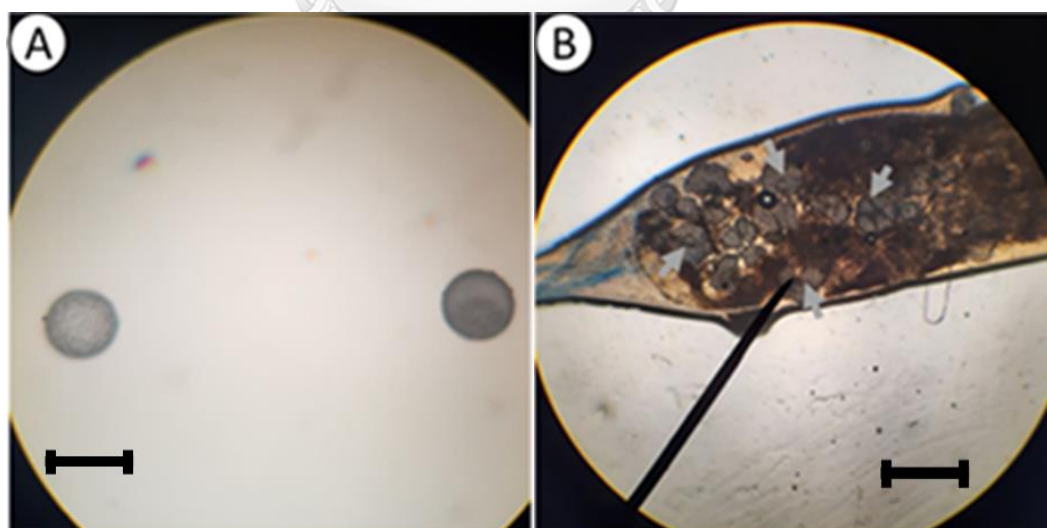
Figure 4.9: An impaction of the microplastic fragment in the gastrointestinal tract. A) HDPE microsphere under a light microscope at 100X (Bar = 55 μm). B) Grey arrows indicate fragments of microplastic in the intestinal tract of shrimp under a light microscope at 10X (Bar = 100 μm).
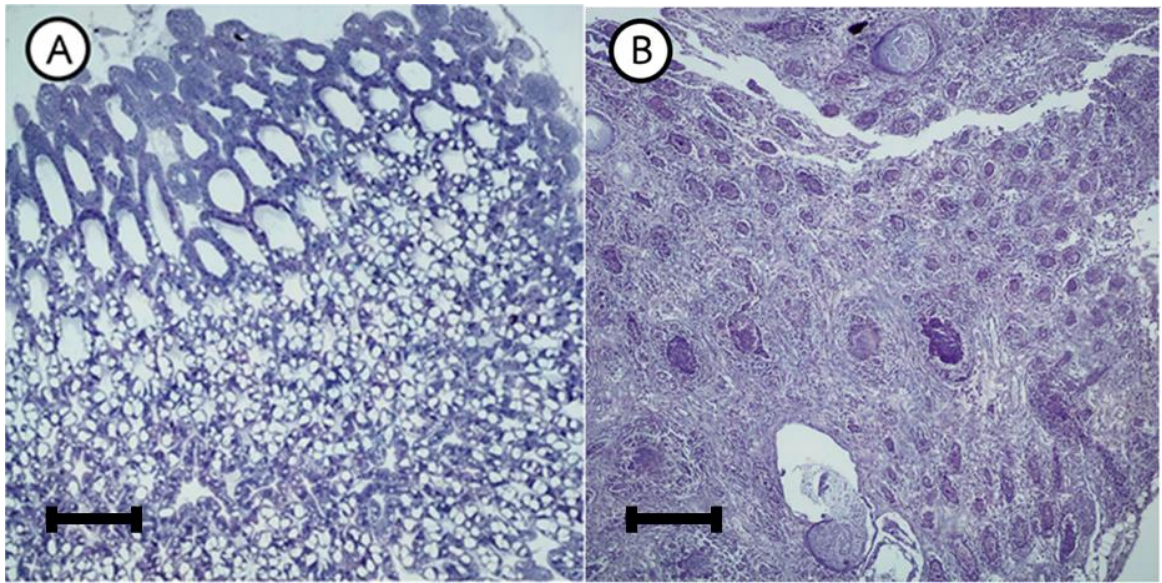
Figure 4.10: The comparison of the histopathological change in the hepatopancreas. (A) the negative control group and (B) shrimp that fed with 3% of HDPE-MP for 28 days. (Bar = 400 µm)
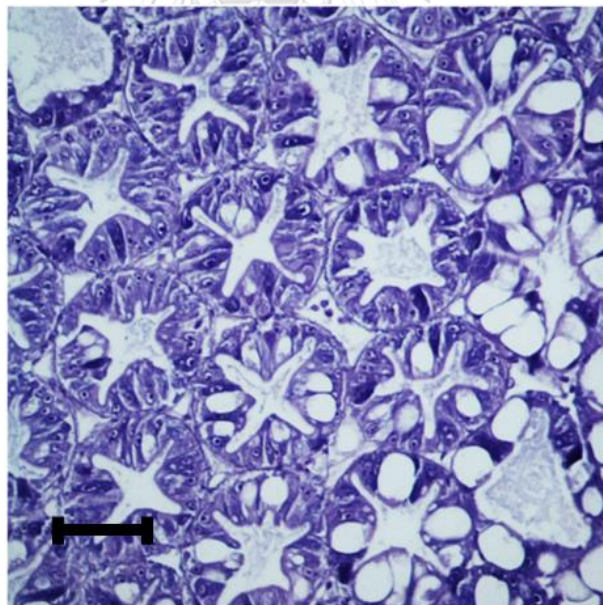
Figure 4.11: The hepatopancreas of shrimp from control with no sign of histopathological lesions in hepatopancreas under a light microscope at 40x (Bar = 100 µm)
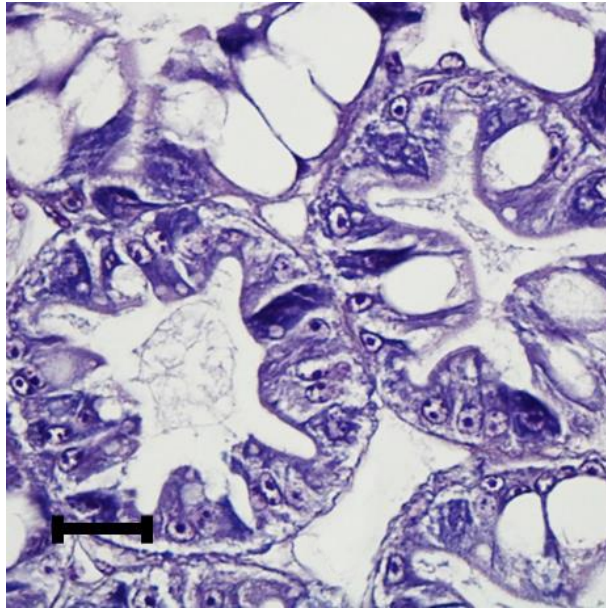
Figure 4.12: The hepatopancreas of shrimp from control with no sign of histopathological lesions in hepatopancreas under a light microscope at 100x (Bar = 40 μm)
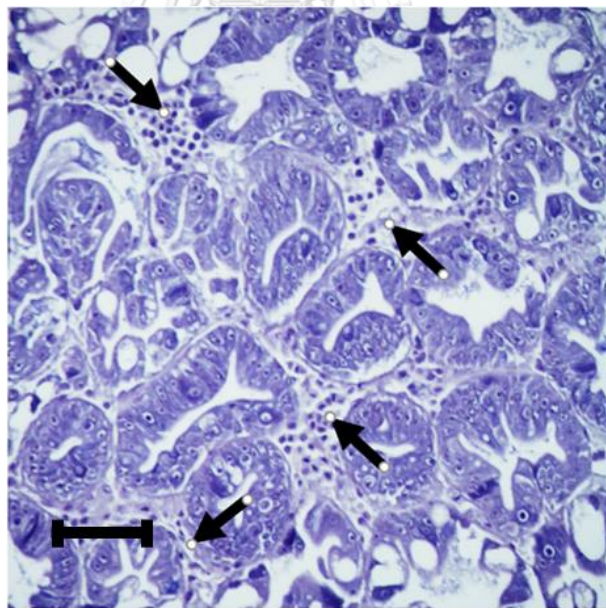
Figure 4.13: The hepatopancreas of shrimp that fed with 3% of HDPE-MP with interstitial hemocyte infiltration (Black arrow) along with hepatopancreatic tubular deformity under a light microscope at 40x (Bar = 100 μm)
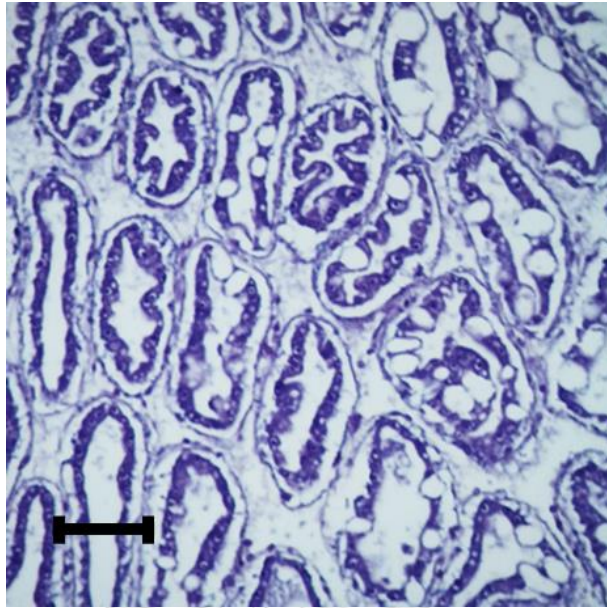
Figure 4.14: Deformity of hepatopancreatic tubular that occurred in the hepatopancreas of shrimp that fed with 0.5% of HDPE-MP under a light microscope at 40x (Bar = 100 μm).
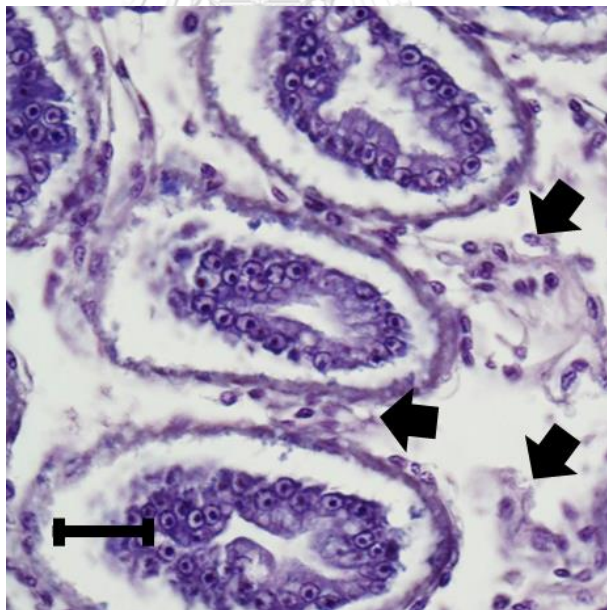
Figure 4.15: The hepatopancreas of shrimp that fed with 0.1% of HDPE-MP with interstitial hemocyte infiltration (Black arrow) and hepatopancreatic tubular deformity under a light microscope at 100x (Bar = 40 μm)
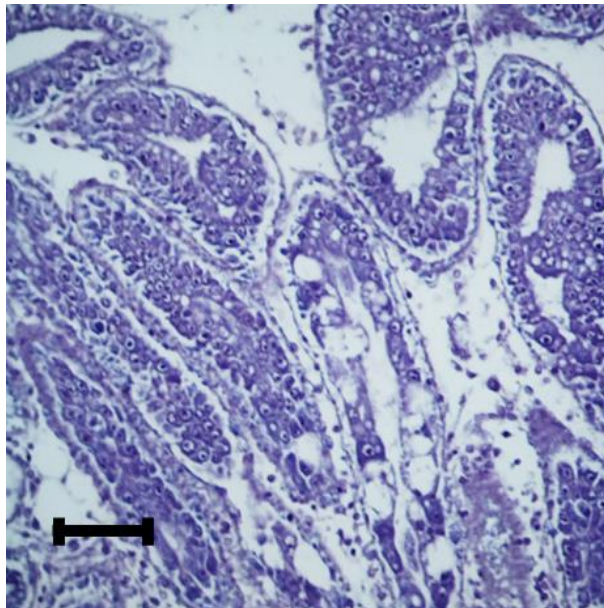
Figure 4.16: Epithelium hyperplasia and hepatopancreatic tubular deformity that occurred in the hepatopancreas of shrimp that fed with 0.5% of HDPE-MP under a light microscope at 40x (Bar = 100 μm).
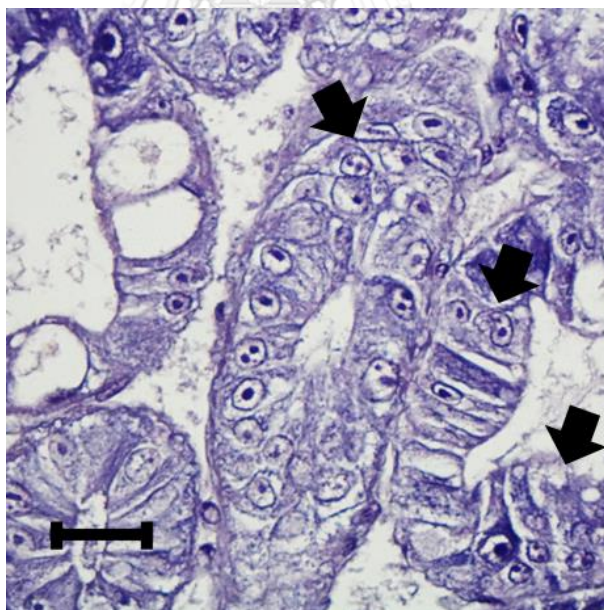
Figure 4.17: The hepatopancreas of shrimp that fed with 0.5% of HDPE-MP with multiple layers of hepatopancreatic epithelium cells (Black arrows) under a light microscope at 100x indicated the sign of epithelial hyperplasia (Bar = 40 μm).
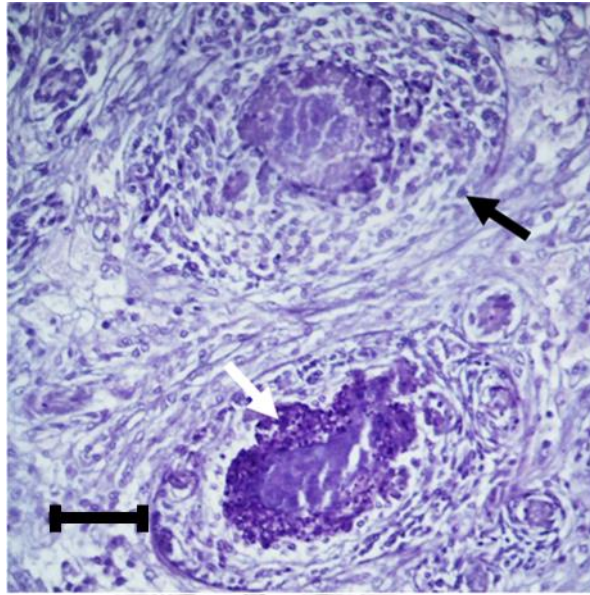
Figure 4.18: The hepatopancreas of shrimp fed with 3% of HDPE-MP with nodule formation (Black arrow) and melanization (White arrow) under a light microscope at 40x (Bar = 100 μm)
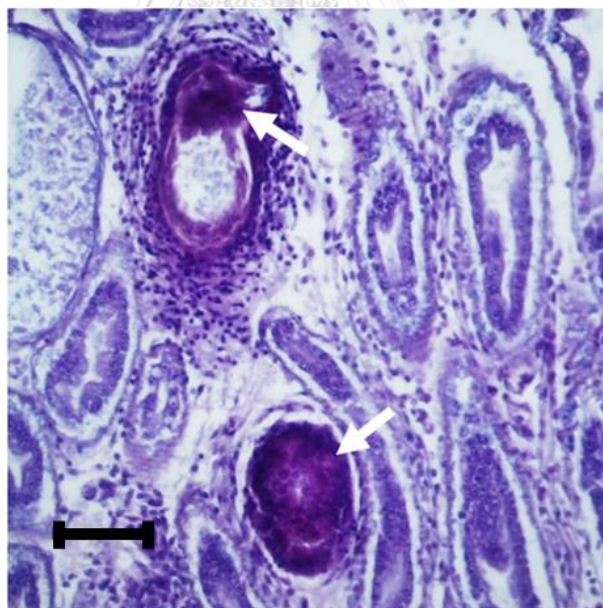
Figure 4.19: Melanization (White arrow) that occurred in the hepatopancreas of shrimp that fed with 1% of HDPE-MP under a light microscope at 40x (Bar = 100 μm)
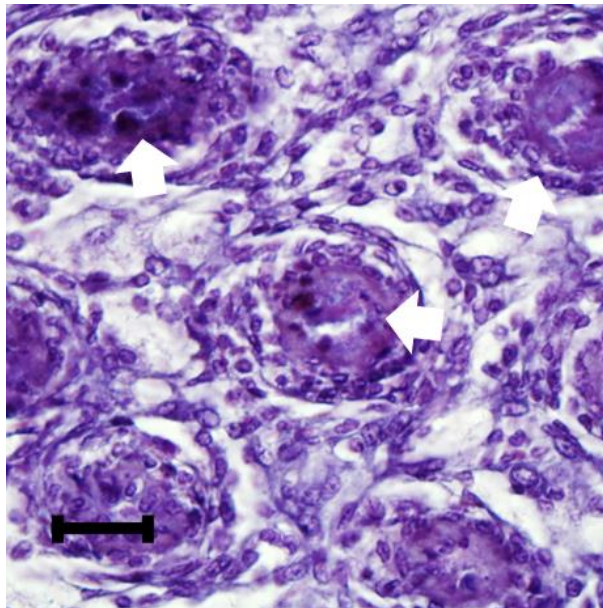
Figure 4.20: The hepatopancreas of shrimp fed with 1% of HDPE-MP with massive interstitial hemocyte infiltration and forming of the nodule with melanized core (White arrow) a under light microscope at 100x (Bar = 40 μm)
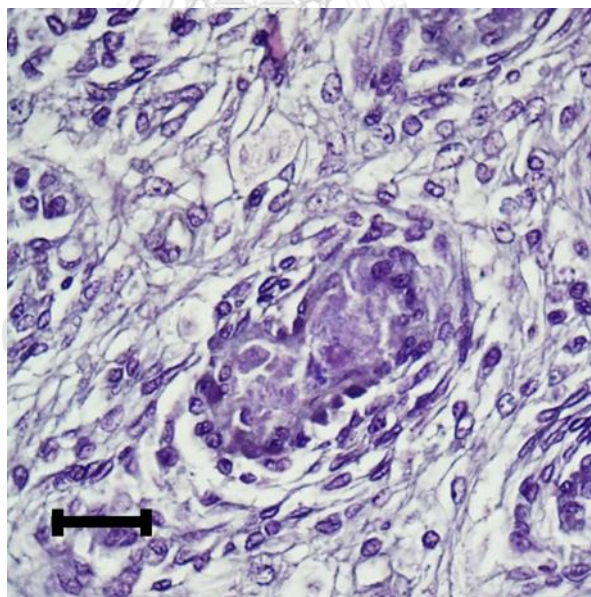
Figure 4.21: Nodule formation with Melanized core that occurred in the hepatopancreas of shrimp that fed with 3% of HDPE-MP under a light microscope at 100x (Bar = 40 μm)
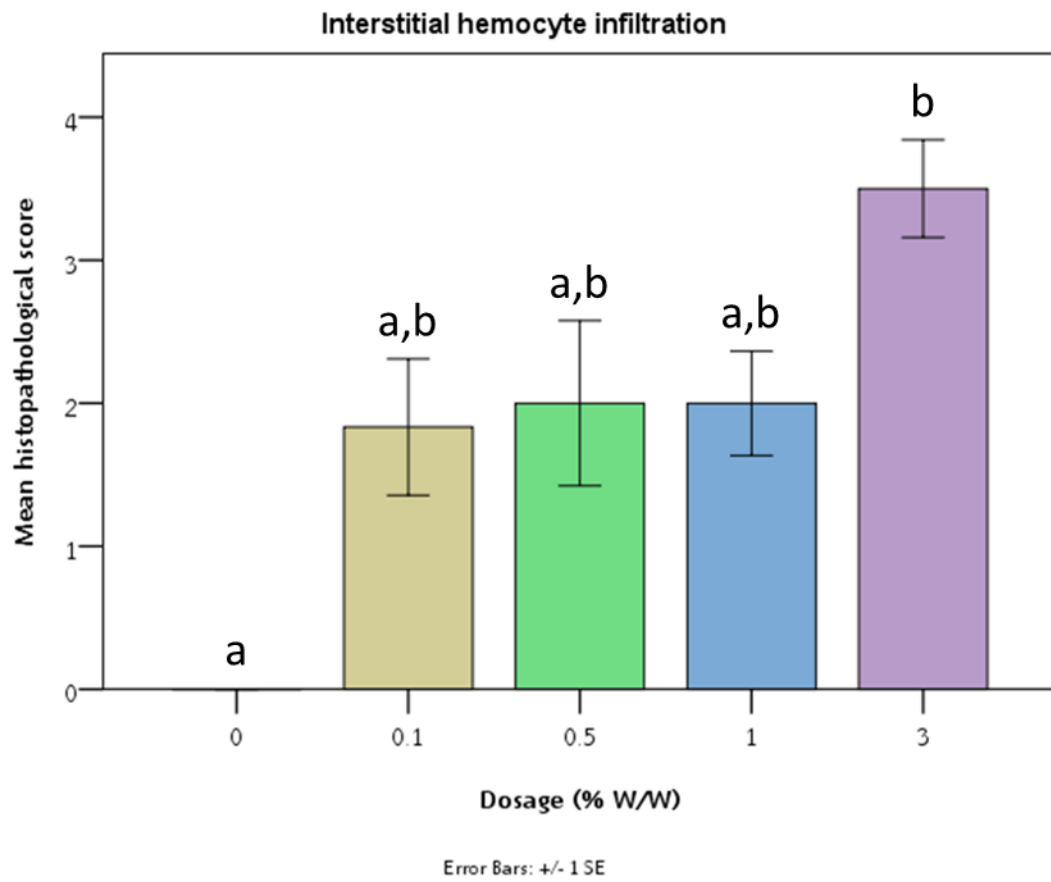
Figure 4.22: The mean histopathological score of interstitial hemocyte infiltration. Different letters above bars of the same series indicate significant differences (P<0.05) among groups.
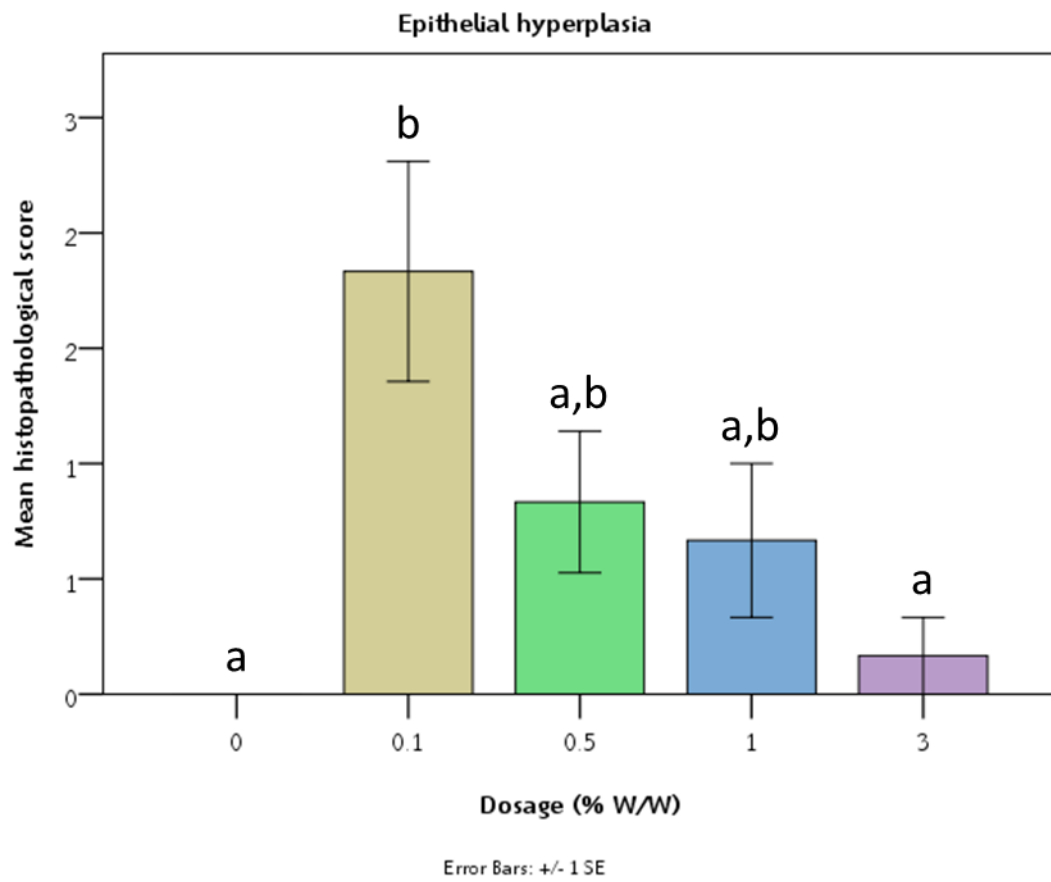
Figure 4.23: The mean histopathological score of epithelium hyperplasia. Different letters above bars of the same series indicate significant differences (P<0.05) among groups.
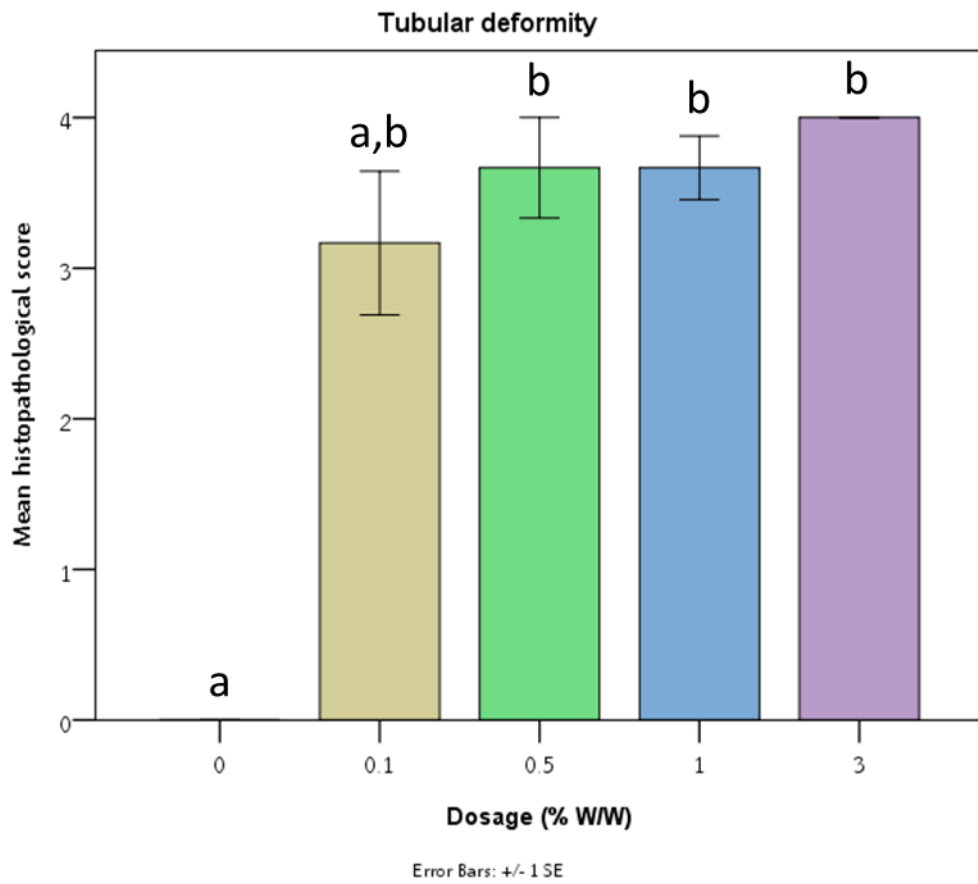
Figure 4.24: The mean histopathological score of tubular deformity. Different letters above bars of the same series indicate significant differences (P<0.05) among groups.

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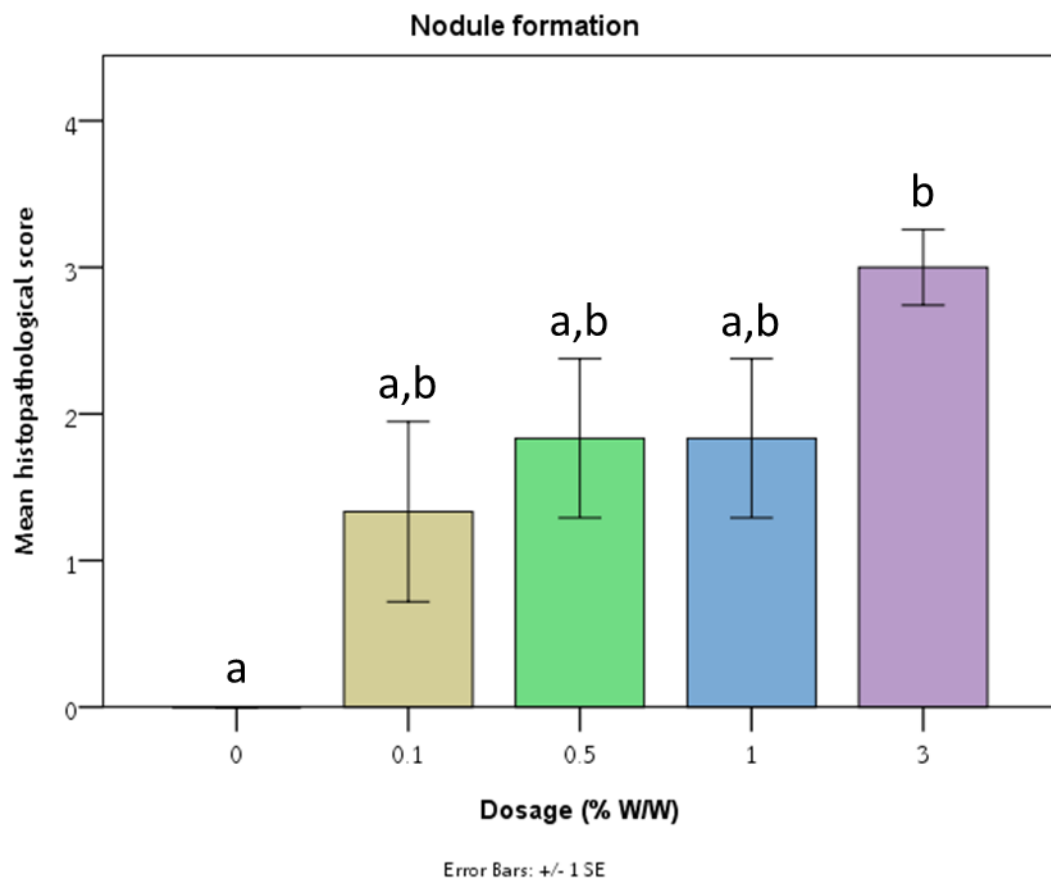
Figure 4.25: The mean histopathological score of nodule formation. Different letters above bars of the same series indicate significant differences (P<0.05) among groups.
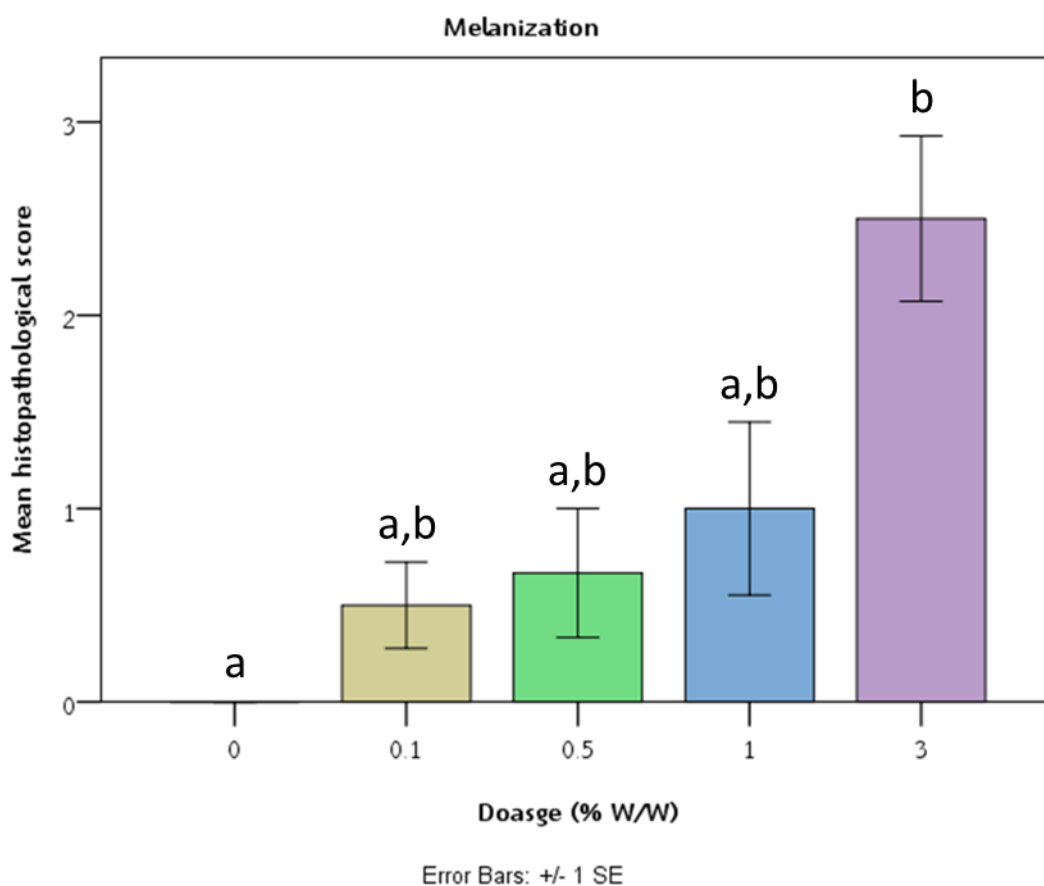
Figure 4.26: The mean histopathological score of melanization. Different letters above bars of the same series indicate significant differences (P<0.05) among groups.

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Table 4.1: The histopathological score in the hepatopancreas of shrimp that fed with HDPE microplastic at 5 different concentrations

Groups (% of HDPE-MP)	Score	Number of shrimp with the histopathological lesion (n=6)				
		Interstitial hemocyte infiltration	Epithelium hyperplasia	Tubular deformity	Nodule formation	Melanization
0 (control)	0	6	6	6	6	6
	1	0	0	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	0
	4	0	0	0	0	0
0.1	0	0	1	0	2	3

	1	3	2	1	2	3
	2	2	2	0	1	0
	3	0	0	2	0	0
	4	1	1	3	1	0
0.5	0	1	2	0	1	3
	1	1	3	0	1	2
	2	2	1	1	3	1
	3	1	0	0	0	0
	4	1	0	5	1	0
1	0	0	3	0	1	2
	1	2	2	0	1	3
	2	2	1	0	3	0
	3	2	0	2	0	1
	4	0	0	4	1	0
3	0	0	5	0	0	0
	1	0	1	0	0	1
	2	1	0	0	1	2
	3	1	0	0	4	2
	4	4	0	6	1	1

Chapter 5

Discussion and conclusion

5.1 Discussion

5.1.1 Toxicity test

This study showed a direct correlation between mortality rate and concentration of HDPE microplastic. The LC_{50} of HDPE microplastic at day 10 in this study might not represent the toxicity of microplastic contaminants in the real environment. This study used a pure HDPE microsphere with a diameter of 50 μm, in contrast with a microplastic contaminant in the environment that comes with different sizes, shapes, polymer, and chemical additives, which affect ingestion, gut retention, and toxicity of microplastics (Zarfl and Matthies, 2010; Velzeboer et al., 2014; Jeong et al., 2016; Frydkjær et al., 2017; Lei et al., 2018a). Microplastics in the marine environment could absorb toxic contaminants from the surrounding environment (Mato et al., 2001; Browne et al., 2007; Rios et al., 2007; Endo et al., 2013; Wang et al., 2015; Llorca et al., 2018). The previous studies demonstrated that toxic contaminants that resided in microplastics could leach into the gut lumen, then be absorbed into the blood circulation of the animals (Tickner et al., 1999; Besseling et al., 2013; Browne et al., 2013; Rochman et al., 2013; Bakir et al., 2014). These contaminants could synergize the toxicity effect of microplastics (Rainieri et al., 2018). Thus, the LC_{50} of the microplastic contaminant in the real environment could be lower than the result of this study. Further research needs to be done to determine the risk of microplastic toxicity in both animals and humans.

Despite only one shrimp with intestinal obstruction being observed in this study, it was suspected to be one of the causes of mortality in shrimp that fed with 20% of HDPE-MP. The previous study on Norwegian lobster showed that long-term ingestion of microplastic fiber could obstruct the gastrointestinal tract and lead to starvation created by false satiation (Welden and Cowie, 2016b; Welden and Cowie, 2016a). Intestinal obstruction was rare in this study because sphere microplastic has the lowest ability to retain in the gastrointestinal tract (Au et al., 2015; Frydkjær et al., 2017).

5.1.2 Nonspecific immunity gene expression

There was evidence of the production and accumulation of reactive oxygen species (ROS) in various organs before microplastic exposure (Jeong et al., 2017; Yu et al., 2018; Kang et al., 2021). The high concentration of ROS is cytotoxic and can induce apoptosis (Kannan and Jain, 2000). Thus, antioxidant enzymes are produced to eliminate ROS and protect cells from oxidative damage. Oxidative stress occurs when ROS was produced excessively and antioxidant enzymes was depleted (Burton and Jauniaux, 2011; Hu and Palić, 2020). The previous study suggested that upregulation of antioxidation enzymes gene expression such as *SOD*, *GPx*, *CAT*, and *GST* prior exposing to microplastic was a sign of responding to ROS (Yu et al., 2018; Hsieh et al., 2021; Kang et al., 2021), therefore, upregulation of SOD and *GPx* gene expression in this study were pieces of evidence of ROS production in the hepatopancreas of shrimp that fed with HDPE microplastic.

This study showed that shrimp that ingested low concentrations of HDPE microplastic (1 and 5%) could respond to ROS induced by microplastics during 21 days of microplastic ingestion. On the other hand, shrimp that ingested high concentrations of HDPE microplastic (1 and 3%) could not respond to ROS for 21 days. This might suggest that shrimp responded to ROS produced by HDPE microplastic in a dose-dependent manner. The down-regulation of *SOD* and *GPx* gene expression suggested the suppression of antioxidant enzymes, which led to oxidative stress in the hepatopancreas (Inoue et al., 2011). This study showed that long-term HDPE ingestion (28 days) could suppress enzyme *SOD* expression in a time-dependent manner. However, shrimp that were fed with 0.1% of HDPE-MP for 28 days did not significantly down-regulated. Therefore, the *SOD* gene expression also behaved in a concentration-dependent manner. The down-regulation of *GPx* gene expression occurred earlier in shrimp that fed with a high concentration of HDPE microplastic (1% of HDPE-MP at day 21 and 3% of HDPE-MP at day 14) than shrimp that fed with a low concentration of HDPE microplastic (1 and 5% at day 28). Thus, the down-regulation of *GPx* gene expression in shrimp that fed with HDPE microplastic was expressed in a concentration-dependent manner.

Expression of the *SOD* and *GPx* genes at day 21 followed the gene expression pattern reported in Mitten crab studies by Yu et al (Yu et al., 2018). Mitten crab exposure to polystyrene microplastic at low concentration (≤ 4 mg/L) for 21 days upregulated SOD and *GPx* gene expression, while this effect was not noticed when a high dose of microplastic (40 mg/L) was used. In our study, shrimp that ingested low concentrations of HDPE-MP (1 and 5% HDPE-MP) could upregulate *SOD* and *GPx* gene expression, providing evidence of shrimp response to reactive oxygen species (ROS) or other forms of oxidative stress induced by HDPE-MP. This is in accordance with recent studies that demonstrated how the upregulation of antioxidant enzymes (*SOD*, *GPx*, *CAT*, and *GST*) in animals exposed to microplastic was indicative of responding to ROS generation (Yu et al., 2018; Hsieh et al., 2021; Kang et al., 2021). ROS increase is cytotoxic and can induce apoptosis in various organs (Kannan and Jain, 2000), and the capacity of an organism for prevention of ROS-induced damages via antioxidant enzymes has limitations (Morel and Barouki, 1999; Parrilla-Taylor et al., 2013). In our study, shrimp fed with a high concentration of HDPE-MP (1 and 3% HDPE-MP) for a longer time could not respond adequately to ROS, as evidenced by suppression of the expression of the antioxidant enzymes. Such suppression resulted in excessive ROS damage to the hepatopancreas (Burton and Jauniaux, 2011).

The alteration of antioxidant gene expression in this study could be a response to stress arising from the ingestion of HDPE-MP. Stress factors such as abnormal temperature, pH changes, poor water quality, and starvation could induce oxidative stress and altered antioxidant gene expression (Wang et al., 2009; Zhou et al., 2010; Lin et al., 2012; Han et al., 2018b). The previous study suggested that microplastic could induce starvation stress. There were reports indicating that fiber of microplastics could disrupt feed ingestion and nutrient absorption, which lead to starvation in the Norwegian lobster (*Nephrops norvegicus*) (Welden and Cowie, 2016b; Welden and Cowie, 2016a). During the toxicity test of this study, the obstruction of HDPE-MP in the intestine of one of the deceased shrimp that fed with

20% of HDPE-MP was observed under a light microscope (**Figure 4.9**) However, this might not suggest the disruption of ingestion and absorption that starvation stress might play a role in alteration of antioxidant gene expression in this study since it was rarely found in this study and correlated with the nature of the microplastic sphere, which was rarely accumulated in the gastrointestinal tract (Au et al., 2015; Frydkjær et al., 2017; Choi et al., 2021). HDPE-MP was observed under a light microscope in the excreta of shrimp that fed with HDPE-MP (**Figure 4.2**). The rediscovered HDPE-MP were not intact and some were broken into pieces, which suspected the ability of Pacific white shrimp that could break down HDPE spheres into smaller microplastics or even nano-plastics that might be translocated into hepatopancreas of shrimp. However ever this study did not have solid evidence of the digestibility of microplastic by shrimp, but the previous study of Antarctic krill (*Euphausia superba*) demonstrated the ability of Antarctic krill that could breakdown microplastic into smaller size or even nano-plastic (Dawson et al., 2018). Thus, the possibility of translocation of microplastic/nano-plastic into hepatopancreas was unlikely to occur in this study suggesting that alteration of *SOD* and *GPx* gene expression was caused by HDPE-MP.

The *LYZ* gene expression in this study was significantly upregulated after ingesting at least 0.5% HDPE-MP for 7 days. The previous studies showed that short-term microplastic exposure could upregulate *LYZ* gene expression and enzyme activity (Liu et al., 2019; Wang et al., 2021). Interestingly, our study also showed that ingestion of at least 0.1% HDPE-MP for 14 days could significantly downregulate *LYZ* gene expression. Since lysozyme is the vital defense mechanism against harmful bacteria such as Vibrio spp. (De-La-Re-Vega et al., 2006; Burge et al., 2007; Ji et al., 2009), downregulation of *LYZ* gene expression can increase shrimp vulnerability to the pathogen causing early mortality syndrome (Lin et al., 2015b). Current literature presents evidence of both reduction and increase of Lysozyme activity and gene expression when the organism is exposed to plastic micro/nanoparticles (Wen et al.,

2018; Yang et al., 2020; Muhammad et al., 2021; Wang et al., 2021). Therefore, besides recognition that variability and seeming contradiction of reported responses can be attributed to differences in study methods including animal species, type/size of plastic particles, and other confounding factors in non-standardized studies, mechanisms and causes of upregulation of *LYZ* gene expression in shrimp exposed to HDPE-MP currently remain unknown.

5.1.3 Histopathology lesions

The study of the acute effect of microplastics in hepatopancreas by Wang et al. (2021) shows that fluorescent red polyethylene microspheres (FRPE) could induce the deformation of hepatopancreatic tubular. The present study focused on the chronic effect of microplastics in the hepatopancreas. However, tubular deformity and other histopathology lesions were also observed in the hepatopancreas in this study with a higher degree of severity. The severity of histopathology lesions that occurred in this study behaved in a dose-dependent manner. The severity of lesions such as tubular deformity, interstitial hemocyte infiltration, nodule formation, and melanization increased when a higher concentration of HDPE microplastic was ingested except for epithelium hyperplasia, which behaved oppositely. Thus, this study suggested that long-term ingestion of low concentrations of HDPE microplastic could induce epithelium hyperplasia.

Granulomatous inflammation can occur when the foreign body was present in tissue (Joyce et al., 2014). The study on textile workers, who were exposed to air-borne microplastic discovered that microplastic could induce granulomatous inflammation in their lungs (Pimentel et al., 1975). Moreover, worn plastic material from the hip prosthesis could induce granulomatous inflammation in the patients (Tallroth et al., 1989). Thus, microplastics are foreign bodies that can induce granulomatous inflammation. Histopathology lesions that were observed in this study such as interstitial hemocyte infiltration, nodule formation, and melanization were signs of granulomatous inflammation (Liu et al., 2004; Soto-Rodriguez et al., 2012) and might be caused by translocated HDPE microplastic. However, this study

did not observe translocation and accumulation of ingested HDPE microplastic in hepatopancreas.

This study demonstrated that a high concentration of HDPE microplastic might lead to damage to the hepatopancreas, which led to cellular dysfunction and resulted in an inhibition of nonspecific immunity gene expression in this study (Khodabandehloo et al., 2016). The hepatopancreas damaged by microplastics could be correlated with the mortality that occurred in this study since the hepatopancreas is a vital organ of crustaceans with an important role in digestion and metabolism regulation (Vogt, 2019).

5.2 Future application

The result of this study demonstrated the potential toxicity of microplastic that could negatively impact the Pacific white shrimp culture and lead to loss of production in the future, since the trend of microplastic contamination in the environment was predicted to be increasing rapidly in the near future (Lebreton et al., 2019). The awareness of toxicity and immunosuppression by microplastic could rise awareness of microplastic contamination that might occur in aquaculture systems such as the usage of microplastics contaminated fish meal (Hanachi et al., 2019), the use of HDPE-pond lining in Pacific white shrimp culture system (Prawitwilaikul et al., 2006), and the source of water that might be contaminated with microplastic (Desforges et al., 2014; Zhang et al., 2015; Cincinelli et al., 2019). This awareness could lead to practice and policy to prevent or minimize the contamination of microplastic in aquaculture systems. This study might lead to further study in the future such as The digestibility and translocation from the digestive tract of microplastic in shrimp, the study of microplastic or nano-plastic accumulation in shrimp, the study of plastic item weathering in the aquaculture environment, the contamination of microplastic in shrimp that rise in an aquaculture environment, the risk assessment of microplastic ingestion by the consumer of aquaculture product, etc. to provide more insight of microplastic toxicity and situation of microplastic contamination in aquaculture system.

5.3 Conclusion

The result of this study suggested that ingestion of HDPE microplastic with a diameter of 50 μm could lead to mortality of Pacific white shrimp and LC_{50} at day 28 is 3.074%. The low concentration of HDPE microplastic could upregulate *SOD*, *GPx*, and *LYZ* gene expression for a short duration of ingestion. On the other hand, the high concentration of HDPE microplastic could down-regulate SOD, *GPX*, and *LYZ* at different duration of ingestion, which resulted in immunosuppression. Histopathological lesions in hepatopancreas were presented in shrimp that were fed with HDPE microplastic for 28 days with histopathological scores correlated to the concentration of HDPE microplastic. This study concluded that ingestion of HDPE microplastic could lead to mortality of Pacific white shrimp and evidence of immunosuppression and damaging of hepatopancreas was presented.
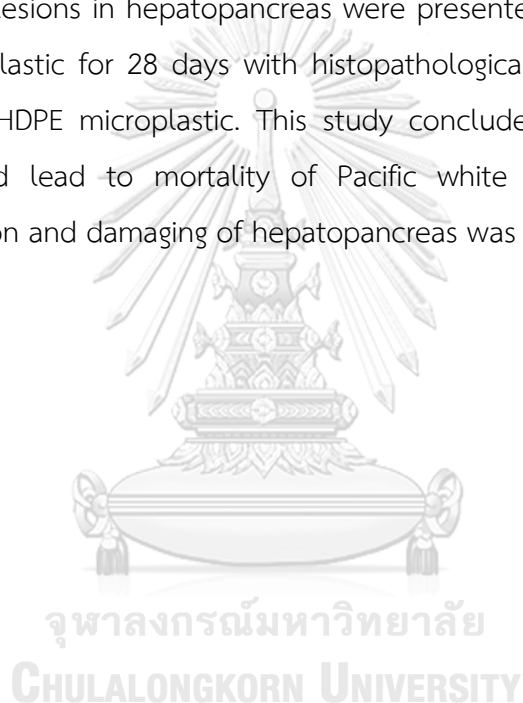
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Appendix

Appendix A: Percentage of nutrients in shrimp feed (Analyzed by the department of animals husbandry, faculty of veterinary science, Chulalongkorn University)

ภาควิชาสัตวบาล

คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย   เลขที่ 17/63

รายงานการวิเคราะห์ตัวอย่างอาหารสัตว์ทางเคมี

ผู้ส่งตัวอย่าง	:	คุณทรงศักดิ์ เนี่ยมเจริญ
วันที่รับตัวอย่าง	:	1 พฤษภาคม 2563
ชนิดตัวอย่าง	:	อาหารกุ้ง
เลขที่ตัวอย่าง	:	S069/63, S070/63, S071/63

ผลการตรวจวิเคราะห์ (หน่วยเป็น %)

ชื่อตัวอย่าง	WG 3% 12 hrs.	WG 4% 12 hrs.	WG 5% 12 hrs.	วิธีวิเคราะห์
ความชื้น	4.19	4.24	4.13	AOAC (2005) 934.01
โปรตีน	33.86	33.26	33.09	AOAC (2005) 2001.11
ไขมัน	8.28	8.12	8.33	AOAC (2005) 920.39
เยื่อใย	13.06	13.79	13.65	AOAC (2005) 978.10
เถ้า	11.10	10.94	10.94	AOAC (2005) 942.05

.................................(ผู้วิเคราะห์)

(น.ส.สุกันดา พลสันต์)

วันที่ 7 พฤษภาคม 2563

.................................(ผู้ควบคุม)

(ผศ.ดร.ทัยรัตน์ พลายมาศ)

วันที่ 7 พฤษภาคม 2563

ผลการตรวจวิเคราะห์ที่ได้รับนี้อ้างถึงเฉพาะตัวอย่างที่ส่งมายังภาควิชาสัตวบาลเท่านั้น

ห้ามแก้ไขข้อมูลหรือนำไปใช้เพื่อโฆษณา

ภาควิชาสัตวบาล

คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

เลขที่ 17/63

รายงานการวิเคราะห์ตัวอย่างอาหารสัตว์ทางเคมี

ผู้ส่งตัวอย่าง	:	คุณทรงศักดิ์ เนี่ยมเจริญ
วันที่รับตัวอย่าง	:	1 พฤษภาคม 2563
ชนิดตัวอย่าง	:	อาหารกุ้ง
เลขที่ตัวอย่าง	:	S072/63, S073/63, S074/63

ผลการตรวจวิเคราะห์ (หน่วยเป็น %)

ชื่อตัวอย่าง	WG 3% 6 hrs.	WG 4% 6 hrs.	WG 5% 6 hrs.	วิธีวิเคราะห์
ความชื้น	9.23	8.36	8.79	AOAC (2005) 934.01

..............................(ผู้วิเคราะห์)

(น.ส.สุกันดา พลสันต)

วันที่ 7 พฤษภาคม 2563

..............................(ผู้ควบคุม)

(ผศ.ดร.หทัยรัตน์ พลายมาศ)

วันที่ 7 พฤษภาคม 2563

Appendix B: Animal protocol permission (IACUC)

บันทึกข้อความ

ส่วนงาน ฝ่ายวิจัยและนวัตกรรม คณะสัตวแพทยศาสตร์ จุฬาฯ โทร.89793

ที่ วจ. IACUC147/2564　　　　　**วันที่** 25 สิงหาคม 2564

เรื่อง ขอส่งใบอนุญาตให้ใช้สัตว์ในงานวิจัยฯ

เรียน รองศาสตราจารย์ สพ.ญ.ดร.นันทริกา ชันซื่อ

สิ่งที่ส่งมาด้วย ใบอนุญาตฯ ให้ใช้สัตว์ในงานวิจัยฯ จำนวน 1 ชุด

　　　　ตามที่ท่านได้ยื่นแบบฟอร์มขออนุญาตใช้สัตว์ทดลองฯ ที่คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย เพื่อเสนอขอต่อใบอนุญาตให้ใช้สัตว์ในงานวิจัย งานทดสอบ งานผลิตชีววัตถุ งานสอน และงานอื่นๆ ให้กับโครงการวิจัย เรื่อง "ผลกระทบของไมโครพลาสติกต่อการแสดงออกของยีนส์ที่เกี่ยวข้องกับ ระบบภูมิคุ้มกันแบบไม่จำเพาะในกุ้งขาว (*Litopenaeus vannamei*)" (เลขที่โครงการ 2031077) นั้น

　　　　บัดนี้ผลการประชุมของคณะกรรมการกำกับดูแลการเลี้ยงและใช้สัตว์เพื่องานทางวิทยาศาสตร์ ประจำเดือน มกราคม 2564 ได้มีผลการพิจารณาอนุมัติให้ออกใบอนุญาตฯ ให้กับโครงการวิจัยของท่าน ฝ่ายวิจัย และนวัตกรรม จึงขอส่งใบอนุญาตฯ ตามสิ่งที่ส่งมาด้วยนี้

　　　　จึงเรียนมาเพื่อโปรดทราบ

(รองศาสตราจารย์ สพ.ญ.ดร.อุตรา จามีกร)
ประธานคณะกรรมการกำกับดูแลการเลี้ยงและ
การใช้สัตว์ทดลองเพื่องานทางวิทยาศาสตร์

เลขที่....2031077......

คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ใบอนุญาตให้ใช้สัตว์ใน

งานวิจัย งานทดสอบ งานผลิตชีววัตถุ งานสอน และงานอื่น ๆ

ใบอนุญาตนี้ให้ไว้เพื่อแสดงว่าคณะกรรมการกำกับดูแลการเลี้ยงและใช้สัตว์เพื่องานทางวิทยาศาสตร์
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ได้พิจารณาได้พิจารณาโครงการวิจัย "ผลกระทบของไมโครพลาสติกต่อ
การแสดงออกของยีนส์ที่เกี่ยวข้องกับระบบภูมิคุ้มกันแบบไม่จำเพาะในกุ้งขาว (Litopenaeus vannamei)" ซึ่งมี
รองศาสตราจารย์ สพ.ญ.ดร.นันทริกา ชันซื่อ เป็นหัวหน้าหรือเจ้าของโครงการแล้วเห็นสมควรอนุญาตให้ดำเนินการตาม
โครงการนี้ได้ โดยมีเงื่อนไขว่าผู้ใช้สัตว์ในความรับผิดชอบของโครงการต้องปฏิบัติต่อข้อมูลที่กรอกในแบบฟอร์มขออนุญาต
ใช้สัตว์ที่คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย สำหรับการวิจัยอย่างเคร่งครัดกรณีที่มีการปฏิบัติอย่างหนึ่งอย่าง
ใดนอกเหนือจากที่ระบุไว้ในแบบฟอร์มขออนุญาตและเสนอในโครงการคณะกรรมการกำกับดูแลการเลี้ยงและการใช้สัตว์เพื่อ
งานทางวิทยาศาสตร์จะดำเนินการงดใบอนุญาตฯ นี้ และแจ้งหน่วยงานที่เกี่ยวข้องทราบ

ลงนาม..

(รองศาสตราจารย์ สพ.ญ.ดร.อุตรา จามีกร)

ประธานคณะกรรมการกำกับดูแลการเลี้ยงและ

การใช้สัตว์เพื่องานทางวิทยาศาสตร์

ลงนาม..

(ศาสตราจารย์ สพ.ญ.ดร.เกวลี ฉัตรดรงค์)

รองคณบดีวิจัย และนวัตกรรม

วันที่ออกใบอนุญาต...........25........สิงหาคม 2564

วันที่หมดอายุ................24........สิงหาคม 2565

Chulalongkorn University Animal Care and Use Committee

Certificate of Project Approval	☑ Original □ Renew
Animal Use Protocol **2031077**	
Protocol Title **Effects of microplastics on gene expression of nonspecific immune system in Pacific white shrimp (*Litopenaeus vannamei*)**	
Principal Investigator **Assoc.Prof.Dr.Nantarika Chansue**	
Certification of Institutional Animal Care and Use Committee (IACUC) This project has been reviewed and approved by the IACUC in accordance with university regulations and policies governing the care and use of laboratory animals. The review has followed guidelines documented in Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes edited by the National Research Council of Thailand.	
Date of Approval **August 25 , 2021**	**Date of Expiration** **August 24 , 2022**
Applicant Faculty/Institution **Faculty of Veterinary Science**	
Signature of Chairperson	**Signature of Authorized Official**
Name and Title **Assoc.Prof.Dr.Uttra Jamikorn** **Chairman**	**Name and Title** **Prof.Dr.Kaywalee Chatdarong** **Associate Dean (Research and Innovation)**
The official signing above certifies that the information provided on this form is correct. The institution assumes that investigators will take responsibility, and follow university regulations and policies for the care and use of animals. *This approval is subjected to assurance given in the animal use protocol and may be required for future investigations and reviews.*	

Appendix C: Water quality during the first phase of the experiment

6/9/2021 (Quarantine)							
Tank	NH_3 (ppm)	NO_2 (ppm)	NO_3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)
1	1.42	0.75	0.35	7.33	160	8.0	27.1
2	1.85	0.96	0.21	7.20	155	8.0	27.0
3	1.73	0.66	0.10	7.47	141	8.0	26.9
4	2.00	0.58	0.25	7.30	155	8.0	26.9
5	1.22	0.74	0.30	7.50	162	8.0	27.2
6	1.65	0.96	0.12	7.51	153	8.0	27.1
Stock	0.40	0.20	0.06	7.48	150	8.0	27.0
9/9/2021 (Quarantine)							
Tank	NH3 (ppm)	NO2 (ppm)	NO3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)
1	2.37	1.49	0.72	7.45	150	8.0	27.0
2	1.79	0.98	0.85	7.53	154	8.0	27.3
3	1.85	1.02	1.10	7.42	146	8.0	27.1
4	2.10	1.45	0.68	7.50	138	8.0	27.1
5	1.92	0.75	1.31	7.52	160	8.0	27.2
6	1.78	1.23	1.35	7.48	143	8.0	27.2
Stock	0.23	0.14	0.24	7.52	159	8.0	27.0
13/9/2021 (Quarantine)							
Tank	NH3 (ppm)	NO2 (ppm)	NO3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)
1	1.58	1.61	1.74	7.42	151	8.0	27.0
2	1.87	1.49	1.52	7.5	145	8.0	27.2
3	1.62	1.18	1.93	7.47	152	8.0	26.8
4	1.93	1.35	1.20	7.52	159	8.0	27.1
5	1.65	1.10	1.69	7.51	147	8.0	26.9
6	2.12	1.58	1.46	7.43	150	8.0	27.3

Stock	0.16	0.17	0.24	7.52	140	8.0	26.9
16/9/2021 (Quarantine)							
Tank	NH3 (ppm)	NO2 (ppm)	NO3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)
1	1.85	1.82	3.96	7.56	160	8.0	26.8
2	2.12	1.76	4.53	7.49	151	8.0	27.0
3	1.65	1.67	3.89	7.50	146	8.0	27.0
4	2.34	2.21	4.51	7.46	148	8.0	27.2
5	1.98	1.81	3.98	7.52	155	8.0	27.1
6	2.36	2.24	3.69	7.53	158	8.0	27.0
Stock	0.12	0.13	0.23	7.49	145	8.0	27.1
20/9/2021							
Tank	NH3 (ppm)	NO2 (ppm)	NO3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)
1	1.82	7.59	7.56	7.53	153	8.0	27
2	1.96	5.6	6.17	7.48	151	8.0	27.1
3	1.97	3.8	7.16	7.42	141	8.0	27.3
4	1.98	4.52	7.91	7.63	149	8.0	27.0
5	2.67	2.95	9.63	7.47	165	8.0	26.9
6	1.45	8.12	6.79	7.51	147	8.0	2.7.1
7	1.06	9.56	8.56	7.49	152	8.0	27.0
8	1.67	7.75	8.45	7.53	142	8.0	26.9
9	2.14	10.31	8.22	7.49	157	8.0	27.2
10	1.58	3.41	5.35	7.51	154	8.0	27.1
11	1.6	9.89	6.88	7.5	150	8.0	27.0
12	1.87	9.52	5.91	7.56	152	8.0	27.2
13	1.79	7.79	7.98	7.49	148	8.0	27.1
14	1.85	7.5	6.45	7.47	157	8.0	27.1
15	2.1	10.23	8.63	7.56	153	8.0	26.9
Stock	0.09	0.05	1.21	7.50	152	8.0	26.8

23/9/2021							
Tank	NH3 (ppm)	NO2 (ppm)	NO3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)
1	1.48	10.22	13.81	7.59	142	8.0	27.1
2	1.75	9.78	14.53	7.52	139	8.0	27.0
3	1.56	15.23	12.02	7.53	145	8.0	26.8
4	1.31	16.23	13.08	7.51	142	8.0	27.0
5	1.55	15.71	12.75	7.61	141	8.0	27.2
6	1.33	12.52	11.36	7.49	160	8.0	27.1
7	1.55	10.14	13.26	7.56	150	8.0	27.3
8	1.68	14.55	11.10	7.59	142	8.0	26.9
9	1.42	16.21	13.45	7.56	140	8.0	27.0
10	1.35	15.50	10.37	7.47	140	8.0	27.1
11	1.75	18.74	13.81	7.63	146	8.0	27.0
12	1.85	12.14	12.42	7.52	150	8.0	26.9
13	1.78	14.74	12.53	7.57	159	8.0	27.1
14	1.95	15.07	13.34	7.55	158	8.0	26.9
15	1.47	18.23	13.12	7.61	153	8.0	27.2
Stock	0.15	0.42	2.64	7.54	150	8.0	27.2
27/9/2021							
Tank	NH3 (ppm)	NO2 (ppm)	NO3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)
1	0.75	21.54	23.00	7.52	145	8.0	27.1
2	0.82	24.16	25.27	7.49	148	8.0	27.2
3	1.42	19.59	19.63	7.51	163	8.0	26.9
4	1.32	21.45	26.74	7.63	140	8.0	27.0
5	1.40	20.16	24.51	7.58	145	8.0	26.8
6	1.35	24.68	19.88	7.57	140	8.0	27.0
7	0.95	26.68	24.53	7.53	148	8.0	27.2
8	1.09	24.57	17.28	7.49	149	8.0	27.1

9	0.77	21.23	19.56	7.59	149	8.0	26.9
10	1.00	17.85	17.24	7.52	145	8.0	26.9
11	1.14	18.95	19.35	7.53	160	8.0	27.1
12	1.36	23.52	19.36	7.61	145	8.0	27.1
13	0.98	29.46	18.54	7.61	152	8.0	26.9
14	0.21	25.68	15.36	7.52	141	8.0	27.2
15	0.85	27.86	19.25	7.57	153	8.0	27.0
Stock	0.15	0.44	4.10	7.49	156	8.0	26.9

30/9/2021							
Tank	NH3 (ppm)	NO2 (ppm)	NO3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)
1	0.11	10.1	10.51	7.61	140	8.0	27.1
2	0.15	7.89	11.10	7.62	154	8.0	27.0
3	0.17	8.94	15.32	7.57	149	8.0	27.0
4	0.19	11.25	15.15	7.53	150	8.0	26.9
5	0.17	9.94	12.88	7.57	149	8.0	27.2
6	0.18	4.63	10.75	7.64	161	8.0	26.9
7	0.19	5.97	4.57	7.51	154	8.0	26.9
8	0.17	1.26	7.94	7.53	160	8.0	27.0
9	0.21	8.78	11.73	7.57	154	8.0	26.9
10	0.21	8.86	9.82	7.56	158	8.0	27.1
11	0.16	9.70	11.25	7.57	148	8.0	27.2
12	0.17	9.95	4.84	7.57	144	8.0	26.8
13	0.15	7.52	12.29	7.51	143	8.0	27.0
14	0.11	8.73	8.94	7.56	158	8.0	27.1
15	0.12	12.76	8.73	7.54	149	8.0	27.2
Stock	0.14	1.89	4.21	7.56	146	8.0	27.2

4/10/2021							
Tank	NH_3	NO_2	NO_3	pH	ALK	DO	Temp.
1	0.16	1.50	8.7	7.52	152	8.0	27.2

2	0.15	1.26	10.2	7.59	154	8.0	26.9
3	0.14	1.05	9.8	7.6	160	8.0	26.9
4	0.19	1.36	11.45	7.51	158	8.0	27.1
5	0.13	0.82	10.5	7.56	137	8.0	27.1
6	0.15	0.69	8.25	7.48	140	8.0	27.0
7	0.16	1.36	6.59	7.5	140	8.0	27.0
8	0.19	0.58	4.17	7.58	158	8.0	27.1
9	0.21	0.9	5.67	7.51	150	8.0	27.0
10	0.13	1.12	10.85	7.53	145	8.0	27.1
11	0.17	0.87	5.64	7.54	142	8.0	27.1
12	0.17	0.9	4.94	7.61	149	8.0	26.8
13	0.19	1.38	8.39	7.49	158	8.0	27.1
14	0.16	1.24	6.54	7.63	148	8.0	27.0
15	0.2	1.2	4.75	7.52	149	8.0	27.2
Stock	0.12	0.054	4.27	7.5	150	8.0	27.0
7/10/2021							
Tank	NH3 (ppm)	NO2 (ppm)	NO3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)
1	0.13	0.08	3.71	7.51	155	8.0	26.8
2	0.16	0.005	3.52	7.60	154	8.0	27.0
3	0.17	0.04	4.95	7.50	149	8.0	27.0
4	0.13	0.12	4.47	7.46	153	8.0	27.1
5	0.1	0.05	3.84	7.51	157	8.0	26.9
6	0.16	0.08	3.29	7.58	160	8.0	27.0
7	0.27	0.09	4.16	7.61	145	8.0	27.0
8	0.21	0.09	5.12	7.59	142	8.0	27.1
9	0.19	0.05	3.94	7.52	149	8.0	27.0
10	0.13	0.12	4.37	7.53	139	8.0	27.2
11	0.31	0.01	3.93	7.54	158	8.0	27.1
12	0.2	0.07	3.76	7.53	161	8.0	27.1

13	0.19	0.12	3.67	7.63	142	8.0	27.2
14	0.14	0.07	4.74	7.58	169	8.0	26.9
15	0.25	0.09	4.71	7.50	159	8.0	26.9
Stock	0.04	0.005	2.26	7.49	145	8.0	27.1

11/10/2021							
Tank	NH3 (ppm)	NO2 (ppm)	NO3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)
1	0.04	0.06	4.87	7.48	157	8.0	26.8
2	0.05	0.051	3.24	7.56	159	8.0	27.0
3	0.04	0.04	3.93	7.62	150	8.0	26.9
4	0.08	0.08	3.60	7.54	154	8.0	27.0
5	0.09	0.01	4.00	7.53	156	8.0	27.1
6	0.05	0.013	3.51	7.61	155	8.0	27.2
7	0.07	0.045	3.94	7.49	159	8.0	27.1
8	0.12	0.06	4.21	7.55	157	8.0	27.3
9	0.11	0.038	3.87	7.67	165	8.0	26.9
10	0.09	0.05	3.80	7.62	153	8.0	26.9
11	0.05	0.10	3.85	7.54	150	8.0	27.2
12	0.09	0.098	5.05	7.61	149	8.0	27.0
13	0.05	0.035	4.51	7.49	167	8.0	27.1
14	0.04	0.09	3.85	7.63	145	8.0	27.0
15	0.1	0.05	4.14	7.48	154	8.0	27.6
Stock	0.08	0.06	2.03	7.57	159	8.0	27.0

14/10/2021							
Tank	NH3 (ppm)	NO2 (ppm)	NO3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)
1	0.06	0.071	3.2	7.52	155	8.0	27.2
2	0.06	0.052	3.1	7.53	156	8.0	27.2
3	0.05	0.047	3	7.53	161	8.0	26.9
4	0.07	0.069	2.8	7.64	146	8.0	27.0

5	0.06	0.043	2.9	7.51	156	8.0	27.1
6	0.07	0.023	3.1	7.61	162	8.0	27.1
7	0.07	0.027	2.5	7.52	151	8.0	27.0
8	0.06	0.029	3.4	7.51	159	8.0	26.9
9	0.12	0.021	2.5	7.57	153	8.0	27.2
10	0.09	0.041	4	7.59	149	8.0	26.9
11	0.9	0.048	2.3	7.53	164	8.0	27.0
12	0.05	0.027	3.8	7.57	157	8.0	26.9
13	0.04	0.035	3.9	7.69	164	8.0	27.2
14	0.07	0.039	3.8	7.51	150	8.0	27.1
15	0.05	0.029	2.4	7.58	154	8.0	27.0
Stock	0.06	0.046	2.7	7.62	157	8.0	27.0

Appendix D: Water quality during the second phase of the experiment

25/10/2021 (Quarantine)							
Tank	NH3 (ppm)	NO2 (ppm)	NO3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)
1	1.61	0.81	0.75	7.50	150	8.0	27.1
2	2.3	1.05	0.1	7.61	140	8.0	27.2
3	2.2	0.72	0.05	7.48	165	8.0	26.9
4	1.78	0.78	0.2	7.52	149	8.0	27.0
5	1.96	0.8	0.23	7.52	135	8.0	27.1
6	2.5	1.14	0.14	7.54	144	8.0	26.8
Stock	0.12	0.1	0.09	7.54	149	8.0	27.0
28/10/2021 (Quarantine)							
Tank	NH3 (ppm)	NO2 (ppm)	NO3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)
1	2.34	1.35	1.26	7.62	142	8.0	27.4
2	1.78	1.85	1.38	7.47	145	8.0	27.2
3	1.99	0.72	0.98	7.52	141	8.0	27.2

Tank	NH3 (ppm)	NO2 (ppm)	NO3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)
4	2.2	1.27	0.63	7.51	135	8.0	27.4
5	2.4	0.98	1.85	7.54	138	8.0	27.3
6	1.13	1.45	1.56	7.49	145	8.0	27.1
Stock	0.14	0.21	0.13	7.46	147	8.0	27.1
1/11/2021 (Quarantine)							
Tank	NH3 (ppm)	NO2 (ppm)	NO3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)
1	1.13	1.87	1.82	7.43	147	8.0	27.2
2	1.56	1.25	2.02	7.56	137	8.0	27.1
3	1.51	0.98	2.05	7.62	146	8.0	27.3
4	2.2	1.24	1.1	7.52	139	8.0	27.1
5	2.29	1.1	2.35	7.54	151	8.0	26.9
6	1.23	2.1	1.75	7.55	140	8.0	27.1
Stock	0.23	0.13	0.11	7.42	140	8.0	27.1
4/11/2021 (Quarantine)							
Tank	NH3 (ppm)	NO2 (ppm)	NO3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)
1	2.61	2.13	3.32	7.47	150	8.0	27.1
2	1.87	1.89	4.23	7.51	149	8.0	27.2
3	1.21	1.12	4.12	7.52	139	8.0	27.1
4	2.27	2.25	5.78	7.49	139	8.0	26.8
5	2.36	2.05	3.89	7.48	147	8.0	27.0
6	1.24	1.96	4.18	7.56	145	8.0	27.1
Stock	0.11	0.23	0.15	7.5	150	8.0	27.30
8/11/2021 (Quarantine)							
Tank	NH3 (ppm)	NO2 (ppm)	NO3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)
1	2.62	2.93	7.31	7.51	152	8.0	27.0
2	2.31	3.75	5.21	7.52	135	8.0	27.0
3	2.33	2.12	4.11	7.48	141	8.0	27.1

4	2.11	3.5	5.69	7.47	143	8.0	27.1
5	1.98	3.34	5.56	7.45	134	8.0	26.9
6	2.23	2.94	7.72	7.55	150	8.0	27.0
Stock	0.21	0.32	0.19	7.53	141	8.0	27.0
11/11/2021 (Quarantine)							
Tank	NH3 (ppm)	NO2 (ppm)	NO3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)
1	1.72	4.35	7.25	7.56	151	8.0	27.0
2	2.1	3.05	4.68	7.52	142	8.0	27.0
3	1.53	2.98	4.21	7.48	148	8.0	27.1
4	1.25	5.23	6.79	7.49	150	8.0	26.9
5	2.1	2.23	3.96	7.5	147	8.0	27.2
6	2.2	6.14	8.21	7.5	145	8.0	27.1
Stock	0.19	0.1	0.13	7.53	139	8.0	26.9
15/11/2021							
Tank	NH3 (ppm)	NO2 (ppm)	NO3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)
1	1.35	5.81	8.5	7.49	140	8.0	27.1
2	1.85	4.05	5.5	7.5	130	8.0	27.2
3	2.5	3.72	4.1	7.52	132	8.0	27.1
4	2.1	6.7	8.3	7.51	139	8.0	27.1
5	2.5	1.8	4.5	7.51	129	8.0	27.1
6	1.15	8.14	10.1	7.52	130	8.0	27.2
7	1.12	8.78	9.5	7.52	135	8.0	27.0
8	1.21	6.91	8.4	7.5	140	8.0	27.1
9	2.5	10.31	8.4	7.49	129	8.0	26.9
10	2.05	3.41	5.6	7.48	131	8.0	27.0
11	1.2	10.17	5.9	7.45	132	8.0	27.0
12	0.95	10.11	8.4	7.5	131	8.0	27.1
13	1.7	6.61	6.2	7.5	130	8.0	27.0

Tank	NH3 (ppm)	NO2 (ppm)	NO3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)
14	1.95	6.99	6.7	7.52	137	8.0	27.1
15	1.6	10.06	7.8	7.53	129	8.0	27.1
Stock	0.13	0.1	0.15	7.52	140	8.0	26.9
18/11/2021							
Tank	NH3 (ppm)	NO2 (ppm)	NO3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)
1	1.5	10.24	13.7	7.59	139	8.0	27.0
2	1.6	9.05	6.7	7.58	135	8.0	27.0
3	1.4	14.53	13	7.53	140	8.0	27.0
4	1.3	15.91	14.3	7.51	140	8.0	27.1
5	1.55	15.12	11.7	7.51	136	8.0	27.1
6	1.3	11.33	10.2	7.62	132	8.0	27.0
7	1.55	10.19	10.6	7.57	139	8.0	27.0
8	1.45	13.21	11.1	7.56	132	8.0	27.0
9	1.3	15.34	13.4	7.65	134	8.0	26.9
10	1.35	14.41	10.3	7.54	140	8.0	26.9
11	0.95	17.68	14.8	7.61	143	8.0	27.0
12	0.95	13.84	12	7.53	144	8.0	26.9
13	0.85	14.05	12	7.49	139	8.0	27.0
14	0.9	15.07	13.3	7.55	136	8.0	27.0
15	1.05	17.25	12.9	7.51	132	8.0	27
Stock	0.18	0.7	3.7	7.62	140	8.0	27
22/11/2021							
Tank	NH3 (ppm)	NO2 (ppm)	NO3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)
1	0.6	20.4	20.1	7.54	142	8.0	27.2
2	0.65	29.6	19.6	7.62	137	8.0	27.2
3	1.9	16.8	23.5	7.73	132	8.0	27.3
4	1.5	33.7	21.4	7.62	135	8.0	27.1
5	1	25.7	23	7.63	140	8.0	27.2

6	1.2	10.67	10.1	7.64	139	8.0	27.3
7	0.5	2.6	4.5	7.65	137	8.0	27.3
8	0.97	4.17	7.7	7.62	140	8.0	27.2
9	0.66	21.2	18.1	7.63	141	8.0	27.1
10	0.98	16.9	16.4	7.61	139	8.0	27.2
11	1	32.8	15.2	7.52	133	8.0	27.2
12	1.3	2	3.9	7.6	132	8.0	27.1
13	0.9	12.8	15.9	7.61	135	8.0	27.1
14	0.34	22.8	14.1	7.59	138	8.0	27.1
15	0.35	23.7	12.4	7.63	131	8.0	27.2
Stock	0.6	0.4	3.2	7.54	142	8.0	27.2

25/11/2021							
Tank	NH3 (ppm)	NO2 (ppm)	NO3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)
1	0.12	10.1	13.2	7.66	139	8.0	27.0
2	0.16	7.8	12.2	7.58	132	8.0	26.9
3	0.18	8.9	16.9	7.61	144	8.0	27.1
4	0.23	11.2	14.5	7.52	150	8.0	27.1
5	0.1	9.9	13.8	7.58	138	8.0	27.0
6	0.17	3.6	9.2	7.59	142	8.0	27.0
7	0.18	0.59	3.3	7.56	141	8.0	27.0
8	0.22	1.2	7.2	7.64	147	8.0	27.1
9	0.15	8.7	14.2	7.55	138	8.0	26.9
10	0.16	7.3	8.8	7.54	136	8.0	26.9
11	0.12	12.3	10.2	7.58	138	8.0	27.1
12	0.11	10	3.2	7.57	134	8.0	27.0
13	0.16	6.3	13.3	7.62	147	8.0	27.1
14	0.15	7.5	9.8	7.6	140	8.0	26.9
15	0.13	13.5	8.2	7.61	138	8.0	27.2
Stock	0.12	1.1	3.2	7.66	139	8.0	27.1

29/11/2021							
Tank	NH3 (ppm)	NO2 (ppm)	NO3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)
1	0.17	1.44	5.7	7.63	145	8.0	27.1
2	0.16	1.16	9.4	7.61	136	8.0	27.1
3	0.16	1.18	10.2	7.62	142	8.0	27.1
4	0.15	1.24	11.5	7.58	136	8.0	27.2
5	0.06	2.44	12.9	7.6	136	8.0	27.1
6	0.17	0.54	4.2	7.64	138	8.0	27.1
7	0.18	0.54	2.5	7.55	140	8.0	27.2
8	0.22	0.46	4.6	7.61	140	8.0	27.3
9	0.15	0.48	3.9	7.64	130	8.0	27.1
10	0.16	1.62	10.8	7.59	126	8.0	27.1
11	0.12	1.19	4.4	7.57	132	8.0	27.3
12	0.11	0.09	2.6	7.6	139	8.0	27.2
13	0.16	1.38	7	763	138	8.0	27
14	0.15	2.36	6.5	7.65	137	8.0	27.1
15	0.13	0.2	3.8	7.62	142	8.0	27.1
Stock	0.17	1.44	5.7	7.63	145	8.0	27.1
2/12/2021							
Tank	NH3 (ppm)	NO2 (ppm)	NO3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)
1	0.12	0.005	2.7	7.61	132	8.0	27.2
2	0.08	0.065	3.9	7.52	138	8.0	27.2
3	0.05	0.41	5.7	7.49	140	8.0	27.3
4	0.06	0.27	4.3	7.63	142	8.0	27.3
5	0.08	0.35	3.6	754	139	8.0	27.2
6	0.12	0.41	3.4	7.56	128	8.0	27.3
7	0.1	0.52	3.9	7.63	140	8.0	27.3
8	0.22	0.13	5.1	7.62	136	8.0	27.1

9	0.12	0.02	4.8	7.65	139	8.0	27.2
10	0.16	0.16	3.6	7.59	138	8.0	27.2
11	0.2	0.021	3.9	7.61	141	8.0	27.2
12	0.51	0.077	3.8	7.6	140	8.0	27.3
13	0.16	0.115	3.1	7.54	137	8.0	27.1
14	0.15	0.066	5.4	7.62	136	8.0	27.1
15	0.15	0.03	3.8	7.54	134	8.0	27.1
Stock	0.12	0.005	2.7	7.61	132	8.0	27.2
6/12/2021							
Tank	NH3 (ppm)	NO2 (ppm)	NO3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)
1	0.05	0.058	1.9	7.5	155	8.0	27.2
2	0.05	0.068	3.3	7.61	154	8.0	27.2
3	0.04	0.033	3.1	7.52	148	8.0	27.2
4	0.1	0.069	4	7.63	149	8.0	27.3
5	0.06	0.02	2.9	7.55	160	8.0	27.2
6	0.08	0.005	3.3	7.59	157	8.0	27.2
7	0.13	0.037	4	7.58	154	8.0	27.1
8	0.09	0.055	4.2	7.62	151	8.0	27.2
9	0.04	0.022	2.5	7.6	160	8.0	27.3
10	0.16	0.044	4.6	7.6	163	8.0	27.2
11	0.1	0.097	1.6	7.54	152	8.0	27.2
12	0.08	0.133	4.4	7.61	140	8.0	27.2
13	0.09	0.045	5	7.58	169	8.0	27.3
14	0.08	0.089	4.7	7.51	168	8.0	27.1
15	0.09	0.046	5.4	7.58	165	8.0	27.2
Stock	0.05	0.058	1.9	7.5	155	8.0	27.2
9/12/2021							
Tank	NH3 (ppm)	NO2 (ppm)	NO3 (ppm)	pH	ALK (ppm)	DO (ppm)	Temp. (C°)

1	0.05	0.063	2	7.49	158	8.0	27.3
2	0.05	0.058	3	7.6	153	8.0	27.2
3	0.06	0.04	2.9	7.53	148	8.0	27.3
4	0.08	0.055	3	7.64	150	8.0	27.2
5	0.05	0.03	2.5	7.59	169	8.0	27.2
6	0.08	0.01	4	7.58	153	8.0	27.4
7	0.08	0.028	5.2	7.69	165	8.0	27.3
8	0.07	0.023	4.5	7.55	162	8.0	27.2
9	0.1	0.015	3.1	7.62	160	8.0	27.2
10	0.12	0.036	4.2	7.64	163	8.0	27.2
11	0.9	0.085	2.3	7.51	158	8.0	27.2
12	0.08	0.093	4.9	7.56	157	8.0	27.2
13	0.08	0.063	5.1	7.54	164	8.0	27.3
14	0.08	0.045	4.6	7.61	165	8.0	27.3
15	0.05	0.023	5.2	7.58	149	8.0	27.2
Stock	0.05	0.063	2	7.49	158	8.0	27.3

Appendix E: Daily mortality during toxicity test

Days	Groups (n = 30)				
	Control	0.5%	5%	10%	20%
1	0	0	0	0	0
2	0	0	0	0	0
3	0	0	0	0	0
4	0	0	0	0	0
5	0	0	0	2	1
6	0	0	0	2	4
7	0	0	1	2	1
8	0	0	2	2	1
9	0	0	1	2	3
10	0	0	1	2	2

11	0	0	1	4	8
12	0	0	0	2	6
13	0	1	0	4	2
14	0	1	2	4	2
15	0	0	2	1	
16	0	0	2	1	
17	0	1	1	1	
18	0	3	0	0	
19	0	0	1	0	
20	0	1	2	1	
21	0	0	1		
22	0	0	1		
23	0	1	2		
24	0	0	1		
25	0	0	0		
26	0	0	1		
27	0	0	0		
28	0	1	1		
Total	0	8	23	30	30

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