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Physico-immunological Characterizations of Exogenous Substances (Palmitoleic Acid and Okadaic Acid) in Bivalves

이매패류에 있어 외인성 화합물(Palmitoleic Acid와 Okadaic Acid)에 의한 생리면역학적 특성 규명

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Abstract

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Palmitoleic acid (PA), an algicidal compound, is used against *Alexandrium tamarense*; however, its impacts on scallops are still unclear. Okadaic acid (OA) is produced by dinoflagellates during harmful algal blooms and is a diarrhetic shellfish poisoning toxin. This study evaluated the effect of harmful algal blooms-related environmental substances on scallops to gain a better understanding of effect of algicide PA and the toxicity of algal toxin OA, and then help improve the intensive breeding and long-term sustainability of scallop farming. Therefore, in the current investigation, various immunological parameters (total hemocyte counts (THC), reactive oxygen species (ROS), malondialdehyde (MDA), glutathione (GSH), lactate dehydrogenase (LDH), and nitric oxide (NO), superoxide dismutase (SOD), acid phosphatase (ACP), alkaline phosphatase (ALP), lysozyme activities,

and total protein content) were assessed in the hemolymph of scallops at 3, 6, 12, 24, and 48 h post-exposure (hpe) to different concentrations of PA (20, 40, and 80 mg/L) and OA (50, 100, and 500 nM). Moreover, the expressions of immune-related genes (*CLT-6*, *FREP*, *HSP90*, *PGRP*, *MT*, *Cu/ZnSOD*, *MnSOD*, *PrxV*, and *BD*) were also investigated after exposing bay scallop for 3, 6, 12, 24, and 48 h to any of three different concentrations of PA and OA.

Results showed that the lysozyme activity was decreased in scallop treated with PA at 12, 24 and 48 hpe, as compared to control. Although, the SOD activity was higher in all PA treated groups but the significant increment was observed only in 20 mg/L of PA treated group at 12, 24 and 48 hpe. The ACP activities were enhanced from 6–48 hpe in 80 mg/L of PA treated group, as compared to control. The total protein level was enhanced in all the PA treated groups, especially in 40 mg/L of PA treatment group at 3 hpe and in 80 mg/L of PA treatment group at 6, 12, 24 and 48 hpe. THC decreased in PA-treated groups, whereas ALP increased significantly in all of the PA treatment groups at 3 hpe, after which it significantly decreased. The LDH and NO levels were significantly enhanced in the high and medium concentration group. Notably, the GSH level increased in all PA treatment groups at each time interval. Our study also revealed that after treatment with different concentrations of PA, variable effects on the expression of genes involved in the immune system response were observed. Our research demonstrated that immersing scallops in PA may disrupt the endocrine system or immune responses. Therefore, the present study highlights the potential risk of using the PA as an algicide to control algal bloom outbreaks in the marine environment.

Moreover, the current investigation revealed the effects of exposure to OA on

the immune responses of bay scallop. Results showed that SOD and ACP activities were decreased between 24–48 hpe. The ALP, lysozyme activities, and total protein levels were also modulated after exposure to different concentrations of OA. The ROS, MDA, and NO levels and LDH activity were enhanced after exposure to different concentrations of OA; however, both THC and GSH decreased between 24–48 hpe. The expression of immune-system-related genes was also assessed at different time points during the exposure period. Therefore, these results provide a better understanding of the response status of bivalves against DSP toxins.

Overall, our investigation suggest that the exposure to algicide PA and algal toxin OA had negative effects on the antioxidant and non-specific immune responses, and even disrupted the metabolism of bay scallops, making them more vulnerable to environmental stress-inducing agents;

Key words: Mollusks, Immunological Response, Bay Scallop, Algicide, Algal Toxin, Palmitoleic Acid, Okadaic Acid

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Abbreviations

PA	Palmitoleic Acid
ACP	Acid Phosphatase
SOD	Superoxide Dismutase
MDA	Malondialdehyde
ROS	Reactive Oxygen Species
HABs	Harmful Algal Blooms
ERA	Environmental Risk Assessment
EC_{50}	Half maximal Effective Concentration
MT	Metallothionein
BD	Big Defensin
AMPs	Antimicrobial Peptides
OD	Optical Density
qPCR	Rreal-time Quantitative PCR
Ct	Threshold cycle
SD	Standard Deviation
THC	Total Haemocyte Counts
ALP	Alkaline Phosphatase Activity
NO	Nitrite Oxide
GSH	Glutathione
LDH	Lactate Dehydrogenase
DTNB	2-Nitrobenzoic Acid

hpe	Hours post-exposure
FREP	Fibrinogen-related Protein
PGRP	Peptidoglycan Recognition Protein
HSP90	Heat Shock Protein 90
Cu/ZnSOD	Copper/Zinc Superoxide Dismutase
MnSOD	Manganese Superoxide Dismutase
ONOO–	Peroxynitrite Anion
NAD+	Nicotinamideadenine Dinucleotide
BkF	Benzo(k)fluoranthene
OA	Okadaic Acid
PSP	Paralytic Shellfish Poisoning
DSP	Diarrhetic Shellfish Poisoning
ASP	Amnesic Shellfish Poisoning
NSP	Neurotoxic Shellfish Poisoning
ASP	Azaspiracid Shellfish Poisoning
DTX	Dinophysistoxins
LPS	Lipopolysaccharide
Mn SOD	Manganese Superoxide Dismutase
Fe SOD	Iron Superoxide Dismutase
CLT	C-type Lectins
DMSO	Dimethyl Sulfoxide
PrxV	Peroxiredoxin Atypical 2-Cys
PGN	Peptidoglycan

General introduction

Harmful algal blooms (HABs), caused by pollution of water bodies and global climate change, can result in ecological and economic losses in coastal areas as they cause mass mortality of cultivated animals from the algal toxins they produce [1]. In order to control HABs, bioactivie componds are considered to be friendly to environment such as palmitoleic acid (PA), which was secreted from *Vibrio* sp. BS02, and recommended as an algicide during HABs as the positive effect on *Alexandrium tamarense*, which is a notorious toxic species of harmful algae, and can cause serious economic loss, human illness, and even death as a result of the production of paralytic shellfish poison [2]. However, the potential impacts of the algicidal compound PA, which is used for controlling a harmful algal bloom in an aquaculture setting, on wind or cultivated animals remains unknown [3].

Moreover, during HABs or after harmful algal cells lysis by algicide, a variety of marine phycotoxins are released into the sea, including paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), amnesic shellfish poisoning (ASP), neurotoxic shellfish poisoning (NSP), and azaspiracid shellfish poisoning (AZP) [4]. The DSP toxins include okadaic acid (OA), the dinophysistoxins-1 (DTX-1), DTX22, DTX-3 and their derivative forms which can be ultimately consumed by humans, causing a variety of gastrointestinal and neurological illnesses through food chain are shellfish toxins [4]. However, the direct impacts of purified toxins OA on physiological responses in marine species have rarely been investigated [5].

Bivalves are particularly affected during HAB events as they accumulate high

levels of algal toxins in their tissues through their sessile and filter-feeding habits [1]. To date, there is little data on the effects of algicide or algal toxins on scallops. Moreover, most of these studies investigated the effects of phycotoxins by feeding or exposing mussels and oysters to harmful algae, which secrete various phycotoxins and other metabolites. However, the direct impacts of purified toxins on physiological responses in scallops have rarely been investigated [4]. Scallops are a cosmopolitan family of molusks, some of which are widely farmed by the aquaculture industry for food and have important economic value. The bay scallop (*Argopecten irradians*) was introduced and has been cultured in the coastal provinces of China for more than 30 years, and now bay scallop farming is also suffering from various substances from the environment as they accumulate high levels of algal toxins in their tissues through their sessile and filter-feeding habits [6].

The multiple factors and complexity of feeding in the effects of exposure to exogenous substances, especially during natural HAB outbreaks, are particularly problematic for the safe and efficient culturing of bivalves, such as scallops, for human consumption. This study evaluated the effect of PA and OA on scallops to gain a better understanding of the toxicity of different exogenous substances and help improve the intensive breeding and long-term sustainability of scallop farming [6]. We compared the immunotoxicity parameters (THC, ROS, MDA, NO, GSH, and LDH) and non-specific immune parameters (THC, SOD, ALP, ACP, lysozyme, phagocytic activities, and total protein level) in the hemolymph of bay scallops following exposure to different concentrations of PA and OA to understand the early physiological and immunological responses of bay scallops to the toxicity of

exogenous substances and provide information on its molecular mechanism of the responses of bay scallops. In addition, we examined the transcription levels of several immune-system-related genes (*PrxV*, *MT*, *PGRP*, *BD*, *CLT-6*, *FREP*, *HSP90*, *MnSOD*, and *Cu/ZnSOD*). To our knowledge, this is the first study comparing the effect of purified marine toxin OA and algicide PA on bay scallop physiological and immunological responses and the expression of immune-system-related genes.

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Literature Review

A. Mollusks

A.1. Bivalve mollusks

Bivalve culture is steadily growing in importance in the aquaculture sector. However, populations of cultivated mollusks, mainly oysters, mussels, clams and scallops, suffer from severe mortalities [1]. Bivalves widely distributed in the seaward end of glaciers, subtropical and temperate estuarine bays, and tropical shallow seas [2], where harmful algal blooms (HABs) frequently happened. Many scallop species are highly prized as a food source, and some are farmed by the aquaculture industry. Recent years, investigations in scallop immunity are increasing continually for their economic importance and their key position in animal phylogeny and evolution. In case of scallop aquaculture, with an annual output of about 1 million metric tons, is one of the most important sectors of China's mariculture industry. Most of the production comes from two species: the local zhikong scallop (*Chlamys farreri*) and the bay scallop *Argopecten irradians* [3], which was successfully introduced to China from North America in 1983, and has become a main marine cultured shellfish species of China. It has been utilized as a cultured human food source for many years and thereby considered as economically important species [4].

Bivalves potentially accumulate different chemicals in their tissues as they filtering large volumes of water, processing microalgae, bacteria, sediments, and particulates. They are regarded as crucial indicators of aquatic pollution. The

accumulation and persistence of toxicity in bivalves is species-dependent and varies according to the concentration of the bloom and rates of feeding and toxin elimination in the shellfish [5]. When a bivalve is exposed to a toxic or noxious particle, shell-valve closure and reduced filtration may constitute the first response and may serve to minimize contact with the soft tissues .

A.2. Innate immune system of bivalves

Mollusks, including bivalves, are unique in terms of health management. They have few means to control and reduce the impact of pathogens. Bivalves are belong to invertebrates, and lack a specific immune response and immunological memory, therefore they rely totally on their innate immune system to defense diseases. As bivalves do not possess lymphocytes and can not produce antibodies, vaccination cannot be used to protect them against infectious diseases. Moreover, the preventive or therapeutic use of drugs is highly restricted, since bivalves farming is carried out in the natural marine environment [1].

Bivalves cellular defense mainly rely on the hemocytes through infiltration, aggregation, encapsulation, cytotoxic reactions, and phagocytosis of foreign particles. Several biologically active molecules have been revealed in the hemolymph of bivalve mollusks. They are generally classified into two categories: serologically active (opsonins, lysins, agglutinins, antimicrobial factors, lysozymes) and lysosomal enzymes (aminopeptidases, β-glucuronidases, acid phosphatase, alkaline phosphatase, α-mannosidase, esterases, and peroxidases) [1].

A.3. Biological costs of adverse environmental changes

Chemico-physical factors and biological interactions continually affect mollusks physiology and behavior. Conversely, the mollusks response to external changes disrupting functional homeostasis depends on the genetic uniqueness of individuals and populations, named finely tuned gene expression [1]. Some of the immediate reactions induced in organisms by physical or chemical stress may include physiological adjustments, recovery from structural damage, the overall metabolism and individual growth rates of bivalve species [1, 6, 7]. Long-term consequences depend on the continuous thermal, chemical, climatic, and biotic changes and are mainly driven by genotype selection, ultimately prevailing on the effects of mutation, migration, and stochasticity [8]. A general problem in obtaining a better understanding of the stress response in bivalves is the complete lack or fragmentary nature of our knowledge about their genes, proteins, and regulatory networks [1].

A.4. Responses to abiotic stress of bivalves

Heat shock proteins are widely exist in a range of living organisms and play a important role in the cellular stress response, acting as protein chaperones in normal development and in response to various thermal and chemical stressors, stabilizing signaling related proteins and assisting protein folding, unfolding, and degradation. During organism development, hsp90 influences morphogenetic pathways, channeling phenotypic variation in spite of destabilizing random events [9]. In the mussel *Mytilus galloprovincialis* and abalone *Haliotis rufescens*, different levels of hsp70 isoforms, hsp60, and hsp90 have been observed following

thermal and chemical stress [10]. Species- and tissue-specificity of heat-shock response and cross-protection against toxicant-induced stress have been reported in bivalves [11], such as the small hsp27 gene significantly down regulated in the digestive gland of mussels after expose to toxicant cocktails in both experimental and field conditions [12].

A.5. Responses to toxicant stress of bivalves

The hysicochemical properties, bioavailability, and potential toxicity of environmental pollutants for organisms with diverse life traits and reproductive strategies is very differently. The most potent toxicants could impact biological function even at very low doses. Exposure to metals, polycyclic aromatic hydrocarbons, organochlorine and organophosphate pesticides, polychlorinated biphenyls, dioxins, and other xenobiotics can increase the physiological levels of such reactive species, disrupt vital processes, and define oxidative stress in living organisms [13]. Transition metals, some pesticides, quinones, and other compounds can undergo redox cycling and consequently enhance the oxidative stress [14]. Superoxide dismutase, GSH peroxidases, reductases, metallothioneins (MTs), and catalase enzyme activity have been measured in bivalve tissues to evaluate oxidative stress [13]. MTs are low molecular weight, display oxyradical scavenging activity and high affinity for free metal ions such as Zn, Cd, Cu, and Hg, and cause metal sequestration and detoxification. Transcription of MTs frequently occurs by trans-activation of metal-response promoter elements in response to anoxia and oxidative stress [15].

Compared to fish and mammals, bivalves have limited functionalization of

xenobiotic molecules, active monoxygenation of aromatic amines and poor monoxygenation of polycyclic aromatic compounds [1, 16]. The existence of CYP4 isoforms has been reported in *M. galloprovincialis* but the expression and AhR-mediated inducibility of cytochrome P450-like proteins in bivalves require further study [17]. Among the phase II conjugating enzymes, gene expression of glutathione S-transferase (GST) has been studied in various bivalves [18, 19]. The detoxifying role of glutathione and GST was confirmed by the substantial gene expression of GST in mussel gills, which is the first target tissue of water contaminants.

B. Algicide

B.1. Algicide classification

The initiation, development, sustaining and die-off of harmful algal blooms (HABs) comprise a complex process involving multiple chemical, physical and biological factors. The control of HABs has been the research focus. HABs control techniques were based on the physical [20, 21], chemical [22] and biological aspects of the harmful algae [23]. Physical control methods include: removing cultivated animals from the HABs zones, or building some form of barrier to separate them from the harmful algae; destroying the algae with electro-magnetic waves or supersonic waves; gathering the algae with pumps [23]. Chemical methods involve: using minerals to absorb and sink the algae; using organic chemicals (for example, compounds of organic amines) or inorganic chemicals (such as bluestone, potassium permanganate, chlorine, sodium hypochlorite, and ozone) as algicides [23]. Biological methods are including using of algicidal

bacteria and macro algae; harmful algae predators; and nutrient competition by other aquatic vegetation [23].

B.2. Special algicidal bacterium

Chemical methods are considered to be potentially dangerous since chemical agents can cause serious secondary pollution, although effective in controlling HABs [24], as indiscriminately killing other aquatic organisms, altering marine food chains and eventually impact natural fish communities [25]. While the high cost of physical approaches may be impractical, therefore, biological agents are now being considered as potential suppressors in controlling the outbreak of algal blooms [26]. Recently, this has become a research hotspot based on the advantages of its species-specificity, efficiency and environment-friendliness [27]. In aquatic ecosystems bacteria act an essential role in nutrient regeneration and energy transformation [24], and previous studies have revealed the existence of bacteria capable of inhibiting or suppressing HABs in aquatic environments [28, 29]. These algicidal bacteria effectively attack on the target algal species directly or indirectly, which the direct attack requires cell-to-cell contact, while the indirect attack involves the secretion algicidal substances [26].

B.3. Bioactive algicidal compound

Some previous investigation reported an algicidal bacterium *Vibrio sp*. BS02 with strong activity against the toxic dinoflagellate *A. tamatense*, which was isolated from a mangrove area in Fujian Province, China. Alga-lysing characterization of this bacterium suggested that the algicidal activity was due to an

extracellular bioactive compound [30]. Therefore, it was demonstrated that the algicidal substance secreted by the BS02 was a fatty acid—the bioactive compound [26]. The algicidal bioactive compound–palmitoleic acid (PA) secreted from *Vibrio* sp. BS02 was recommended used as a novel algicide. As an algicide, it has a half maximal effective concentration (EC_{50}) value of 40 mg/L until 48 h and suppresses algal growth at concentrations higher than 20 mg/L. PA almost completely inhibited algal growth at 80 mg/L, and the lysis of *A. tamarense* cells was observed after exposure to PA under light microscopy, and it also can effectively inhibit the growth of other harmful algaes such as *A. minutum*, *Asterionella japonica* and *Heterosigma akashiwo* [26]; however, further information about the potential impacts of the algicidal compound PA, which is used for controlling a harmful algal bloom in an aquaculture setting, on wind or cultivated animals remains unclear.

C. Marine toxins

C.1. Shellfish poisoning classification

HABs result from a combination of physical, chemical, and biological mechanisms and their interactions with other components of the food web that are mostly poorly understood, and also have various negative impacts on public health and threaten aquaculture industry, as they cause mass mortality of cultivated animals from the algal toxins they produce [31]. The main marine phycotoxins which can be ultimately consumed by humans, causing a variety of gastrointestinal and neurological illnesses through food chain are shellfish toxins, including paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), amnesic

shellfish poisoning (ASP), neurotoxic shellfish poisoning (NSP), and azaspiracid shellfish poisoning (AZP) [32, 33].

C.2. Amnesic shellfish poisoning

Domoic acid, which was isolated from the red algae *Chondria armata*, was identified as a neurotoxic agent causing ASP, and it is produced by some strains of diatoms, such as *Pseudo-nitzschia spp.*, *Nitzschia navis-varingica*, and *Amphora coffeaeformis* [34]. Domoic acid from algal blooms may accumulate from algal blooms not only in shellfish but also in fish such as sardines and anchovies, even through the food chain, it can be transferred into sea birds, sea lions, and other animals, causing diseases and death after eating toxic fish.

C.3. Neurotoxic shellfish poisoning

NSP results from accumulation of microalgal toxins known as brevetoxins and analogous natural products in shellfish and other marine species. People ingesting toxins from contaminated shellfish do not die but exhibit gastrointestinal and neurological symptoms such as nausea, vomiting, slurred speech, loss of coordination, and paresis of facial muscles. Neurological symptoms can progress to partial paralysis. Brevetoxins can also suppress the immune systems of humans and other animals, and then increase the danger of infections and other secondary diseases [34].

C.4. Paralytic shellfish poisoning

PSP toxin is another of the most potent toxins. Saxitoxin was first isolated

from the butter clam, *Saxidomus giganteus*, which were found in many species of edible mollusk and pufferfish [35]. PSP-toxins could permeate into edible mollusks from dinoflagellates *Gymnodinium, Alexandrium,* and *Pyrodinium spp*. through the food web, and then accumulate in certain body components of mollusks, where they are preserved for several weeks.

C.5. Diarrhetic shellfish poisoning

DSP is induced by a whole series of microalgal toxins, including okadaic acid, pectenotoxins, dinophysistoxins, and yessotoxins, which contain polyether structures and can accumulate in shellfish. Okadaic acid was first isolated from the sponges *Halichondria okadai* and *H. melanodocia* [36]. While it was later established that it enters sponges from the dinoflagellates of the *Prorocentrum* and *Dinophys genera* [37], which it often participate in symbiotic relationships with different macrophytic algae and benthic marine invertebrates. That is the reason why okadaic acid easy infiltrate into planktivorous mollusks and other marine animals through the food chain. Okadaic acid has an ability to inhibit protein serine/threonine phosphatases due to the interaction with the PP1- and PP2A-catalytic domains of these enzymes. Okadaic acid also inhibits protein phosphatases of both human and animal enzymes, as well as yeasts and plants equally [34]. The half-maximal inhibitory concentrations (IC_{50}) against the PP2A and PP1 types of protein phosphatases are 0.2–1.0 and 60–500 nM, respectively [38]. In addition, okadaic acid is known as powerful tumor promoter.

C.6. Azaspiracid shellfish poisoning

ASP was discovered when it occurred in people in the Netherlands after consumption of mussels from Ireland. As a separate group of toxins, azaspiracids were isolated from contaminated mollusks when one of their spirocycles was found to contain nitrogen instead of oxygen [34]. It was found that these compounds are produced by a newly identified microalgal species *Azadinium spinosus* gen. et sp *nov*. [39] and related species of Dinophyceae belonging to the same genus (*Azadinium*). Although azaspiracids have never been included in the DSP group, they cause severe and protracted diarrhea in humans,.

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Chapter I

Effect of the Algicide Palmitoleic Acid on Immune Functions of Bay Scallop (*Argopecten Irradians*)

Abstract

Palmitoleic acid (PA), an algicidal compound, is used against *Alexandrium tamarense*; however, its impacts on scallops are still unclear. Therefore, we investigated the impacts of effective algicidal concentrations (20, 40, and 80 mg/L) of PA on immune responses in *Argopecten irradians.* Various immune parameters including acid phosphatase (ACP) activity, superoxide dismutase (SOD), lysozyme, phagocytic activity, total protein, malondialdehyde (MDA) level, and reactive oxygen species (ROS) production and the expression of immune-related genes (*PrxV*, *CLT-6*, *MT,* and *BD*) were measured at 3, 6, 12, 24, and 48 h post-exposure (hpe) to PA. Lysozyme activity was lower in scallops at 12–48 hpe to 80 mg/L. SOD, ACP activity, ROS production, the total protein, and MDA level was higher at 12 to 48 hpe with different concentrations of PA. Phagocytic activity increased at 6-12 hpe to 40–80 mg/L of PA, but decreased at 24–48 hpe. The expressions of genes *PrxV*, *CLT-6*, *MT* and *BD* down-regulated at 3 hpe were observed, while differential expressions from 6–48 hpe with different concentrations of PA. The present study demonstrated that immersing *A. irradians* in PA at effective concentrations could result in differential effects on non-specific immune responses and expressions of immune-related genes.

Keywords: Algicide, *Argopecten irradians*, Immune response, Palmitoleic acid, Harmful algal blooms.

1.1. Introduction

Since the 20th century, population explosions and the rapid development of agriculture and industry have accompanied an apparent global increase in the occurrence, area, and harm (both ecological and anthropogenic) caused by harmful algal blooms (HABs). As chemical approaches are considered potentially dangerous for aquatic life and create environmental hazards, biological agents, including protozoa, viruses, and macrophytes, bacteria (e.g: bacterial secondary metabolites),are now being considered as potential suppressors for controlling the outbreak of HABs [1-3]. Several studies also revealed the existence of certain bacteria capable of inhibiting or degrading algal blooms in marine environments [4, 5]. Very few studies have focused on the isolation of algicidal compounds from these bacteria. *Alexandrium tamarense* as a harmful algal species in marine environments can produce paralytic shellfish toxin. This toxin have heavily affected shellfish resources which leads to serious economic loss in shellfish aquaculture industry, and also causes human illness, even death [1, 6]. Recently, an algicidal bioactive compound–palmitoleic acid (PA) secreted from *Vibrio* sp. BS02 was used for controlling *A. tamarense* in Xiamen Sea Area, China [1]. As an algicide, it has a half maximal effective concentration (EC_{50}) value of 40 mg/L until 48 h and suppresses algal growth at concentrations higher than 20 mg/L. PA almost completely inhibited algal growth at 80 mg/L, and the lysis of *A. tamarense* cells was observed after exposure to PA under light microscopy, and it also can effectively inhibit the growth of other harmful algaes such as *A. minutum*, *Asterionella japonica* and *Heterosigma akashiwo* [1]; however, the potential impacts of the algicidal compound PA, which is used for controlling a harmful

algal bloom in an aquaculture setting, on wind or cultivated animals remains unknown.

Suspension-feeding bivalves naturally ingest most microalgae, and, thus, are exposed to a variety of toxic components. The accumulation and persistence of toxicity in bivalves is species-dependent and varies according to the concentration of the bloom and rates of feeding and toxin elimination in the shellfish [7]. When a bivalve is exposed to a toxic or noxious particle, shell-valve closure and reduced filtration may constitute the first response and may serve to minimize contact with the soft tissues [4]. Scallops are a cosmopolitan family of bivalves, widely distributed in some water areas including the seaward end of glaciers, subtropical and temperate estuarine bays, and tropical shallow seas [8], where HABs frequently happened. Many scallop species are highly prized as a food source, and some are farmed by the aquaculture industry. In recent years, interest in scallop immunity has continued to increase due to their economic importance and their key position in animal phylogeny and evolution. Therefore, scallops are good candidates for immunological studies. Scallop aquaculture, with an annual output of about 1 million metric tons, is one of the most important sectors of China's mariculture industry. Most of the production comes from two species: the local zhikong scallop (*Chlamys farreri*) and the bay scallop *Argopecten irradians* [9], which was successfully introduced to China from North America in 1983, and has become a main maricultured shellfish species of China. It has been utilized as a cultured human food source for many years and thereby considered as economically important species [6].

It is generally assumed that scallop, an invertebrate, lacks the complexity of

the adaptive immune system, relying solely on innate immunity mediated by both cellular and humoral components [10]. Cellular immune reactions, including encapsulation and phagocytosis, are performed by different haemocytes, and humoral immune responses consist of reaction cascades for microbe recognition, signal transduction, and immune effector productions [11]. During phagocytosis, large amounts of reactive oxygen species (ROS) are generated to kill the internalized bacteria, which is important for invertebrate survival [12], but a series of damage will occur when the generation of ROS is too much [13]. Production of excessive ROS and other pro-oxidants damages unsaturated lipids, breaks DNA bonds, as well proteins, amino-acids and carbohydrates [14]. Malondialdehyde (MDA) represents membrane lipid peroxidation status and always is used as a marker for the extent of oxidative damage [14]. However, in scallops, several antioxidant enzymes, such as superoxide dismutase (SOD) which is considered to be the first and most important line of defence against ROS and protects tissues from oxidative damage [15], catalase and extra cellular glutathione peroxidase have been identified, and all of them are involved in the host response against microbe challenge and environmental stress [8]. In addition, some antimicrobial compounds in humoral immune responses, like lysosomal enzymes, acid phosphatase (ACP) also participate in degradation of foreign proteins, carbohydrates and lipids [1]. Therefore, phagocytosis, ACP, and SOD are essential components in the understanding of the immune response of scallops [12]. Total protein also plays a significant role in the immune response, and the modification of total protein level can provide information from a whole-scope viewpoint on the processes of protein synthesis, post translation modifications, protein degradation,

and the interaction between proteins or other molecules, in response to environmental stress in marine animals [16, 17].

To our knowledge, the effect of algicide PA on the immune responses of bay scallop has not been reported. Therefore, the present study investigated the interaction between PA and scallop immune responses, and aimed to reveal impacts and potential risk of using algicide PA on non-specific immune responses of bay scallops *A. irradians*. Healthy scallops were stimulated by immersion in graded levels of PA for short durations, and temporal profiles of immune parameters were assessed. Furthermore, we studied the effect of PA on the expression of immune-related genes.

1.2. Materials and methods

1.2.1. *Palmitoleic Acid*

Analytical-grade palmitoleic acid (PA) was obtained from Sigma-Aldrich Co. LLC (Sigma, USA) and stored at 4 ℃ in a refrigerator until use.

1.2.2. *Animals*

Bay scallops, *A. irradians*, averaging 60–70 mm in shell length, were collected from the Noryangjin fisheries wholesale market (Seoul, South Korea) and maintained in lantern nets suspended in 800-L-capacity tanks containing filtered and aerated sea water to acclimatize to laboratory conditions (temperature: 10 ± 1°C; salinity: 30 ± 0.1‰) for two weeks. Seawater was changed every day. Scallops were fed commercial shellfish diet (Instant Algae® Shellfish Diet; Reed Mariculture Inc., Campbell, CA, USA) at a rate of approximately 1.2×10^{10} algae

cells per scallop per day.

Bay scallops were randomly divided into a control group (without PA) and three treatment groups (with PA). There were three replicates for each treatment and control group. The treatment groups were treated with three concentrations (20, 40, and 80 mg/L) of PA that were reported as the minimum algicidal dose, EC_{50}, and maximum effective algicidal dose against *A. tamarense*, respectively [1]. Three scallops from each replicate were randomly collected at 3, 6, 12, 24, and 48 h post-exposure (hpe). One mililiter of haemolymph was collected from adductor muscle using a 1-mL sterile syringe fitted with a 22-gauge needle within 1 min of removing a scallop from the tank. At each time point, an equal volume of haemolymph from three scallops in each replicate was pooled to reduce inter-individual variation. Individual scallops were sampled once to avoid repeated bleeding and/or handing stress. A 100-μL sample of haemolymph from each replicate was used for RNA extraction. Then, another 100-μL haemolymph from each replicate was centrifuged at 750 × *g* for 3 min to collect the serum, which was then stored at –80 ℃ until determining humoral immune parameters. The rest of haemolymph maintained on ice until use for the measurement of phagocytic activity and reactive oxygen species production.

1.2.3. *Measurement of Non-Specific Immune Responses*

1.2.3.1. Measurement of superoxide dismutase activity

Superoxide dismutase (SOD) activity was determined according to the method described by Ōyanagui [18] using SOD kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) following the manufacturer's instructions. The optical

density value (OD value) was measured at 550 nm. One unit of SOD was defined as the amount required to inhibit the rate of xanthine reduction by 50% in a 1-mL reaction system. Specific SOD activity was expressed as SOD units per mL of serum.

1.2.3.2. Measurement of Malondialdehyde

Malondialdehyde (MDA), a degrading product of lipid peroxidation known as thiobarbituric acid-reactive substance, was determined according to the thiobarbituric acid method using a MDA test kit (Nanjing Jiancheng Bioengineering Institute, China).

1.2.3.3. Measurement of acid phosphatase activity

Acid phosphatase activity (ACP) in serum was spectrophotometrically measured using disodium phenyl phosphate as a substrate [19] using an acid phosphatase detection kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). One unit of ACP activity was defined as the amount of enzyme in 100 mL of serum necessary to produce 1 mg of nitrophenol for 30 min at 37 ℃.

1.2.3.4. Measurement of lysozyme activity

Lysozyme activity was measured using lysozyme kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) following the manufacturer's instructions. One unit of lysozyme activity was defined as the amount of serum lysozyme that caused a decrease in absorbance by 0.001 per min at 530 nm.

1.2.3.5. Measurement of the total protein content

The total protein concentration in scallop serum was determined using kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) following the manufacturer's instructions.

1.2.3.6. Measurement of phagocytic activity

Phagocytic activity of phagocytic haemocytes was determined using the previously described method of Xue *et al.* [20]. Two hundred haemocytes were counted. Phagocytic activity, defined as the phagocytic rate (PR), was expressed as: PR = (phagocytic haemocytes / total haemocytes) × 100.

1.2.3.7 Measurement of reactive oxygen species

Reactive oxygen species (ROS) production was measured using reactive oxyg en species kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) follow ing the manufacturer's instructions. Fluorescence, quantitatively related to the ROS production of haemocytes without any stimulation, was measured at 500–530 nm by a fluorescence microplate reader. Fluorescence was expressed in arbitrar y units (A.U.).

1.2.4. *RNA Extraction and Reverse Transcription*

Total RNA was extracted from haemolymph using TRIzol Reagent (CWBio, Beijing, China). The quality and purity of RNA was assessed by spectrophotometry, and the 260:280 ratios were 1.8–2.0. Afterwards, genomic DNA contamination was removed using DNase I (Promega, Madison, WI, USA). cDNA was synthesized

using a PrimeScript™ RT Reagent Kit (TaKaRa Bio, Japan) following the manufacturer's instructions. The resulting cDNA was stored at –80 ℃.

1.2.5. *Real-Time Quantitative PCR Analyses of Gene Expression*

The expression of immune-related genes, *PrxV*, *CLT-6*, *MT*, and *BD*, was carried out using real-time quantitative PCR (qPCR) (Qiagen, Hilden, Germany). All qPCR reactions were performed using SYBR Premix Ex Taq™ Perfect Real-Time Kits (TaKaRa Bio, Japan) and were conducted using a QiagenRotor-Gene Q RT-PCR Detection System (Qiagen, Hilden, Germany). Gene expression was normalized using the housekeeping gene *β-actin*. PCR primer sequences used for qPCR are listed in Table 1 [21-24]. The reaction mixture included 10-μL SYBR Premix Ex Taq™, 1 μL of the forward and reverse primer (10 mM), and 1-μL cDNA. The remaining volume was filled with ultra-pure water to a final total volume of 20 μL. The reaction conditions and cycle index were conducted at 95°C for 10 min followed by 40 cycles at 95 ℃ for 45 s, 56 ℃ for 45 s and 72 ℃ for 30 s. After the amplification phase, a melting curve analysis was conducted to eliminate the possibility of non-specific amplification or primer dimer formation. A standard curve was created from serial dilutions of sample cDNA. A standard curve was drawn by plotting the natural log of the threshold cycle (Ct) against the number of molecules. Standard curve of each gene was run in duplicate and three times for obtaining reliable amplification efficiency. The correlation coefficients (R^2) of all standard curves were >0.99 and the amplification efficiency were between 90 and 110%. The relative expression ratios of the target gene in the treatment group versus those in the control group were calculated according to the following

formula: Fold changes = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct$ = [Ct (treatment group) – Ct (treatment *β-actin*)] – [Ct (control group) – (control *β-actin*)] [25]. In all cases, each PCR was performed using three replicates.

1.2.6. *Statistical Analysis*

Normality and homogeneity of variance were tested using Kolmogrov–Smirnov and Cochran's tests, respectively. All percentage data were arcsine-transformed, and the data were subjected to one-way ANOVA. Values are expressed as the arithmetic mean ± standard deviation (SD). Differences were determined using Duncan's test in SPSS statistical software version 19.0 (IBM Corp., Armonk, NY, USA) with $P<0.05$ indicating statistical significance.

1.3. Results

1.3.1 *Non-Special Immune Responses*

1.3.1.1. SOD activity

In the present work, SOD activity was increased ($P<0.05$) only at 20 mg/L of PA at 12, 24, and 48 h post-exposure (hpe) compared to control (Figure 1.1 A).

1.3.1.2. MDA level

The MDA level at 3 and 6 hpe was significantly increased in all PA treated groups, however from 12 to 48 hpe only in 40 and 80 mg/L treated groups (Figure 1.1 B).

1.3.1.3. ACP activity

Although ACP activities at 6 hpe were higher ($P<0.05$) only in the group received 80 mg/L of PA, at 12, 24, and 48 hpe were higher ($P<0.05$) in all PA treated groups than control (Figure 1.1 C).

1.3.1.4. Lysozyme activity

Lysozyme activity was lower ($P<0.05$) in 80 mg/L of PA treatment group after 12, 24, and 48 hpe (Figure 1.1 D).

1.3.1.5. The phagocytic activity

The phagocytic activity was significantly different only at the two higher concentrations (40 and 80 mg/L of PA) with an increase at 6 and 12 hpe and a decrease at 24 and 48 hpe (Figure 1.1 E)

1.3.1.6. ROS level

Reactive oxygen species (ROS) production in current investigation was increased ($P<0.05$) at 12, 24, and 48 hpe with 20, 40 and 80 mg/L of PA, at 6 hpe with 40 and 80 mg/L of PA and at 3 hpe only with 80 mg/L of PA (Figure 1.1 F)

1.3.1.7. The protein level

As shown in the Figure 1.1 G, the protein levels were higher ($P<0.05$) in groups treated with 80 mg/L of PA at each time interval and with 40 mg/L at 3 hpe .

1.3.2. *Expression of immune-system-related genes*

1.3.2.1. Expression of PrxV gene

PrxV expression was down-regulated (*P<0.05*) at the initial and final hour of PA treatment; however, *PrxV* expression was higher (*P<0.05*) in PA treatment groups at 6–24 h, except 6 hpe with 20 mg/L of PA, than in the control group, and declined rapidly at 48 hpe (Figure 1.2 A)

1.3.2.2. Expression of CLT-6 gene

Scallops stimulated with 20 mg/L of PA had significantly higher (*P<0.05*) *CLT-6* expression at each time interval; however exposure with 40 mg/L of PA exhibited higher *CLT-6* expression only at 6 and 12 hpe than control, and the highest expression was observed at 12 hpe. The 80 mg/L of PA attenuated (*P<0.05*) the expected expression of *CLT-6* mRNA at 3, 6, 24, and 48 h (Figure 1.2 B)

1.3.2.3. Expression of MT gene

In the present study, similar to the *CLT-6* gene expression, scallops treated with 20 mg/L of PA had a higher (*P<0.05*) *MT* expression from 6–48 hpe and with 40 mg/L of PA only at 12 hpe than was seen in the control, and the highest expression was observed at 12 hpe (Figure 1.2 C).

1.3.2.4. Expression of BD gene

BD expression in haemolymph of scallops treated with 20 mg/L of PA was significantly higher (*P<0.05*) at 6 to 24 hpe, and then decreased (*P<0.05*) rapidly at 48 hpe. However, *BD* expression was higher at 12 and 24 hpe, but attenuated (*P<0.05*) at 3, 6, and 48 h in groups treated with either 40 or 80 mg/L of PA (Figure 1.2 D).

1.4. Discussion

Nowadays, environmental risk assessment (ERA) is widely accepted and consists of four main steps: hazard identification, a dose-response assessment, an exposure assessment, and risk characterisation. In earlier study, palmitoleic acid (PA) was shown to have an EC_{50} value of 40 mg/L until 48 h against *A. tamarense*, and suppressed algal growth at a concentration higher than 20 mg/L [1]. PA almost completely inhibited algal growth at 80 mg/L, and selectively inhibited the growth of other harmful algaes. An effective immune response is essential for maintaining the health of an organism, and may subsequently affect growth, reproduction, and ultimately, survival [26, 27]. Therefore, the present study investigated how exposure to effective algicidal concentrations of an algicidal compound–PA alters non-specific immune responses and the expression of immune-related genes in *A. irradians*. The results showed that PA could modulate non-specific immune responses and the expression of a series of immune-related genes in bay scallop haemolymph. Therefore, the present work contributes towards the first step in the ERA procedure through the identification of effects of algicide PA on the bay scallop. This is an essential part of establishing the risks associated with developing and using algicides for controlling and inhibiting HABs.

The rapid adaptation of a species to sudden changes in environmental oxygen content depends on its ability to increase its antioxidant production capacity [28]. Among these antioxidant systems, SOD is the first and most important defensive parameter [29]. SOD has been used as a biomarker for monitoring environmental pollution [30]. Malondialdehyde (MDA) is closely related to membrane lipid

peroxidation status, therefore MDA content assay is always used indirectly to evaluate the extent of oxidative damage [23]. In the present work, SOD activity was significantly increased only at 20 mg/L of PA at 12, 24, and 48 h post-exposure (hpe) compared to control. The MDA level at 3 and 6 hpe was significantly increased in all PA treated groups, however from 12 to 48 hpe only in 40 and 80 mg/L treated groups, which indicated that the haemolymph was suffered from serious oxidative stress. The current results were consistent with the earlier investigation that the MDA levels were significantly higher in *C. farreri* after exposure to ammonia-N [31]. Thus, as an indicator of oxidative damage, significantly higher MDA content in 20 mg/L of PA exposed group at 3 and 6 hpe indicated that the low concentration of PA could activate the antioxidant system and acute phase response system in scallops; however, it turned to normal level at 12, 24 and 48 h with SOD activity increasing. In contrary, the higher concentrations of PA suppressed the activation of SOD during the experimental period. Therefore, the higher MDA content in 40–80 mg/L of PA exposure groups may be due to the related lower SOD activity than low concentration. The higher level of MDA even leaded to the peroxidation and damage of the cells, as it was considered to be a result of increased oxidative damage levels. Pan *et al.* [32] also reported the similar results that exposure to chemical compounds benzo(α)pyrene and benzo(κ)fluoranthene induced SOD activity in low concentration, but restrained from certain time in higher concentrations with the MDA level increasing in the haemolymph of *C. ferrari*. These phenomenon reflected that in higher concentrations of PA groups, the related lower SOD activities could not eliminate the oxidative stress effectively, and thus leading to the increment in MDA

level, and eventually leading to DNA damage or apoptosis of haemocyte. Finally it seriously affected the immune functioning of bay scallops.

Acid phosphatase activity (ACP) is an important hydrolytic enzyme in phagocytic lysosomes [29]. In our work, although ACP activities at 6 hpe were significantly higher only in the group received 80 mg/L of PA, at 12, 24, and 48 hpe were significantly higher in all PA treated groups than control. Stronger ACP activity could enable the phagocyte to destroy and clear pathogens more effectively, conferring to scallops an increased resistance against long-term pathogen invasion. Measurement of ACP, a typical lysosomal enzyme sensitive to environmental stresses [29], showed that ACP activities in pre-stimulated scallops were significantly higher than those of un-stimulated scallops exposed to different concentrations of PA at various time intervals. Our results are consistent with previous work reported by Jing *et al.* [30] that increased ACP activity in *Pinctada fucata* in response to copper exposure. In light of earlier reports, our results indicated that ACP activity was modulated to protect against PA immersion.

In the present study, lysozyme activity was significantly lower in 80 mg/L of PA treatment group after 12, 24, and 48 hpe. These results suggest that long time exposure to the high concentration of PA can reduce lysozyme activity of scallop. Phagocytosis of haemocytes as well as the lysosomal enzymes usually acted as important parameters to evaluate the immunotoxicity of environmental stimuli or pollutions to bivalve [13]. In the present study, the phagocytic activity was significantly different only at the two higher concentrations (40 and 80 mg/L of PA) with an increase at 6 and 12 hpe and a decrease at 24 and 48 hpe, which suggested negative relationship with the PA concentrations and exposure time. These results

implied that a short time PA exposure could stimulate phagocytosis of haemocytes of scallops, but more than 24 h exposure with PA may inhibit the phagocytic activity even DNA damage and apoptosis due to the sequential accumulation of ROS. Therefore, our results suggested that lysozyme and phagocytic activity in the bay scallop are useful parameters for monitoring the potential impact of environmental hazards or aquatic toxins on bay scallop non-specific immunity.

Reactive oxygen species (ROS) production in current investigation was significantly increased at 12, 24, and 48 hpe with 20, 40 and 80 mg/L of PA, at 6 hpe with 40 and 80 mg/L of PA and at 3 hpe only with 80 mg/L of PA. ROS production is another important mechanism of bivalve cellular immunity and most is the superoxide ion radical O_2^-, hydrogen peroxide H_2O_2, and the hydroxyl radical OH [29]. Although a small amount of ROS is necessary to enhance the internal defence against pathogens, a series of damage will occur when the generation of ROS is too much [13]. In this work, PA was found to enhance ROS production compared with the control group, and this was in line with the results showed by Liu *et al*. [13] that Aroclor1254 could increase ROS generation in scallop *C. farreri*. In addition, the higher ROS production was observed in the higher PA concentrations as well as over the increased exposure time. These suggested that the higher concentration and longer of PA exposure had caused accumulation of reactive oxygen species (ROS) with stronger potentially toxic effect on bay scallops.

Tomanek [17] revealed that a common set of stress-induced proteins including molecular chaperones that stabilize denaturing proteins during cellular stress increase. In the present work, the protein levels were significantly higher in groups

treated with 80 mg/L of PA at each time interval and with 40 mg/L at 3 hpe. Our results were similar to those reported by Hannam *et al.* [27] that the significant enhancement of plasma protein concentration was observed in *C. islandica* following dispersed oil exposure. The current results may correlate with cytolysis in lysosome-enriched cells such as haemocytes caused by high concentration of PA.

Under normal conditions, the intracellular level of ROS is strictly maintained and controlled by peroxiredoxins (Prxs) and other enzymes. Prxs comprise a family of ubiquitously expressed proteins, such as 2-Cys (PrxI-IV), atypical 2-Cys (PrxV) and 1-Cys (PrxVI) [33]. PrxV is an indispensable part of an integrated cellular antioxidant defence network, which prevents ROS-mediated damage and ensures that cells respond appropriately to increasing levels of oxidative stress through H_2O_2-mediated signalling pathways [23]. Our results showed that *PrxV* expression was significantly down-regulated at the initial and final hour of PA treatment; however, *PrxV* expression was significantly higher in PA treatment groups at 6–24 h, except 6 hpe with 20 mg/L of PA, than in the control group, and declined rapidly at 48 hpe. This phenomenon suggests that PA might be able to inhibit *PrxV* expression immediately following a short exposure time; although the higher concentration of PA may stimulate greater ROS production, which induces higher *PrxV* expression to protect against damage from ROS, as exposure time increasing it could inhibit the *PrxV*-mRNA expression significantly.

C-type lectins act as a first line of defence against pathogens. They can recognize and bind to terminal sugars on glycoproteins and glycolipids, and play significant roles in non-self recognition and the clearance of foreign particles, either as cell surface receptors for microbial carbohydrates or as soluble proteins

existing in scallop tissue fluids [21]. We found that scallops stimulated with 20 mg/L of PA had significantly higher *CLT-6* expression at each time interval; however exposure with 40 mg/L of PA exhibited higher *CLT-6* expression only at 6 and 12 hpe than control, and the highest expression was observed at 12 hpe. The 80 mg/L of PA attenuated significantly the expected expression of *CLT-6* mRNA at 3, 6, 24, and 48 h. A previous study reported that *Listonella anguillarum* could induce significant up-regulation of the mRNA level of C-type lectins in scallop haemocytes [21]. This result suggested that *CTL-6* in bay scallop haemolymph could effectively be induced by a lower concentration of PA, and strong transcription of *CTL-6* was needed to synthesize and recruit proteins to defend against environmental stress and/or an invading pathogen; however, higher PA concentrations could inhibit *CLT-6* expression, which is a negative influence on immune responses of bay scallops.

Metallothionein (MT) is another group of molecules chiefly involved in the response to oxidative stress, especially from toxic metals [34]. MT induction has also been found to occur dramatically in response to tissue injury, infection and inflammation; therefore, MT is probably of great importance to the immune defence system of scallops [22, 35]. In the present study, similar to the *CLT-6* gene expression, scallops treated with 20 mg/L of PA had a significantly higher *MT* expression from 6–48 hpe and with 40 mg/L of PA only at 12 hpe than was seen in the control, and the highest expression was observed at 12 hpe. Moreover, the highest concentration of PA (80 mg/L) attenuated significantly the expected expression of *MT*-mRNA, except at 12 h. MT could protect cells from oxidative stress not only through metal binding/release dynamics, but also by acting as a

scavenger of free radicals and reactive oxygen metabolites [22, 36]. PA might be able to increase the disorder of oxidation/reduction reactions and provoke oxidative stress in scallops or induce an oxidative burst in scallop haemocytes. The increase of *MT* mRNA expression in scallops indicated that MT is inducible by immune infection and it activates protective defence mechanisms against the rise of ROS and possible oxidative stress produced by the invading pathogens; however, the high PA concentration (80 mg/L) could inhibit *MT* expression, and drop the ability of response to oxidative stress, especially from toxic metals.

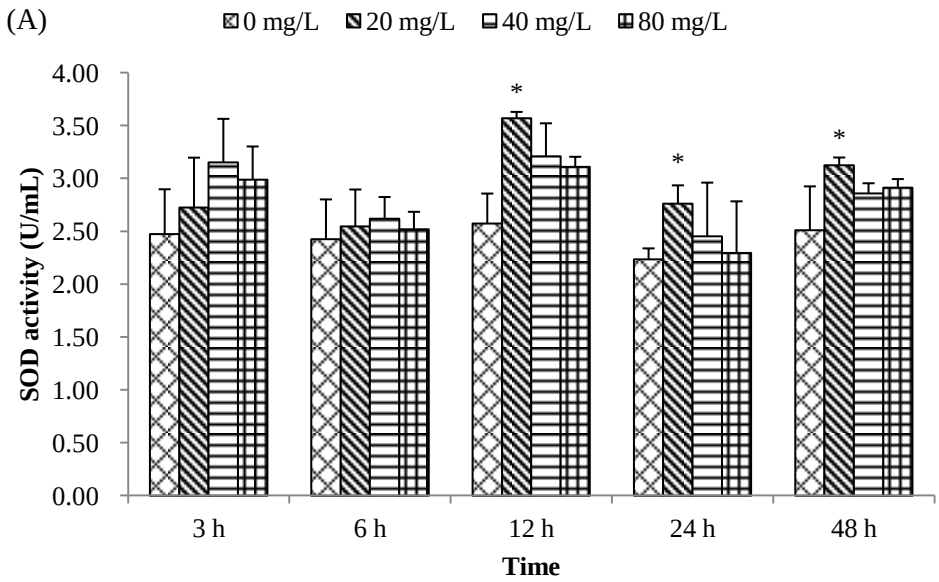
Antimicrobial peptides (AMPs) are often small cationic molecules widely distributed in all organisms, and they are thought to be a common feature of non-specific immunity in animals [37]. Big defensin (BD) is an AMP with remarkable microbicidal activity against gram-positive and gram-negative bacteria, and fungi in scallops [24]. In the present study, *BD* expression in haemolymph of scallops treated with 20 mg/L of PA was significantly higher at 6 to 24 hpe, and then decreased rapidly at 48 hpe. However, *BD* expression was higher at 12 and 24 hpe, but significantly attenuated at 3, 6, and 48 h in groups treated with either 40 or 80 mg/L of PA. This may be suggesting higher concentrations of PA suppressed *BD* mRNA transcription in the early stage of suddenly exposure to PA, and as time progressed, haemocytes were also mobilised to synthesise *BD* mRNA; perhaps other AMPs were expected to act as protector proteins during the late stage of this environmental stimulation. Moreover, the higher concentration may easily inhibit *BD* expression in the early stage of exposure, which restrained rapidly after short-term up-regulation during the late stage.

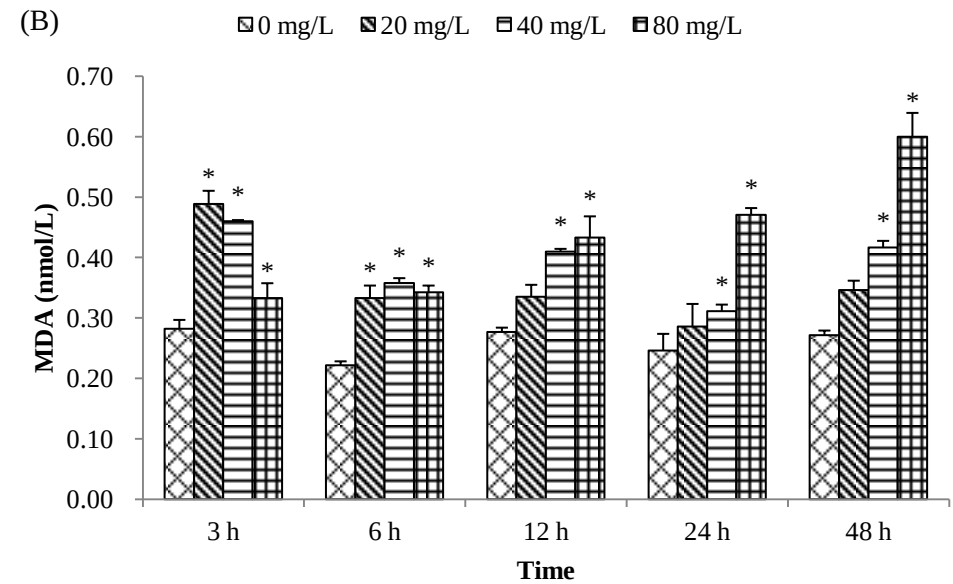
In general, the results of the present investigation demonstrated that exposure

to different concentrations of PA modulates various immune parameters. Low concentration of PA had no significant effect on the immune parameters (SOD, ACP, phagocytic, lysozyme activities, total protein and ROS levels) at initial exposure time (at 3–6 hpe). However, at the highest concentration of PA, significantly higher activities in ACP, phagocytic, ROS, and total protein were observed at initial times point(s). Treatment with higher concentration of PA for longer period (24–48 hpe) resulted in significant increment of ACP, ROS and total protein levels whereas result was vice versa for lysozyme and phagocytic activities. These results indicated that low concentration of PA exposure for shorter period had less effect on immune functions of bay scallop than the higher concentrations for longer time. Further, the MDA level was always higher in the treatment groups at the initial time points (3–6 hpe); however, at higher time points (12–48 hpe) significantly increased level of MDA was observed in 40–80 mg/L treated groups only. This result suggests that exposure to higher concentrations of PA for longer period had more stress on immune function of bay scallop. Our results suggest that the effect of PA on immune function of bay scallop is related to its dose and exposure time.

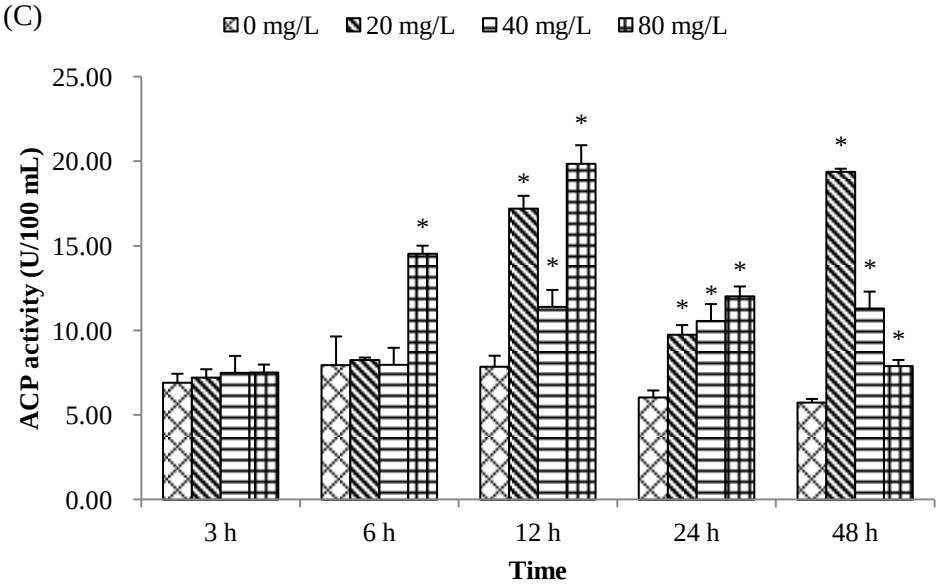
Table 1.1. Primers used for the analysis of mRNA expression by qRT-PCR.

Genes	**Primer sequence**	**Accession No.**
β-actin	F: 5′CAAACAGCAGCCTCCTCGTCA 3′	AY335441
	R: 5′CTGGGCACCTGAACCTTTCGTT 3′	
PrxV	F: 5′AATCAAGGAGCGGCTGGCA 3′	HM461987
	R: 5′TCAACTTCTCAATCTTCCCGTCAT 3′	
CTL-6	F: 5′CAGTTGCTACAGGGTTCG 3′	GQ202279
	R: 5′GGGCGTTATCTGGCTCAT 3′	
MT	F: 5′AACTTGCTGTAGTGGGAATG 3′	EU734181
	R: 5′AGGCTGGAAACTGCTGTGGT 3′	
BD	F: 5′CGTGCCATACCCATTGCTTA 3 ′	DQ334340
	R: 5′ATGATTGTCGTTGCTCCTTGAT 3 ′	

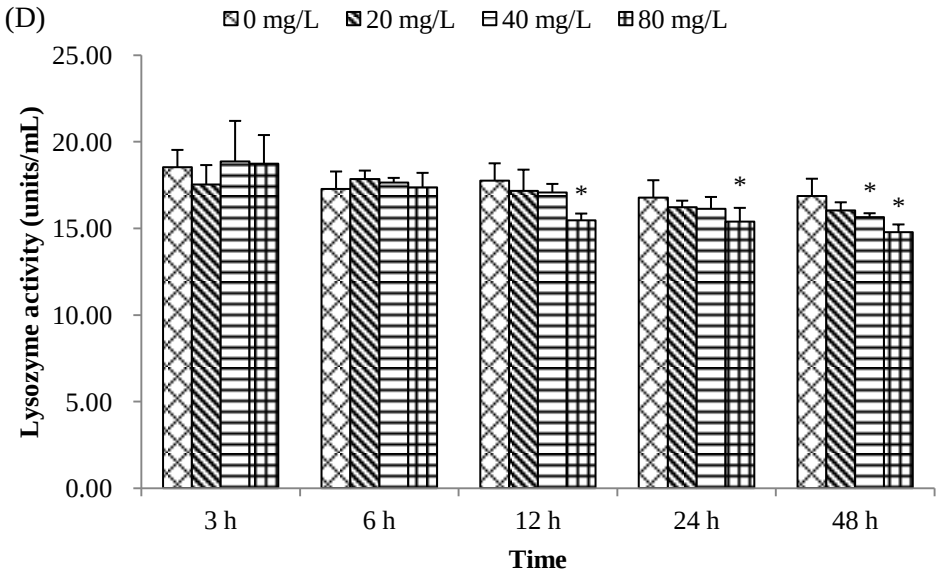

(A)
0 mg/L
20 mg/L
40 mg/L
80 mg/L
SOD activity (U/mL)
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
3 h
6 h
12 h
24 h
48 h
Time


(B)
0 mg/L
20 mg/L
40 mg/L
80 mg/L
MDA (nmol/L)
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
3 h
6 h
12 h
24 h
48 h
Time

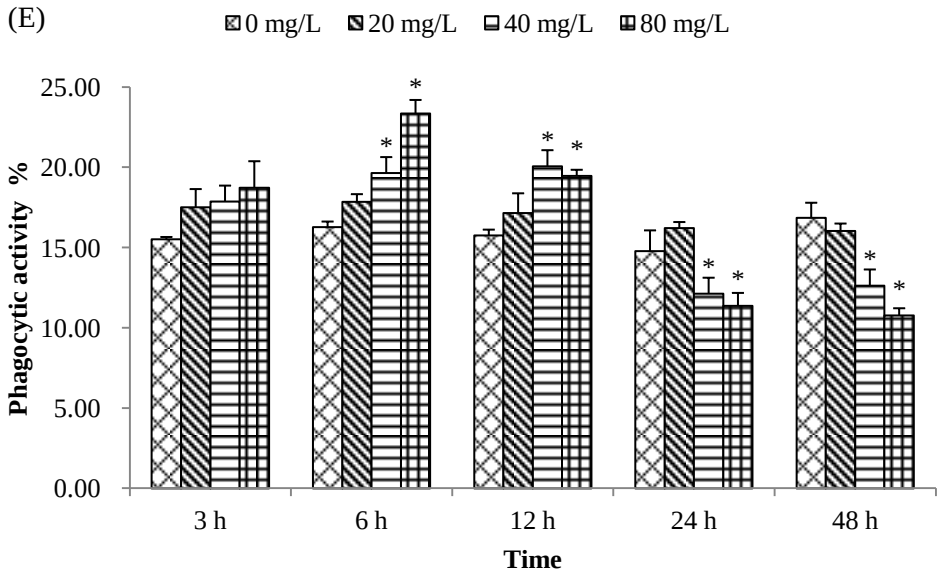
(C)


0 mg/L
20 mg/L
40 mg/L
80 mg/L
25.00
20.00
15.00
10.00
5.00
0.00
ACP activity (U/100 mL)
3 h
6 h
12 h
24 h
48 h
Time

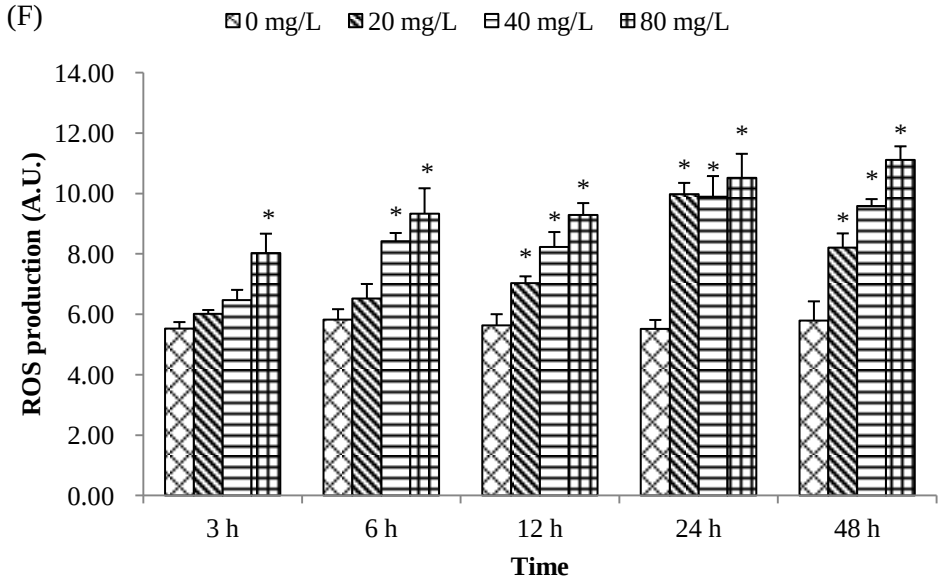
(D)


0 mg/L
20 mg/L
40 mg/L
80 mg/L
25.00
20.00
15.00
10.00
5.00
0.00
Lysozyme activity (units/mL)
3 h
6 h
12 h
24 h
48 h
Time

(E)


0 mg/L
20 mg/L
40 mg/L
80 mg/L
Phagocytic activity %
25.00
20.00
15.00
10.00
5.00
0.00
3 h
6 h
12 h
24 h
48 h
Time

(F)


0 mg/L
20 mg/L
40 mg/L
80 mg/L
ROS production (A.U.)
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
3 h
6 h
12 h
24 h
48 h
Time

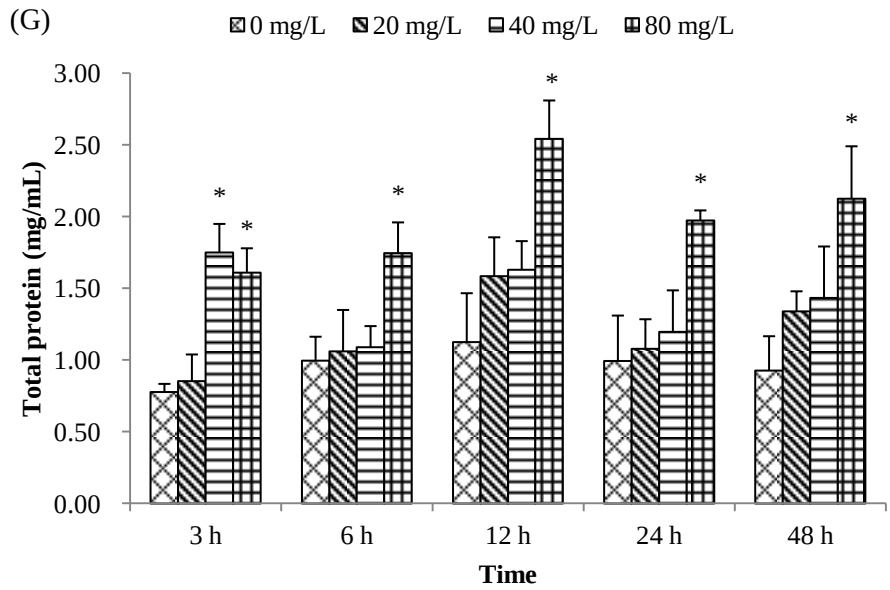
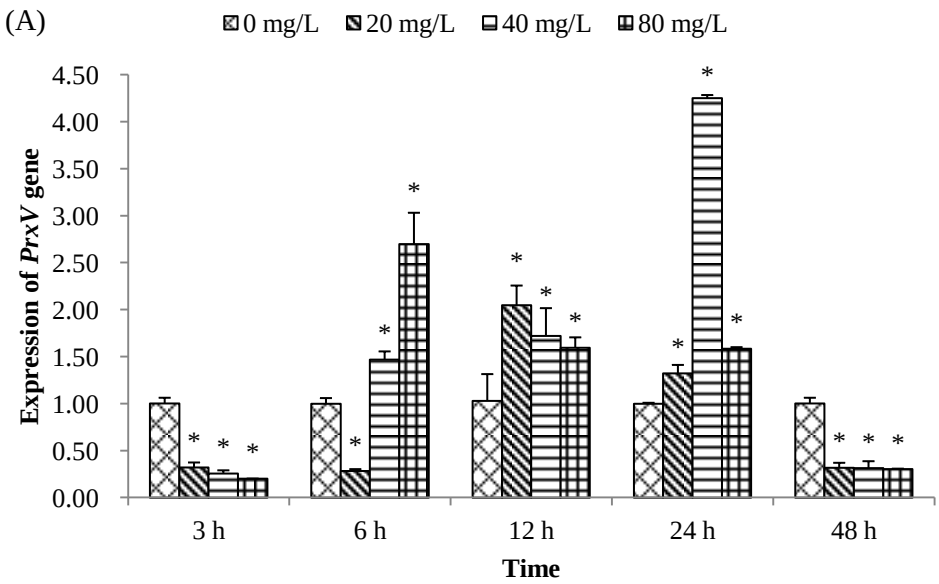
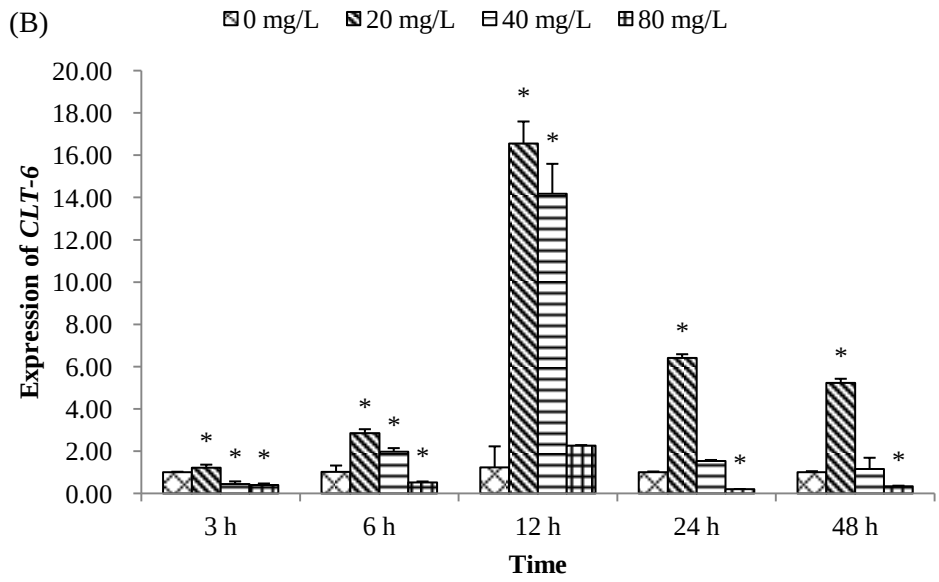


Figure 1.1. Effects of PA on non-special immune responses in bay scallop *A. irradians* at different time points after exposure to three concentrations (20, 40, and 80 mg/L). (A) SOD activity; (B) MDA content; (C) ACP activity; (D) lysozyme activity; (E) phagocytic activity; (F) ROS production (G) total protein; Data represent mean ± SD values ($n = 3$) at the same sampling time with different letters denoting significant differences ($P<0.05$).


(A)
0 mg/L
20 mg/L
40 mg/L
80 mg/L
Expression of PrxV gene
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
3 h
6 h
12 h
24 h
48 h
Time


(B)
0 mg/L
20 mg/L
40 mg/L
80 mg/L
Expression of CLT-6
20.00
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
3 h
6 h
12 h
24 h
48 h
Time

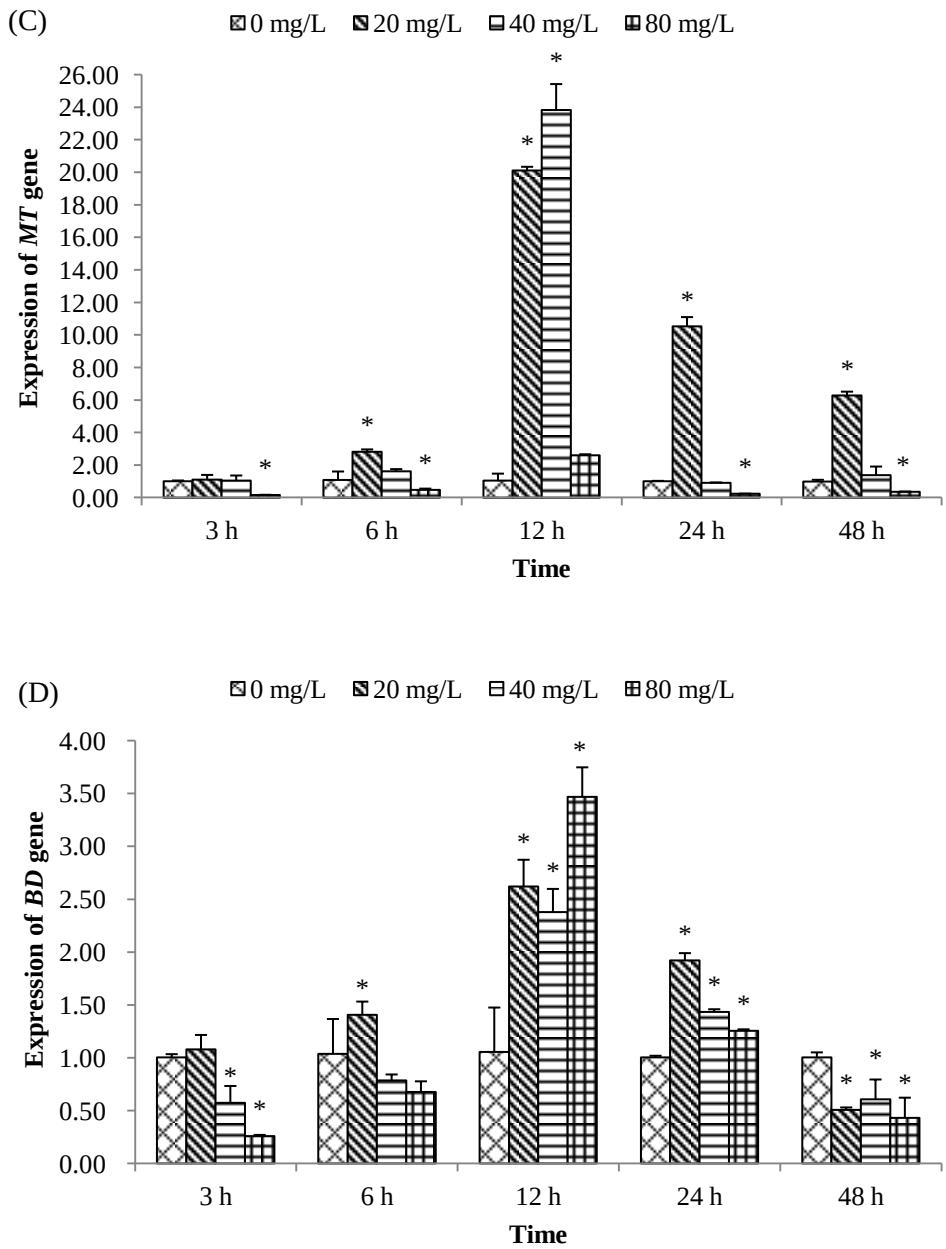


Figure 1.2. Effects of PA on immune related genes in bay scallop *A. irradians* at different time points after exposure to three concentrations (20, 40, and 80 mg/L). (A) *PrxV* gene; (B) *CLT-6* gene; (C) *MT* gene; (D) *BD* gene; Data represent mean

± SD values ($n = 3$) at the same sampling time with different letters denoting significant differences ($P<0.05$).

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Chapter II

Physico-immunological Response of the Bay Scallop (*Argopecten irradians*) Exposed to the Algicide Palmitoleic Acid

Abstract

Palmitoleic acid (PA) is an effective algicide against the toxin-producing dinoflagellate *Alexandrium tamarense*; however, its effects on the immune system of the edible bay scallop *Argopecten irradians* are unclear. Therefore, we investigated the effects of PA on the immune response in *A. irradians* by assessing total haemocyte counts (THC), alkaline phosphatase activity (ALP), nitrite oxide (NO), glutathione (GSH), and lactate dehydrogenase (LDH) levels, as well as the expression of immune-related genes (*FREP*, *PGRP*, *HSP90*, *MnSOD*, and *Cu/ZnSOD*) at various hours post-exposure (hpe) to the compound. THC decreased in PA-treated groups, whereas ALP increased significantly in all of the PA treatment groups at 3 hpe, after which it significantly decreased. The LDH and NO levels were significantly enhanced in the high and medium concentration group. Notably, the GSH level increased in all PA treatment groups at each time interval. Our study revealed that after treatment with different concentrations of PA, variable effects on the expression of genes involved in the immune system response were observed. The results of our study demonstrate that immersing scallops in PA at effective concentrations could result in differential effects on immune system responses and expression of immune-related genes. Specifically, PA may disrupt the endocrine

system or affect signal transduction pathways in the scallops. Therefore, the present study highlights the potential risk of using the PA as an algicide to control algal bloom outbreaks in the marine environment.

Keywords: Algicide, *Argopecten irradians*, Immune response, Palmitoleic acid, Harmful algal blooms.

2.1. Introduction

Harmful algal blooms (HABs) caused by pollution and global climate change are well known to cause ecological and economic losses in coastal areas [1]. HABs also negatively impact public health and threaten the aquaculture industry [2], as they can cause mass mortality of cultivated animals due to the toxins they produce [1]. Therefore, attempts to control HABs have received much attention in recent years. Several effective measures to inhibit HABs have been developed, including the implementation of chemical methods [3], physical manipulation [4], and use of biological agents [2]. Although chemical approaches are effective in controlling blooms, chemical agents may lead to serious secondary pollution [5], and indiscriminately kill other organisms in the aquatic ecosystem, which may alter marine food chains and ultimately impact natural biotic communities [6].

Biological agents, including bacteria [7], protozoa [6], viruses [8] and macrophytes [9], are now being considered potential suppressors to control HABs. Herbal based bioactive molecules including α-linolenic, oleic, linolic, and palmitic acids have been reportedly used as effective algicides to control various harmful algae, e.g. *Heterosigma akashiwo* and *Botryococcus braunii* [10, 11]. Bacteria have been speculated to be potential suppressors to control HAB outbreaks and harbour various advantages since they are efficient, species-specific, and environmentally friendly [2]. Recently, studies have revealed that some algicidal bacteria have the ability to attack target algal species [12]; however, few researchers have isolated the relevant algicidal compounds. In a recent study, Li *et al*. [2] reported that the algicidal bioactive compound palmitoleic acid (PA), isolated from the bacterium *Vibrio* sp. BS02, is effective against *Alexandrium tamarense* and functions as an

algicide during HABs. As an algicide, it has a 48 h half maximal effective concentration (EC_{50}) value of 40 mg/L and suppresses algal growth at concentrations higher than 20 mg/L [2]. PA almost completely inhibited algal growth at 80 mg/L, and lysis of *Alexandrium tamarense* cells was observed using light microscopy after PA exposure. Furthermore, PA effectively inhibits the growth of other harmful algae such as *Alexandrium minutum*, *Asterionella japonica*, and *Heterosigma akashiwo* [2]. However, how these bioactive molecules affect other marine species is not clear, as they may cause secondary pollution of the water. Previous studies reported the toxic effects of PA on murine and human melanoma cell lines, which revealed that PA causes loss of membrane integrity and leads to DNA fragmentation [13]. Although PA has been used as algicide [2], the potential impact of PA on marine species (e.g. fish, scallops, etc.) is not yet understood fully. The toxicity of this compound may induce stress responses, have toxic effects, and potentially lead to metabolic disturbance or oxidative stress of the non-target marine species [14].

Among non-target marine species, marine bivalves are particularly impacted by HABs due to their sessile and filter-feeding habits [1]. Scallops accumulate pollutants in their tissues to a greater extent than other bivalves since they have a low metabolic rate and are a cosmopolitan family of bivalves, with certain species widely farmed in the aquaculture industry as a food source, ensuring that they have a high economic value [15]. Therefore, scallops are ideal candidates for immunotoxicology studies. The bay scallop (*Argopecten irradians*) was introduced from America, and has been cultured in the coastal provinces of China since the 1980s. Currently, bay scallop farming in China is suffering from various problems

related to the marine environment [16]. An improved understanding of bay scallop immune system mechanisms may offer an efficient solution to aquaculture related problems, as well as enhance intensive breeding and the long-term sustainability of scallop farming [16].

Haemocytes are the circulating cells in scallops and are involved in both physiological processes and immune functions [17]. Short-term biomarkers for stress, such as alkaline phosphatase (ALP), which acts as a transphosphorylase at an alkaline pH, is a polyfunctional phosphomonoester hydrolase enzyme [18]. Molecules involved in antioxidant defence, such as glutathione (GSH), are also well-known to be produced in response to chemical threats, and frequently used as biomarkers in aquatic species including scallops [18, 19]. Lactate dehydrogenase (LDH) is a soluble cytosolic enzyme and is widely used as a biomarker for toxicology studies [20]. Any changes observed in the level of LDH activity suggests metabolism has been altered in the affected tissues [21]. Additionally, nitric oxide (NO) is involved in a series of pathogenic diseases and physiological processes in invertebrates, including the immune defence system [22]. However, NO can also react indirectly with reactive oxygen species (ROS) to produce the more powerful oxidant peroxynitrite, which inhibits DNA repair and can lead to apoptosis [22]. Further, analysing molecular and enzymatic variations in the expression of specific biomarkers is frequently used to assess the biological impact of environmental pollutants [23]. Thus, variation in mRNA levels can be monitored to identify the up- or down-regulation of complex biochemical systems or metabolic processes, which implicitly assumes that shifts in expression directly affects cell function [23].

Our previous study investigated the interaction between PA and the scallop immune response, in which we monitored various parameters including the activity of acid phosphatase (ACP), superoxide dismutase (SOD), lysozyme, phagocytes, as well as measured total protein content, malondialdehyde (MDA) level, the production of reactive oxygen species (ROS), and the expression of immune-related genes (*PrxV*, *CLT-6*, *MT*, and *BD*) [14]. In order to expand upon our previous work and further reveal the impact and any potential risk of using PA on the non-specific immune response of *A. irradians*, healthy scallops were immersed in various concentrations of PA over short periods of time, and the temporal profiles of immune parameters were assessed. To determine the impact of PA on the immune response of the bay scallop, its effects on the expression of immune-related genes were also monitored.

2.2. Materials and methods

2.2.1. Palmitoleic acid

Analytical-grade palmitoleic acid (PA) was obtained from Sigma-Aldrich Co. LLC (Sigma, USA) and stored at 4 ℃ in a refrigerator until use.

2.2.2. Animals

A total of 360 bay scallops (*A. irradians*), averaging 60–70 mm in shell length and 46.02 ± 2.67 g in weight, were collected from the Noryangjin fisheries wholesale market (Seoul, South Korea) and maintained in lantern nets suspended in 800 L capacity tanks containing filtered and aerated seawater in order to acclimatize the specimens to laboratory conditions (temperature: 10 ± 1 ℃; salinity:

30 ± 0.1‰) for 2 weeks. Half of the total volume of seawater was changed daily. Scallops were fed commercial shellfish diet (Instant Algae® Shellfish Diet; Reed Mariculture Inc., Campbell, CA, USA) at a rate of approximately 1.2×10^{10} algae cells per scallop per day.

The bay scallops were then randomly divided into a control group (without PA) and experimental groups (with PA). The experimental groups were treated with three concentrations (20, 40, or 80 mg/L) of PA corresponding to the minimum algicidal dose, EC_{50}, and the maximum effective algicidal dose against *Alexandrium tamarense*, respectively [2]. Three scallops from each treatment condition were randomly collected 3, 6, 12, 24, and 48 h after treatment. One mL of haemolymph was collected from the adductor muscle using a 1-mL sterile syringe fitted with a 22-gauge needle within 1 min of removing scallops from the tank. At each time point, an equal volume of haemolymph from three scallops in each replicate was pooled to account for any sample variation. Individual scallops were sampled once to avoid repeatedly drawing blood and/or handling stress. A 100-μL sample of haemolymph from each replicate was used for RNA extraction. Each 20-μL haemolymph sample was diluted 1:3 with Baker's Formol Calcium (2% sodium chloride, 1% calcium acetate, 4% formaldehyde) to fix cells and prevent aggregation in order to facilitate total haemocyte count [15]. Then, the remainder of the haemolymph sample from each replicate was centrifuged at $750 \times g$ for 3 min to collect the serum, which was then stored at –80 ℃ to later determine humoral immune parameters. Three replicates were obtained for each concentration and control group.

2.2.3. *Non-specific immune parameters assay*

2.2.3.1. Total haemocyte count

Total haemocyte counts (THC) were then carried out using an improved Neubauer haemocytometer under 40 × magnification [24].

2.2.3.2. Measurement of alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was determined using a chemical detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The unit definition of ALP enzymatic activity corresponded to 1 mg of phenol liberated per 100 mL haemolymph.

2.2.3.3. Measurement of nitric oxide

Nitrite oxide (NO) level was estimated enzymatically using a commercial test kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

2.2.3.4. Measurement of glutathione

The reduced glutathione (GSH) content in the haemolymph was measured with an assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) using 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB).

2.2.3.5. Measurement of lactate dehydrogenase

Lactate dehydrogenase (LDH) released from the haemolymph was measured using a lactate dehydrogenase assay kit (Nanjing Jiancheng Bioengineering

Institute, Nanjing, China) according to the manufacture's protocol.

2.2.4. *RNA Extraction and Reverse Transcription*

Total RNA was extracted from the haemolymph using TRIzol Reagent (CWBio, Beijing, China). The quality and purity of RNA were assessed by spectrophotometry by determining the 260:280 ratio. Afterward, genomic DNA was purified using DNase I (Promega, Madison, WI, USA) to remove any contamination. cDNA was then synthesized using the PrimeScript™ RT Reagent Kit (TaKaRa Bio, Ostu, Japan) following the manufacturer's instructions, and was stored at –80 ℃ [25].

2.2.5. *Real-time quantitative PCR analyses of gene expression*

The expression of genes involved in the immune response (*FREP*, *PGRP*, *HSP90*, *MnSOD*, and *Cu/ZnSOD*) was monitored using real-time quantitative PCR (qPCR) (Qiagen, Hilden, Germany). All qPCR reactions were performed using SYBR Premix Ex Taq™ Perfect Real-Time Kits (TaKaRa Bio, Ostu, Japan) and were conducted using a QiagenRotor-Gene Q RT-PCR Detection System (Qiagen, Hilden, Germany). Gene expression was normalized using the housekeeping gene for *β-actin*. The PCR primer sequences used for qPCR are listed in Table 1 [16, 26-29]. The reaction mixture included 10 μL SYBR Premix Ex Taq™, 1 μL of the forward and reverse primers (10 mM), and 1 μL cDNA. Ultra-pure water was then added to the reaction to bring it to a final total volume of 20 μL. The reaction conditions and cycle index were conducted at 95 ℃ for 10 min followed by 40 cycles of 95 ℃ for 45 s, 56 ℃ for 45 s, and 72 ℃ for 30 s. After the amplification

phase, a melting curve analysis was conducted to account for the possibility of non-specific amplification or primer dimer formation [30]. A standard curve was created from serial dilutions of sample cDNA and was drawn by plotting the natural log of the threshold cycle (Ct) against the number of molecules. The standard curve of each gene was run in duplicate and triplicate to obtain a reliable measure of amplification efficiency. The correlation coefficients (R^2) of all standard curves were > 0.99 and amplification efficiencies were between 90% and 110%. The relative expression ratios of the target gene in the treatment group versus those in the control group were calculated according to the following formula: Fold changes = $2^{-\Delta\Delta}$Ct, where $\Delta\Delta$Ct = [Ct (treatment group) – Ct (treatment *β-actin*)] – [Ct (control group) – (control *β-actin*)] [31]. In all cases, each PCR was carried out in triplicate.

2.2.6. *Statistical analyses*

Normality and homogeneity of variance were tested utilizing the Kolmogorov–Smirnov and Cochran's tests, respectively. All percentage data were arcsine-transformed, and subjected to a one-way ANOVA. Values are expressed as the arithmetic mean ± standard deviation (SD). Differences were determined using LSD test in SPSS statistical software version 19.0 (IBM Corp., Armonk, NY, USA) with *P*-values < 0.05 indicating statistical significance.

2.3. Results

2.3.1. *Non-specific immune responses*

2.3.1.1. THC

The THC (Figure 2.1 A) value obtained after the highest level (80 mg/L) of PA exposure decreased ($P<0.05$) after each time interval, with a medium concentration (40 mg/L) at 12–48 hours post-exposure (hpe). However, no significant changes in the low concentration (20 mg/L) treatment group were observed compared to the control.

2.3.1.2. ALP activity

ALP activity (Figure 2.1 B) was greater ($P<0.05$) in the 20 mg/L of PA treatment group at 3–24 hpe, and then returned to a normal level at 48 hpe, as compared to that in the control group. However, a significant increase ($P<0.05$) of ALP activities after the 40 mg/L PA treatment was observed at 3 and 6 hpe, after which the results were inversed at 12–48 hpe, and in particular, significantly diminished at 24 hpe. In the 80 mg/L PA treatment group, ALP activity was higher ($P<0.05$) only at 3 hpe, then diminished and significantly decreased ($P<0.05$) at 24 and 48 hpe.

2.3.1.3. NO level

The NO level (Figure 2.1 C) was affected by exposure to PA compared to the control, and significantly increased at the medium concentration of PA at 12–48 hpe, as well as the high concentration at 6–48 hpe. However, no significant change was identified at the low concentration of PA at any time point.

2.3.1.4. GSH level

The level of GSH was higher ($P<0.05$) in all of the PA treated groups at all

time points (Figure 2.1 D) with the highest level at 3 hpe, after exposure to 80 mg/L, followed by exposure to 40 and 20 mg/L at any time interval.

2.3.1.5. LDH activity

LDH activity (Figure 2.1 E) was significantly higher in the groups treated with the greatest concentration of PA at any time point, and in the group treated with 40 mg/L, a higher LDH concentration was only recorded at 12, 24, and 48 hpe. However, LDH activity in the group treated with 20 mg/L PA stabilized over the entire exposure period compared to the control group.

***2.3.2** Expression of immune-related genes*

2.3.2.1. Fibrinogen-related protein (*FREP)*

The expression of the *FREP* gene (Figure 2.2 A) after the 80 mg/L treatment was significantly up-regulated at 3–48 hpe, while in the 40 mg/L treatment expression gradually increased with the exposure time and was significantly up-regulated 6–48 hpe, peaking at 48 hpe. However, gene expression in the 20 mg/L treatment group only significantly increased at 24 hpe. For this gene as well as the others monitored, the 260:280 ratios, which are an indication of purity, were all between 1.8 and 2.0.

2.3.2.2. Peptidoglycan recognition protein (PGRP)

The *PGRP* gene expression (Figure 2.2 B) in all of the PA treated groups was significantly down-regulated ($P<0.05$) at the initial 3 hpe, after which it was up-regulated ($P<0.05$) after exposure to 40–80 mg/L PA. However, gene expression in

the 20 mg/L treatment group returned to the same level at 12 hpe as the control, and then increased significantly ($P<0.05$) at 24 and 48 hpe.

2.3.2.3. Heat Shock Protein 90 (HSP90)

Similar to the *PGRP* gene, *HSP90* gene expression (Figure 2.2 C) in the three PA treatments was lower ($P<0.05$) than that of control at 3 hpe, but returned to the same level in the 20 mg/L PA treatment group until 12 hpe, after which it increased notably at 24 and 48 hpe. The expression of *HSP90* in the 40 and 80 mg/L of PA treatment groups gradually increased after 6 hpe, specifically the 80 mg/L group showed significant up-regulation from 6–48 hpe, compared to the control.

2.3.2.4. Copper/zinc superoxide dismutase (Cu/ZnSOD)

The expression of *Cu/ZnSOD* mRNA in the haemolymph treated with PA is shown in Figure 2.2 D. The expression of the *Cu/ZnSOD* gene in the 20 mg/L PA treatment group was higher ($P<0.05$) at 12–48 h than the control group. The expression levels in the group treated with 40 mg/L PA ($P<0.05$) were higher at each time interval than the control, with the highest expression values observed at 24 hpe. Moreover, the group treated with 80 mg/L PA was up-regulated ($P<0.05$) from 3–24 hpe with the highest level at 12 hpe, after which it returned to the same level as the control at 48 hpe.

2.3.2.5. Manganese superoxide dismutase (MnSOD)

Analogous to the *Cu/ZnSOD* gene, the expression of *MnSOD* in the haemolymph treated with PA is presented in Figure 2.2 E. The haemolymph treated

with 20 mg/L of PA had higher ($P<0.05$) *MnSOD* expression levels 12–48 hpe than measured in the control. Moreover, the medium concentration of PA attenuated ($P<0.05$) the expected expression of *MnSOD*-mRNA (except at 3 hpe), which reached its highest level at 24 hpe, and diminished at 48 hpe. The group treated with 80 mg/L PA had enhanced expression levels ($P<0.05$) from 3–24 h with the highest values measured at 12 h, after which they returned to the same level as the control.

2.4. Discussion

Environmental risk assessment (ERA), consisting of the following four main steps, is widely acknowledged to be a standard method: hazard identification, dose-response assessment, exposure assessment, and risk characterisation. Additionally, various contaminants have been reported to exert immunotoxic effects on organisms [15]. To mitigate the negative impact of HABs on public health and the aquaculture industry, biological approaches are considered environment-friendly alternative methods to traditional chemical applications. Palmitoleic acid (PA), isolated from the bacterium *Vibrio* sp. BS02, was reported to be a novel environmentally friendly algicidal bioactive compound against HABs [2]. However, in our previous study, we found that PA affected ACP, SOD, lysozyme, phagocytic activity, total protein content, MDA level, and ROS production as well as the expression of specific genes (including *PrxV*, *CLT-6*, *MT*, and *BD*) [14]. In order to further assess the effects of PA on immune system function in the bay scallop, we investigated how exposure to this effective algicide alters physiological and immune responses as well as the expression of immune-related genes in *A.*

irradians. The results highlight that PA could modulate a series of physiological and non-specific immune responses as well as the expression of immune-related genes (*FREP*, *PGRP*, *HSP90*, *MnSOD*, and *Cu/ZnSOD*) in the haemolymph of bay scallops. Therefore, here we highlight the potential risks of using PA as an algicide to control and inhibit HABs.

Haemocytes are important to the immune response in bivalves, and are involved in the inflammatory response, respiratory bursts, wound recovery, phagocytosis, and encapsulation [32]. We found that THC levels decreased after exposure to high concentrations of PA, which is consistent with previous findings, which revealed that exposure to the organic pollutant Aroclor1254 (a highly chlorinated PCB mixture) decreased THC in *Chlamys farreri* [33]. The reason behind this phenomenon may be due to cell or tissue damage in areas including the gills and digestive glands of scallops. Therefore, haemocytes may participate in the restoration of injured tissue by infiltrating damaged connective tissue in order to repair it, reducing the number of haemocytes circulating in the haemolymph [33].

ALP is involved in the degradation and breakdown of invading non-self material, which has been reported in numerous bivalve species [15]. Hannam *et al.* [15] found that dispersed oil exposure did not significantly alter ALP activity in *Chlamys islandica*. However, in this study, low concentrations of PA significantly increased ALP activity from 3 to 24 hpe, and the medium concentration (40 mg/L) of PA enhanced levels measured at 3 and 6 hpe with a significant decrease observed at 24 hpe, after which the values recovered to the same level as the control at 48 hpe. Moreover, after exposure to high PA concentrations (80 mg/L), ALP activity was greater only at 3 hpe with lower activities at 24 and 48 hpe. Jing

et al. [34] also revealed that exposure to the contaminant copper resulted in differential ALP activities in *Pinctada fucata* at different exposure times. The results of our study suggest that ALP activity is sensitive to changes in the concentration and length of PA exposure. Lower concentrations of PA may improve ALP activity and allow for adaptation after 48 hpe, while higher concentrations may inhibit ALP activity after 24 hpe.

In bivalves, NO is an essential molecule related to normal physiological functions [35], such as the regulation of neural signal transmission, vascular tone, and the general immune defence system [22]. While NO is not toxic alone, during phagocytosis in combination with superoxide anions, it generates a peroxynitrite anion (ONOO–), which is highly toxic [35]. Here, we found that the NO level was significantly greater at the medium concentration of PA at 6–48 hpe, as well as the highest concentration at 12–48 hpe, although no changes were observed at low concentrations. The highest NO level was found at the greatest PA concentrations, and this enhancement was also noteworthy after prolonging the exposure time. These results suggest that high concentrations of PA and longer periods of exposure have potentially strong toxic effects on bay scallops.

Through biotransformation, pollutants can produce free radical O_2 and small amounts of H_2O_2, both of which may lead to oxidative stress if promptly metabolized [36]. GSH could initiate the catalysis of H_2O_2 to molecular water, playing a crucial role in protecting organisms from oxidative stress. The GSH content of the haemolymph in bay scallops increased across all PA treatment groups during the experiment, and revealed patterns related to changes in PA concentration. It is apparent that PA may be found in the haemolymph, and specific

tissues and organs are able to release antioxidants and enzymes to counter the toxic effects of this compound

LDH is a soluble cytosolic enzyme present in the majority of living cells, and catalyses the reversible oxidation of L-lactate to pyruvate using nicotinamideadenine dinucleotide (NAD+) as the hydrogen acceptor in the final step of anaerobic glycolysis [20, 37]. Therefore, it is widely used as a biomarker in toxicology assessments and clinical chemistry to diagnose cell, tissue, and organ damage, with any changes in the LDH activity level reflecting metabolic shifts in affected tissues [37]. In the present study, although no significant changes in LDH activity were observed at low PA concentrations, significant enhancement at the medium concentration was found at 12–48 hpe, as well as at all time points in the high PA treatment group. These data indicate that exposure to higher concentrations of PA might cause cellular membrane damage or increase membrane leakage in some affected tissues, allowing PA to move into the haemolymph.

It is well known that HSPs are present in almost all living organisms and play a crucial role in the cellular stress response. Specifically, they act as protein chaperones in both normal development and in the response to thermal and chemical stressors, stabilizing signalling related proteins and assisting in protein folding, unfolding, and degradation [23]. In particular, HSP90 is involved in the immune response, and regulated by a range of stressors such as polychlorinated biphenyl [38], arsenates [39], heavy metals [40], and disease [35, 41]. In the present study, the expression of the *HSP90* gene initially diminished after exposure to PA, although high concentrations of PA enhanced expression at 6–48 hpe as well as at low and medium concentrations at 24–48 hpe, suggesting that HSP90 protects

the bay scallop against higher PA concentrations as exposure time increases. Wang *et al.* [42] also reported that the expression of the *HSP90* gene increased significantly after exposure for 24 h to environmental ammonia-N. Therefore, PA may induce *HSP90* expression, which was found to protect the host from stress-induced cellular damage, and perhaps even repair damaged DNA and proteins.

SOD is an important enzyme, which functions as an antioxidant and catalyses the conversion of superoxide to oxygen and hydrogen peroxide, and has been considered a suitable biomarker for use in ecological risk assessments [36]. In the haemolymph of the bay scallop, both the *MnSOD* and *Cu/ZnSOD* genes exhibited similar expression patterns after PA exposure, and expression was strongly induced from 3–24 hpe with the highest levels identified at 12 and 24 hpe after exposure to high and medium concentrations of PA, respectively, after which expression rapidly dropped at 48 hpe. Pan *et al.* [36] revealed that SOD activities in the scallop, *Chlamys farreri*, were induced after exposure to high (10 μg/L) and medium (1 μg/L) concentrations of the environmental pollutant benzo(k)fluoranthene (BkF) at both 12 and 24 hpe, after which they returned to basal levels at approximately 48 and 120 hpe, respectively. Kim *et al.* [43] reported that after exposure to 1 μg/L 4-nonylphenol, the expression level of both *MnSOD* and *Cu/ZnSOD* dramatically increased. These findings indicated that modulation of *MnSOD* and *Cu/ZnSOD* expression after exposure to PA might vary depending on exposure concentration and total time. Higher concentrations and longer exposure periods may more easily induce oxidative stress, and require a protective cellular capacity to scavenge superoxide.

The main function of PGRP is to recognize and bind PGN as well as activate

the downstream immune response [29]. The expression of the *PGRP* gene was significantly down-regulated after exposure to all three PA concentrations at 3 hpe, suggesting that this compound negatively affects bay scallops. FREP plays an important role as a pattern recognition receptor in innate immune responses, especially in the clearance of non-self invaders such as single celled microorganisms and late apoptotic cells, using the lectin pathway [28]. The expression of the *FREP* gene in the haemolymph of scallops was strongly induced after exposure to high concentrations of PA at each time interval assessed, specifically at the medium concentration level at 6–48 hpe, as well as at the low concentration level only at 24 hpe, indicating a concentration-dependent response. Zhang *et al.* [28] reported that the *FREP* gene was up-regulated in the bay scallop after exposure to *Listonella anguillarum*. These results suggest that not only bacterial invasion but also the bioactive compound PA, can modulate *FREP* gene expression, which may cause apoptosis of haemocytes and cells of affected tissue.

We previously reported, as an adaptation to ROS overproduction, expression levels of *PGRP*, *FREP*, *MnSOD*, *Cu/ZnSOD*, and *HSP90* as well as ALP activity in the haemolymph of PA treated scallops increased significantly at 24 and 48 hpe [14]. However, the notable enhancement of LDH, NO, and GSH content presented here, in addition to changes in MDA and ROS concentration identified in previous work, was also observed in the haemolymph of scallops exposed to PA, despite the significant increase in immune parameters observed previously [14]. This implies that PA exposure could induce immune responses and cause further oxidative stress, as well as possibly even increase instances of bacterial infection in scallops. In agreement with our hypothesis, limited repair capacity of the immune system after

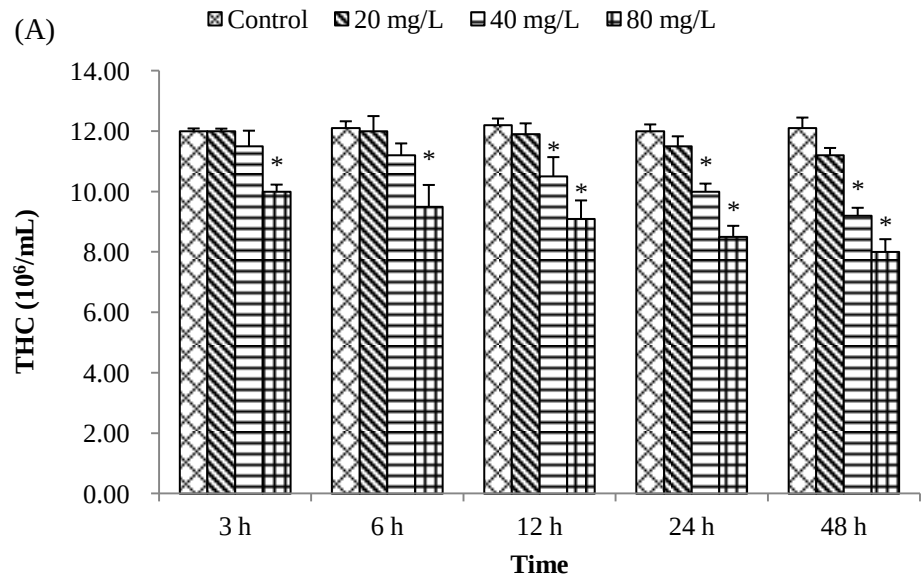
environmental stress has also been observed in the Zhikong scallop and American white shrimp (*Litopenaeus vannamei*). Moreover, during the experimental period, the health of several scallops diminished or specimens died after exposure to relatively higher concentrations of PA, and the activity of scallops decreased in comparison to groups exposed to low concentrations of PA as well as the control. These data suggest that higher PA concentrations lead to oxidative damage in scallops.

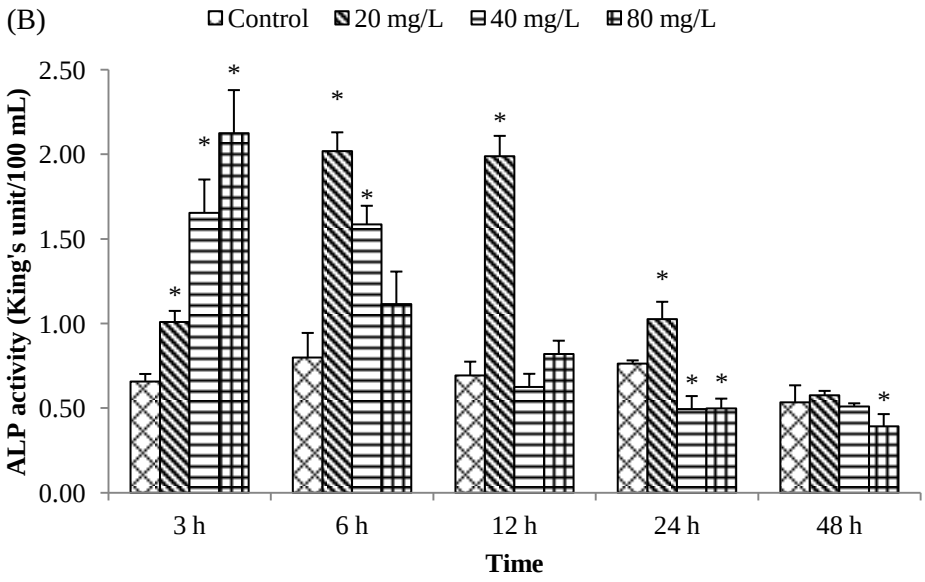
Here, we identified a shift in bay scallop immune responses, induced by exposure to effective concentrations (20, 40, and 80 mg/L) of the algicide PA. The significant changes found in several of the immune-related parameters we monitored (THC, ALP, LDH, NO, and GSH) as well as the expression of immune response related genes (*PGRP*, *FREP*, *MnSOD*, *Cu/ZnSOD*, and *HSP90*) in scallops after exposure to PA can affect the ability of detoxification mechanisms to mitigate damage in scallops experiencing oxidative stress. However, the metabolic process and immunotoxic mechanism induced by PA are complicated. This algicide might act as an environmental endocrine disruptor, which could negatively affect the endocrine system, or affect the signal transduction pathway in scallops, both of which would influence immune system function. Therefore, this study, in addition to earlier research, further verifies that utilizing PA as an algicide poses a potential risk to scallop production. According to our results, the oxidative stress and immune responses induced by PA on bay scallop showed expose time and concentration dependence. Therefore, reducing the exposure time and concentration of PA may have less adverse effects on scallops. In addition, the use of suitable immunostimulants synergistically with PA in scallop culture might be an

effective alternative method to boost scallop immune system function as well as reduce the negative effect of PA. Furthermore, the results of this study highlight the need to investigate the impact of other algicides used to control HABs in the marine environment, as well as address secondary pollution caused by PA.

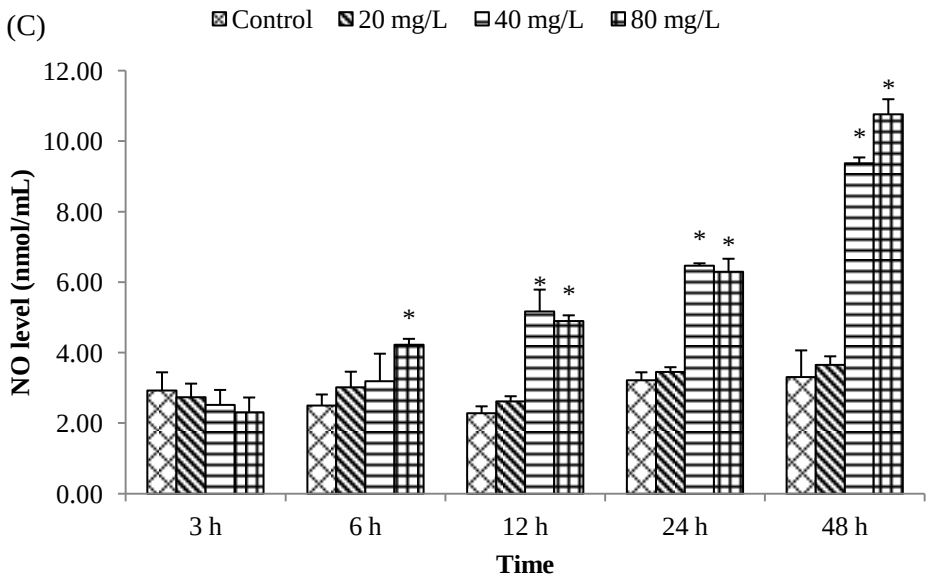
Table 2.1. Primers used for the analysis of mRNA expression by qRT-PCR.

Genes	Primer sequence	Accession no.
β-actin	F: 5′CAAACAGCAGCCTCCTCGTCA 3′ R: 5′CTGGGCACCTGAACCTTTCGTT 3′	AY335441
FREP	F: 5′CGTCGCAAATGCTGAAGATG 3′ R: 5′TAAGTTGTGGTCGGTCCTGAGA 3′	EU399719
PGRP	F: 5′GGGCAAGTGTATGAGGGAAGAG 3′ R: 5′TCCGATGAAGGAGACAGCGTAG 3′	AY437875
HSP90	F: 5′TCAGTATGGTTGGTCCGCTAA 3′ R: 5′CGGTTGCCTTTTCCTTCAGA 3′	EF532406
Cu/ZnSOD	F: 5′GTATTGAAAGGTGATTCGGAGG 3 ′ R: 5′ATGCACATGAAAGCCATGTAGG 3 ′	EU563958
MnSOD	F: 5′AATAGGGATTTTGGCTCGTTTG 3 ′ R: 5′TGGTTGAAGTGGGTCCTGGTTA 3 ′	EU137676

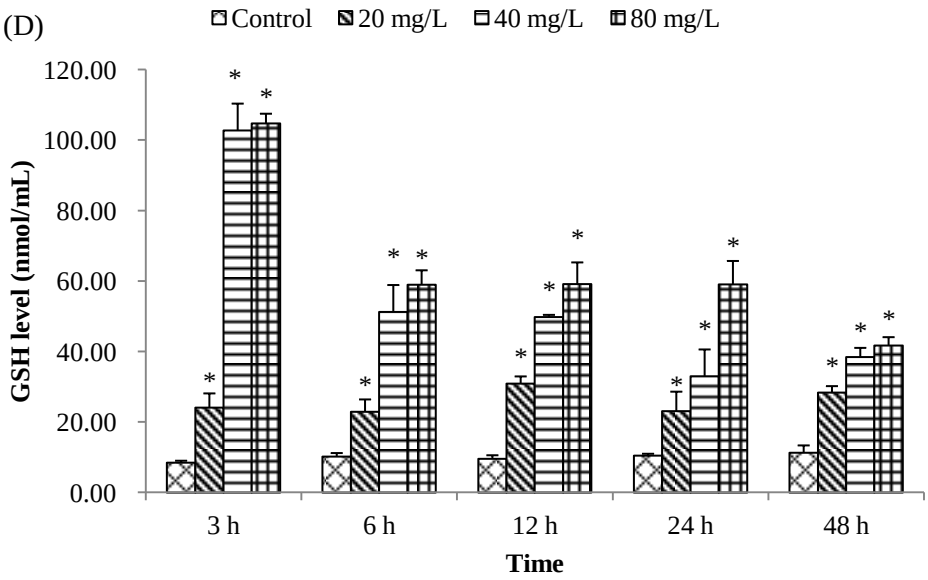

(A)
Control
20 mg/L
40 mg/L
80 mg/L
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
THC (10⁶/mL)
3 h
6 h
12 h
24 h
48 h
Time


(B)
Control
20 mg/L
40 mg/L
80 mg/L
2.50
2.00
1.50
1.00
0.50
0.00
ALP activity (King's unit/100 mL)
3 h
6 h
12 h
24 h
48 h
Time

(C)


Control
20 mg/L
40 mg/L
80 mg/L
12.00
10.00
8.00
6.00
4.00
2.00
0.00
NO level (nmol/mL)
3 h
6 h
12 h
24 h
48 h
Time

(D)


Control
20 mg/L
40 mg/L
80 mg/L
120.00
100.00
80.00
60.00
40.00
20.00
0.00
GSH level (nmol/mL)
3 h
6 h
12 h
24 h
48 h
Time

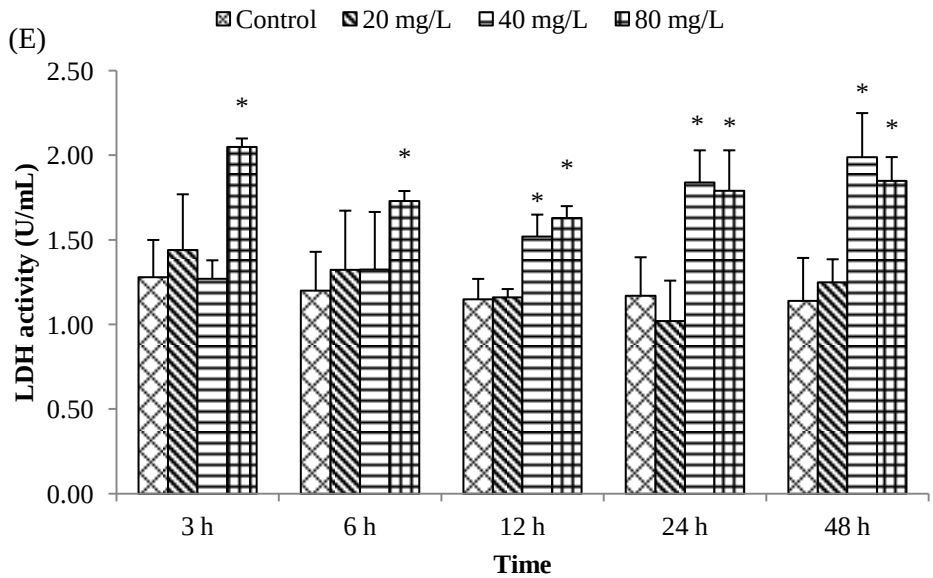
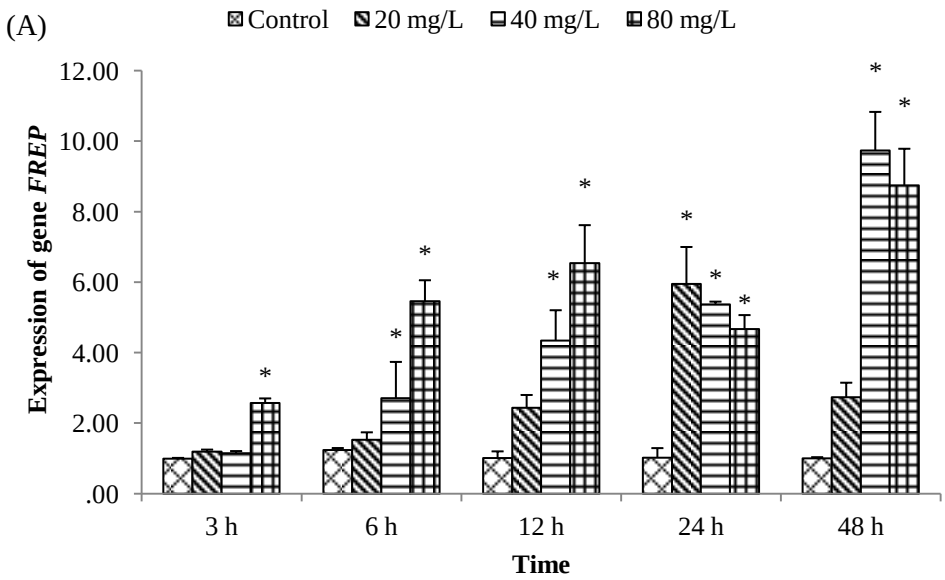
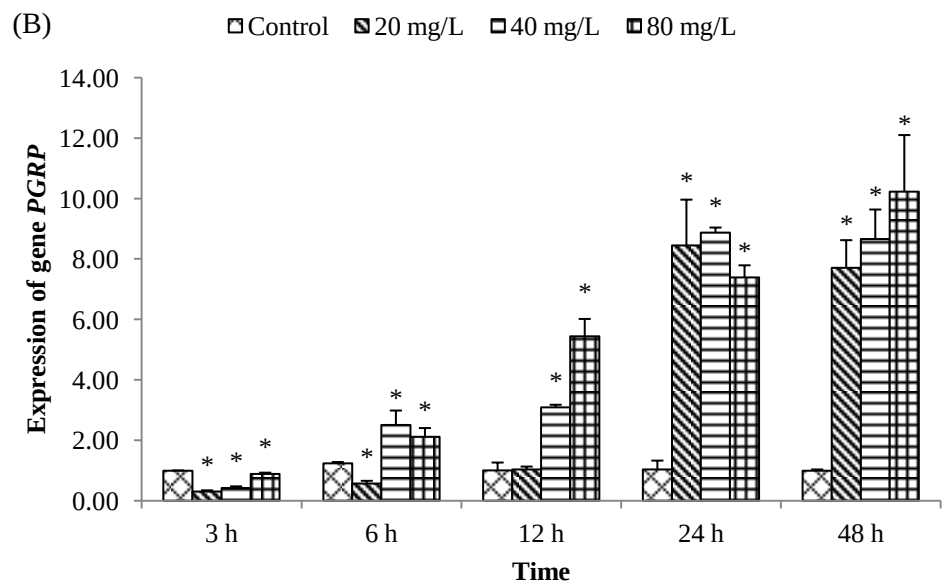
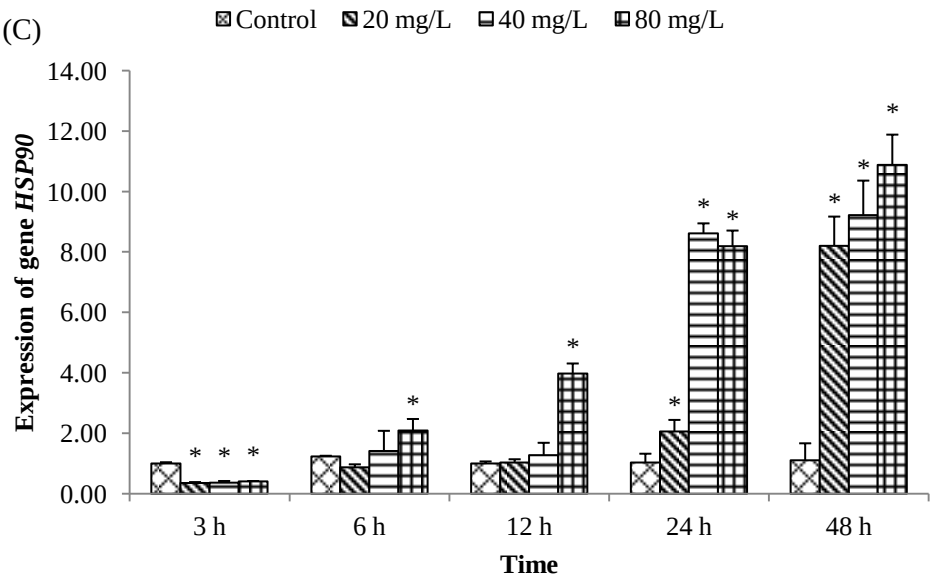


Figure 2.1. Effects of palmitoleic acid (PA) on non-specific immune responses in the bay scallop *Argopecten irradians* at different time points after exposure to three concentrations (20, 40, and 80 mg/L) of PA. (A) total haemocyte counts (THC); (B) alkaline phosphatase (ALP) activity; (C) nitrite oxide (NO) level; (D) glutathione (GSH) level; (E) lactate dehydrogenase (LDH) activity; Data represent mean ± SD values ($n = 3$) at the same sampling time with "*" denoting significant differences ($P<0.05$).

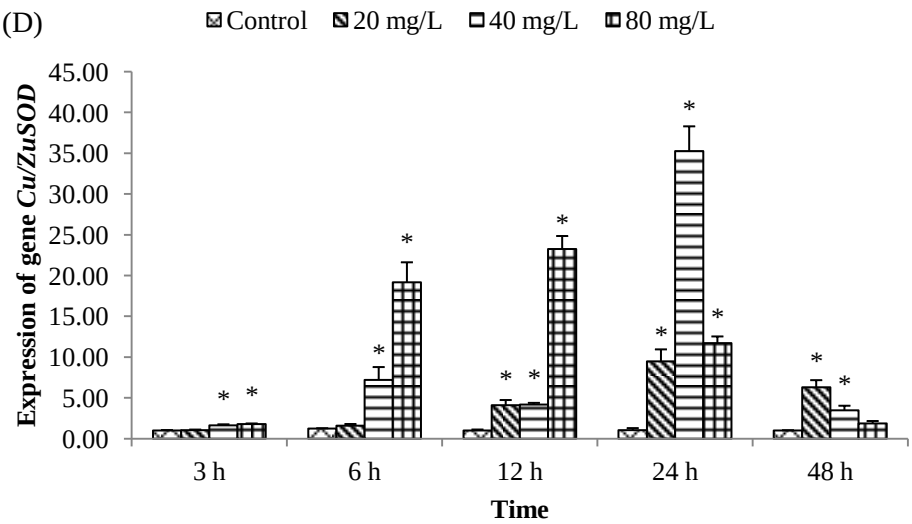

(A)
Control
20 mg/L
40 mg/L
80 mg/L
Expression of gene FREP
12.00
10.00
8.00
6.00
4.00
2.00
.00
3 h
6 h
12 h
24 h
48 h
Time


(B)
Control
20 mg/L
40 mg/L
80 mg/L
Expression of gene PGRP
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
3 h
6 h
12 h
24 h
48 h
Time

(C)


Control
20 mg/L
40 mg/L
80 mg/L
Expression of gene HSP90
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
3 h
6 h
12 h
24 h
48 h
Time

(D)


Control
20 mg/L
40 mg/L
80 mg/L
Expression of gene Cu/ZuSOD
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
3 h
6 h
12 h
24 h
48 h
Time

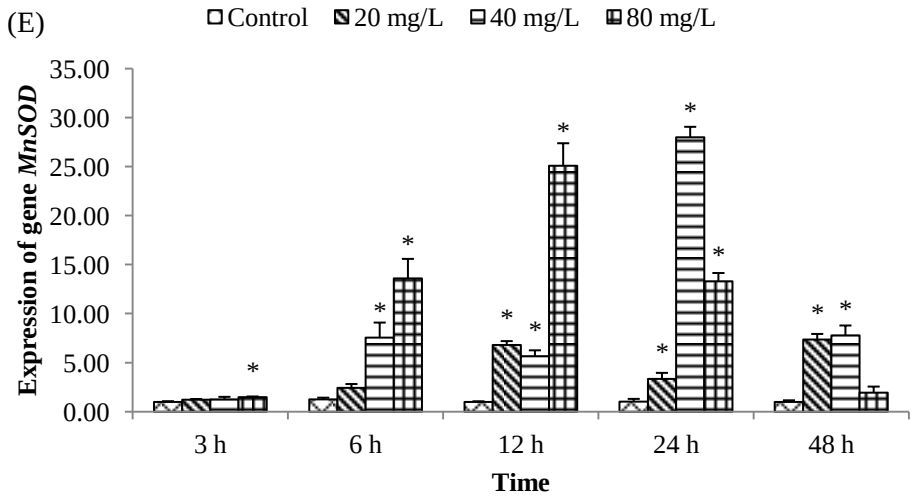


Figure 2.2. Effects of palmitoleic acid (PA) on immune-related genes in bay scallop *Argopecten irradians* at different time points after exposure to three concentrations (20, 40, and 80 mg/L). (A) *FREP* gene; (B) *PGRP* gene; (C) *HSP90* gene; (D) *Cu/ZuSOD* gene; (E) *MnSOD* gene; Data represent mean ± SD values (*n = 3*) at the same sampling time with "*" denoting significant differences (*P<0.05*).

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Chapter III

Immune Toxicological Effect of Marine Toxin Okadaic Acid in Bay Scallop (*Argopecten irradians*)

Abstract

Okadaic acid (OA) is produced by dinoflagellates during harmful algal blooms and is a diarrhetic shellfish poisoning toxin. This toxin is particularly problematic for bivalves that are cultured for human consumption. This study aimed to reveal the effects of exposure to OA on the immune responses of bay scallop, *Argopecten irradians*. Various immunological parameters were assessed (total hemocyte counts (THC), reactive oxygen species (ROS), malondialdehyde (MDA), glutathione (GSH), lactate dehydrogenase (LDH), and nitric oxide (NO) in the hemolymph of scallops at 3, 6, 12, 24, and 48 h post-exposure (hpe) to different concentrations of OA (50, 100, and 500 nM). Moreover, the expression of immune-system-related genes (*CLT-6*, *FREP*, *HSP90*, *MT*, and *Cu/ZnSOD*) was also measured. Results showed that ROS, MDA, and NO levels and LDH activity were enhanced after exposure to different concentrations of OA; however, both THC and GSH decreased between 24–48 hpe. The expression of immune-system-related genes was also assessed at different time points during the exposure period. Overall, our results suggest that exposure to OA had negative effects on immune system function, increased oxygenic stress, and disrupted metabolism of bay scallops.

Keywords: Okadaic Acid; Harmful Algal Blooms; Bay Scallop; *Argopecten*

irradians; Immune Response.

3.1. Introduction

Harmful algal blooms (HABs), caused by pollution of water bodies and global climate change, can result in ecological and economic losses in coastal areas [1]. HABs also have various negative impacts on public health and threaten aquaculture industry [2], as they cause mass mortality of cultivated animals from the algal toxins they produce [1]. The main marine phycotoxins which can be ultimately consumed by humans, causing a variety of gastrointestinal and neurological illnesses through food chain are shellfish toxins, including paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), amnesic shellfish poisoning (ASP), neurotoxic shellfish poisoning (NSP), and azaspiracid shellfish poisoning (AZP) [3]. The DSP toxins include okadaic acid (OA), the dinophysistoxins-1 (DTX-1), DTX22, DTX-3 and their derivative forms [4]. These are produced by some microalgae of the genera *Dinophysis* and *Prorocentrum*, such as *Prorocentrum lima*, *P. concavum*, *P. maculosum*, *D. acuminata*, *P. rhathymum*, and *D. fortii* etc. [3]. These toxins can accumulate in the fatty tissue of bivalves [4]. Among them, OA and its derivatives are the best representative of DSP toxins [5], which inhibit serine-threonine protein phosphatase 1 and phosphatase 2A, leading to metabolic process deregulation and hyperphosphorylation of many cellular proteins [6]. Previous studies have reported that exposure to OA or *P. lima* cause damage to hemocyte function and the viability of carpet shell clams (*Ruditapes decussatus*) [6]. Huang *et al.* [3] also revealed that *P. lima*, a dinoflagellate producing OA, induced cytoskeleton disorganization, oxidative stress, and dysfunction of metabolism in mussels.

Bivalves are particularly affected during HAB events as they accumulate high

levels of algal toxins in their tissues through their sessile and filter-feeding habits [1]. Therefore, previous studies of the impact of algal toxins have focused on oysters, mussels, or clams shells following exposure to toxic algae [1, 3, 6-11]. To date, there is little data on the effects of algal toxins on scallops. Moreover, most of these studies investigated the effects of phycotoxins by feeding or exposing mussels and oysters to harmful algae, which secrete various phycotoxins and other metabolites. However, the direct impacts of purified toxins on physiological responses in scallops have rarely been investigated [3, 7-11].

Scallops are a cosmopolitan family of bivalves, some of which are widely farmed by the aquaculture industry for food and have important economic value. The bay scallop (*Argopecten irradians*) was introduced to China from America and has been cultured in the coastal provinces of China for more than 30 years, and now bay scallop farming is also suffering from HABs [12]. Scallops accumulate toxins in their tissues to a greater extent as they have a low metabolic rate [13]. Hemocytes are important in the immune responses of bivalves as they are involved in the inflammatory response, respiratory burst, wound recovery, phagocytosis, and encapsulation [14]. During phagocytosis, large amounts of reactive oxygen species (ROS) are generated to kill the internalized bacteria, which is important for invertebrate survival [12]; however, damage occurs as a result of excessive generation of ROS [13]. Excessive production of ROS and other pro-oxidants damages unsaturated lipids, and breaks DNA bonds, proteins, amino acids, and carbohydrates [14]. In scallops, superoxide dismutase (SOD) is considered as the first and most important line of defense against ROS and protects tissues from oxidative damage [15]. Glutathione (GSH) is another well-known antioxidant

defense. Both GSH and SOD are frequently used as biomarkers in aquatic species, including scallops [15, 16]. GSH can directly neutralize several reactive species by being oxidation to oxidized glutathione (GSSG), and also acts as a cofactor of several antioxidant glutathione-dependent enzymes. Lactate dehydrogenase (LDH) is a soluble cytosolic enzyme and is widely used as a biomarker in toxicology and chemical threats to evaluate the status of cell, tissue, and organ damage [17]. Any changes in the level of LDH activity suggest metabolic changes in the affected tissues [18]. Nitric oxide (NO) is a crucial gaseous signaling molecule that is involved in a series of disease pathogenesis and physiological processes in invertebrates, including immune defense [19]. However, NO can also react indirectly with ROS to produce a more powerful oxidant peroxynitrite, which prevents DNA repair and is closely related to apoptosis [19]. In addition, malondialdehyde (MDA) levels represent membrane lipid peroxidation status and are also used as a marker to determine the extent of oxidative damage [14].

The multiple factors and complexity of feeding in the effects of exposure to toxic microalgae, especially during natural HAB outbreaks, are particularly problematic for the safe and efficient culturing of bivalves, such as scallops, for human consumption. This study evaluated the effect of OA on scallops to gain a better understanding of the toxicity of DSP toxins and help improve the intensive breeding and long-term sustainability of scallop farming [12]. We compared the immunotoxicity parameters (THC, ROS, MDA, NO, GSH, and LDH) in the hemolymph of bay scallops following exposure to different concentrations of OA to understand the early physiological and immunological responses of bay scallops to the toxicity of DSP toxins and provide information on its molecular mechanism

of the responses of bay scallops. In addition, we examined the transcription levels of several immune-system-related genes (*CLT-6*, *FREP*, *HSP90*, *MT*, and *Cu/ZnSOD*). To our knowledge, this is the first study comparing the effect of purified OA on bay scallop physiological and immunological responses and the expression of immune-system-related genes.

3.2. Materials and Methods

3.2.1. Okadaic acid

Okadaic acid (OA), 92–100% (HPLC), was obtained from Sigma-Aldrich Co. LLC (Sigma, USA) and stored at 4 ℃ in a refrigerator until use.

3.2.2. Animals

Bay scallops, *A. irradians*, averaging 60–70 mm in shell length, were collected from the Noryangjin fisheries wholesale market (Seoul, South Korea) and maintained in lantern nets suspended in 800-L-capacity tanks containing filtered and aerated sea water to acclimatize to laboratory conditions (temperature: 10 ± 1 °C; salinity: 30 ± 0.1‰) for 2 weeks. Half of the seawater was changed every day. Scallops were fed commercial shellfish diet (Instant Algae® Shellfish Diet; Reed Mariculture Inc., Campbell, CA, USA) at a rate of approximately 1.2×10^{10} algae cells per scallop per day.

Three hundred and sixty bay scallops (mean 46.02 ± 2.67g) were randomly divided into a control (without OA) and treatment groups (with OA). Each group consist of 30 scallops with three replicates (30×3=90 per group) The OA treatment groups were treated with one of three concentrations (50, 100, and 500 nM) of OA.

The three OA concentrations were selected based on previous studies reporting cytotoxic and genotoxic effects of OA on different cell lines [6]. Three scallops from each replicate treatment group were randomly collected at 3, 6, 12, 24, and 48 h after exposure to OA. Two mL of hemolymph were collected from each adductor muscle using a 1-mL sterile syringe fitted with a 22-gauge needle within 1 min of removing a scallop from the tank. Individual scallops were sampled once to avoid repeatedly drawing blood and/or handling stress. A 100-μL sample of hemolymph from each replicate treatment group was used for RNA extraction. A 20-μL sample of hemolymph was diluted 1:3 with Baker's Formol Calcium (2% sodium chloride, 1% calcium acetate, 4% formaldehyde) to fix cells and prevent aggregation for total hemocyte count [13]. The remaining hemolymph from each replicate treatment group was centrifuged at 750 × *g* for 3 min to collect the serum, which was then stored at –80 ℃ until testing for humoral immune parameters.

3.2.3. *Measurement of Non-Specific Immune Responses*

3.2.3.1. Total hemocyte count

A sample of 100 μl hemolymph was first fully mixed with an equal volume of Tris–EDTA (18 mM Tris; 0.45 M NaCl; 13 mM KCl; 16 mM d-glucose; 20 mM EDTA; pH 7.5) to avoid haemocyte agglutination, and then added to a haemacytometer, and then total hemocyte count (THC) was calculated as cells per mL using an improved Neubauer hemocytometer under 40 × magnification [20]. Counts were performed in triplicate and mean and standard deviation calculated.

3.2.3.2. Measurement of Reactive Oxygen Species production

Reactive oxygen species (ROS) production was measured using reactive oxygen species kits (Nanjing Jiancheng Bioengineering Institute) following the manufacturer instructions. Fluorescence, quantitatively related to the ROS production of hemocytes without any stimulation, was measured at 500–530 nm by a fluorescence microplate reader. Fluorescence was expressed in arbitrary units (A.U.).

3.2.3.3. Measurement of malondialdehyde content

Malondialdehyde (MDA), a degradation product of lipid peroxidation known as thiobarbituric acid-reactive substance, was determined according to the thiobarbituric acid method using a MDA test kit and following manufacturer instructions (Nanjing Jiancheng Bioengineering Institute). The MDA in decomposed products of lipid peroxidation can have condensation reaction with thio-barbituric acid, generate red product which has a maximum absorption peak at 532 nm (wavelength).

3.2.3.4. Measurement of nitric oxide assay

Nitrite oxide (NO) level was estimated enzymatically using a commercial test kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer instructions. NO is oxidized by oxygen to nitrate and nitrite which reacts with chromogenic agent to generate azo dyes and can be measured spectrophotometrically.

3.2.3.5. Measurement of glutathione assay

The content of reduced glutathione (GSH) in hemolymph was measured with an assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). DTNB (5,5′-Dithiobis(2-nitrobenzoic acid)) was developed for the detection of thiol compounds. DTNB and glutatuhione react to generate 2-nitro-5-thiobenzoic acid and glutathione disulfide (GSSG). Since 2-nitro-5-thiobenzoic acid is a yellow colored product, GSH concentration in a sample solution can be determined by the measurement at 420 nm absorbance.

3.2.3.6. Measurement of lactate dehydrogenase assay

Lactate dehydrogenase (LDH) released from hemolymph was measured using a lactate dehydrogenase assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacture instructions. LDH can catalyze lactic acid to pyruvic acid, pyruvic aci reacts with 2,4-dinitrophenylhydrazine to produce pyruvic dinitrophenylhydrazone. The product appeara red-brown in alkaline solution, enzyme activity can be worked out by measuring absorbances.

3.2.4. *RNA extraction and reverse transcription*

Total RNA was extracted from hemolymph using TRIzol Reagent (CWBio, Beijing, China). The quality and purity of RNA was assessed by spectrophotometry, and the 260:280 ratios were 1.8:2.0. Genomic DNA contamination was removed using DNase I (Promega, Madison, WI, USA). cDNA was synthesized using a PrimeScript^{TM} RT Reagent Kit (TaKaRa Bio, Japan) following the manufacturer instructions. The cDNA so obtained was stored at –80 ℃.

3.2.5. *Real-time quantitative PCR analyses of gene expression*

The expression of immune-system-related genes *CLT-6*, *FREP*, *HSP90*, *MT*, and *Cu/ZnSOD* was performed using real-time quantitative PCR (qPCR) (Qiagen, Hilden, Germany). All qPCR reactions were performed using SYBR Premix Ex Taq^{TM} Perfect Real-Time Kits (TaKaRa Bio, Japan) and were conducted using a Qiagen Rotor-Gene Q RT-PCR Detection System (Qiagen, Hilden, Germany). Gene expression was normalized using the housekeeping gene *β-actin*. PCR primer sequences used for qPCR are listed in Table 1 [12, 21-24]. The reaction mixture included 10 μL SYBR Premix Ex Taq^{TM}, 1 μL of the forward and reverse primer (10 mM), and 1 μL cDNA. The remaining volume was filled with ultra-pure water to a final total volume of 20 μL. The reaction conditions and cycle index were conducted at 95 ℃ for 10 min, followed by 40 cycles at 95 ℃ for 45 s, 56 ℃ for 45 s, and 72 ℃ for 30 s. After the amplification phase, a melting curve analysis was conducted to eliminate the possibility of non-specific amplification or primer dimer formation. A standard curve was created based on serial dilutions of sample cDNA. A standard curve was drawn by plotting the natural log of the threshold cycle (Ct) against the number of molecules. Standard curves for each gene were run in duplicate and triplicate to obtain reliable amplification efficiency. The correlation coefficients (R^2) of all standard curves was > 0.99 and the amplification efficiency was between 90 and 110%. The relative expression ratios of the target genes in the treatment groups versus the control group were calculated according to the following formula: Fold changes = $2^{-\Delta\Delta}$Ct, where ΔΔCt = [Ct (treatment group) – Ct (treatment *β-actin*)] – [Ct (control group) – (control *β-actin*)] [25]. In all cases, each PCR was carried out with three replicates.

3.2.6. *Statistical analysis*

Normality and homogeneity of variance were tested utilizing Kolmogrov–Smirnov and Cochran's tests, respectively. All percentage data were arcsine-transformed, and the data were analysed with one-way ANOVA. Values are expressed as the arithmetic mean ± standard deviation (SD). Differences were determined using the LSD test in SPSS version 19.0 (IBM Corp., Armonk, NY, USA) with *P*-values < *0.05* indicating statistical significance.

3.3. Results

3.3.1. *Non-Specific Immune Responses*

3.3.1.1. Total hemocyte count (THC)

The THC (Figure 3.1 A) decreased in groups exposed to 100–500 nM of OA for 12–48 hours post-exposure (hpe) (*P<0.05*). However, significant reduction of THC was also observed in the group exposed to 50 nM OA at 48 hpe.

3.3.1.2. Reactive oxygen species (ROS) level

The ROS level (Figure 3.1 B) was increased *(P<0.05*) at all time intervals following exposure to three concentrations of OA, and gradually increased with increasing exposure time.

3.3.1.3. Malondialdehyde (MDA) level

The level of MDA was higher (*P<0.05*) in all of the OA treated groups at 6–48 hpe (Figure 3.1 C) with a significant increase at 3 hpe at 500 nM of OA. In each

time interval, the highest level of MDA was observed at 500 nM of OA, followed by exposure to 100 nM, and 50 nM of OA.

3.3.1.4. Nitric oxide (NO) level

The level of NO was significantly increased in all of the OA-treated groups at all time intervals (Figure 3.1 D) with the highest level at 12 hpe at 100–500 nM of OA.

3.3.1.5. Glutathione (GSH) level

The level of GSH (Figure 3.1 E) showed no significant changes in any of the OA-treated groups at 3–6 hpe; however, a higher GSH level in 100–500 nM OA-treated groups was observed at 12 hpe. Thereafter, the GSH level in all of the OA-treated groups sharply decreased ($P<0.05$) at 24–48 hpe.

3.3.1.6. Lactate dehydrogenase (LDH) activity

The LDH activity (Figure 3.1 F) was greater ($P<0.05$) in the 500 nM OA treatment group at all time intervals compared to the control group; however, significant increments ($P<0.05$) of LDH activities in scallops treated with 50 and 100 nM of OA were observed at 12–48 hpe.

3.3.2. *Expression of immune-system-related genes*

3.3.2.1. Expression of CTL-6 gene

CTL-6 gene expression (Figure 3.2 A) in the three OA treatment groups was lower ($P<0.05$) than that in the control group at 3–48 hpe, except in the 50 nM OA

treatment group at 12–48 hpe, which returned to normal levels compared to the control.

3.3.2.2. Expression of FREP gene

FREP gene expression (Figure 3.2 B) in the 50 nM of OA treatment group was down-regulated ($P<0.05$) at 3–48 hpe. However, *FREP* gene expression in the 100 and 500 nM of OA treatment groups significantly decreased at 3 hpe, and sharply increased at 6 hpe, and thereafter sharply decreased from 12–48 hpe.

3.3.2.3. Expression of HSP90 gene

The expression of *HSP90* mRNA (Figure 3.2 C) in the hemolymph treated with three concentrations of OA gradually increased ($P<0.05$) up to 48 h with increasing exposure time, reaching the highest levels at 48 hpe.

3.3.2.4. Expression of MT gene

Scallops treated with 50 and 100 nM of OA had lower ($P<0.05$) MT expression from 3–6 hpe, but higher ($P<0.05$) MT expression at 12–48 hpe (Figure 3.2 D). Conversely, MT expression was strongly induced at 3–6 hpe in the 500 nM of OA group, and then returned to normal (control) level at 12 hpe, thereafter, it was obviously suppressed at 24–48 hpe.

3.3.2.5. Expression of Cu/ZnSOD gene

Expression of the *Cu/ZnSOD* gene (Figure 3.2 E) in the hemolymph treated with the three concentrations of OA showed no significant changes at 3 hpe;

however, it was continuously down-regulated ($P<0.05$) from 6–48 hpe, compared to the control group.

3.4. Discussion

Okadaic acid (OA) is the main marine toxin responsible for the DSP that causes gastrointestinal symptoms in humans following consumption of contaminated bivalves. OA, a potent and non-selective inhibitor of serine/threonine phosphatases, has been shown to be cytotoxic in a variety of cell lines [26]. Previous reports revealed that constant contact with OA induced chromosome loss, apoptosis, DNA damage, and inhibited phosphatases in contaminated bivalves. In this study, immune-system responses and expression of immune-system-related genes were determined in the hemolymph of bay scallops following exposure to different concentrations of OA.

Several immune parameters have been successfully employed to evaluate the immune status of bivalves subjected to various stresses [27]. The total hemocyte count (THC) is one of the most widely used parameters to assess bivalve health status as the number of circulating hemocytes changes under stressful conditions [9]. In this study, THC decreased significantly in the medium to high concentrations of OA-treatment groups after 12 hpe. A similar situation was observed in the clam, *Ruditapes philippinarum*, where the THC decreased upon exposure to the dinoflagellate *Prorocentrum minimum*, which produces algal diarrheic shellfish toxins, such as OA and dinophysistoxins [9, 28]. Exposure to higher concentrations of OA for longer periods resulted in higher apoptosis. This suggests that OA induced hemocyte death in a dose and time-dependent manner.

Similarly, previous reports have revealed that OA induced apoptosis even in human monocytic U-937 cells [26]. Therefore, this result suggests that OA exposure could disturb and restrain the immune response of bay scallops. ROS production is another important mechanism of bivalve cellular immunity.Although a small amount of ROS is necessary to enhance the internal defense against pathogens, serious damage to lipids, proteins, and DNA occurs when the generation of ROS is excessive [13]. A significant increase in ROS in OA-treated scallops was observed during all time intervals, and ROS generation was time and dose-dependent. This is in agreement with the previous report of OA-induced ROS generation in human monocytic U-937 cells [26]. Therefore, our results indicated that exposure to OA generated ROS in scallops.

Malondialdehyde (MDA) is closely related to the membrane lipid peroxidation status, therefore MDA content assay is used to indirectly evaluate the extent of oxidative damage [29]. In this study, exposure to OA resulted in significant increases in MDA content in all time intervals, which continued to increase as the exposure time increased. The current results are agreement with previous study, which reported that MDA level in the haemolymph of the temperate scallop *Pecten maximus* significantly increase with exposure to phenanthrene [30]. This phenomenon indicated that the hemolymph suffered from serious oxidative stress and oxidative damage to macromolecules. In addition, the NO level was significantly increased following exposure to OA, and sharply increased from 12 hpe in the group exposed to highest concentration of OA. In bivalves, NO is an essential molecule that is related to normal physiological functions [31], such as the regulation of neural transmission, vascular tone, and

immune defense [19]. Although NO is not toxic in itself, during phagocytosis in combination with superoxide anions synthesis, it generates the highly toxic peroxynitrite anion (ONOO–) [31]. Recently, it has been shown that production of the physiological messenger NO increased after treatment with the polyunsaturated aldehyde decadienal produced by diatoms in embryos of the sea urchin *Paracentrotus lividus* [32]. Moreover, a significant increase in NO production was reported in sea urchin embryos and in the different developmental stages of the offspring derived from females of the sea urchin exposed to heavy metal (e.g: cadmium and manganese) contamination [33, 34]. C.M. de Barros *et al.* [35] also reported that zymosan A and lipopolysaccharide (LPS) enhanced NO production in hemocytes of the ascidian *Phallusia nigra*. Our results of the present investigation are in accordance with the report of Migliaccio O. *et al.* [36] that adults of sea urchins showed high nitric oxide (NO) levels, along with a low fertilization rate after toxic blooms of *Ostreospsis cf. ovata*. These previous studies indicated that Zymosan A, LPS, or other soluble or particulate non-self materials could stimulate NO production in mollusk. Therefore, the results of our study suggests that NO mediates the stress response of bay scallop against the toxic effects of OA.Lactate dehydrogenase (LDH) is a soluble cytosolic enzyme present in most living cells. This enzyme catalyzes the reversible oxidation of L-lactate to pyruvate, with nicotinamide adenine dinucleotide (NAD+) as a hydrogen acceptor in the final step of the metabolic chain of anaerobic glycolysis [17, 37]. Therefore, LDH is widely used as a biomarker in toxicology and clinical chemistry to diagnose cell, tissue, and organ damage. In this study, bay scallops treated with different concentrations of OA produced notable increases in LDH activity in the hemolymph. This

increase in LDH activity reflected OA damage to tissues or apoptosis. Ravindran *et al.* [26] also reported a significant increase in LDH activity in U-937 cells from OA exposure after 4 h, reaching a maximum at 16 h. Traoré *et al.* [38] reported that 15 ng/mL of OA increased the release of LDH in Vero cells.

Pollutants can produce free radical O_2 and H_2O_2 through biotransformation, both of which may cause oxidative stress if not metabolized quickly [39]. Glutathione (GSH), which catalyzes H_2O_2 to molecular water, plays a crucial role in protecting organisms from oxidative stress. Although a significant increase in GSH level was observed in the OA treatment groups at 12 hpe, which reflects an up regulation of antioxidant defences, and thereafter it gradually decreased in all treatment groups may be due to overwhelming of the antioxidant capacity that can lead to mass oxidation of GSH resulting in excretion of the oxidised molecule (GSSG) from the cell leading to a reduced intracellular concentration of GSH level [40]. Ravindran *et al.* [26] also reported a significant decrease in GSH in OA-treated U-937 cells. Moreover, Zhang *et al.* [41] revealed that GSH levels in one-month-old mice reduced at 24 h after intraperitoneal injection of OA. In addition, a similar reduction in total glutathione has been reported in scallop exposed to the acute oil after 48 h [40]. Superoxide dismutase (SOD) is another important antioxidant enzyme that catalyzes conversion of superoxide to oxygen and H_2O_2, and has been considered as a suitable indicator for ecological risk assessments [38]. Based on the metal ion cofactor in the active site, SODs are classified into iron SOD (Fe SOD), manganese SOD (Mn SOD), nickel SOD, and copper-zinc SOD (Cu/Zn SOD); the Cu/ZnSODs have two forms: cytosol dimmers and extracellular tetramers [42]. In the hemolymph of bay scallops, the expression of the *Cu/ZnSOD*

gene was significantly down-regulated in all of the OA-treated groups at 6–48 hpe. This indicated that OA inhibited the expression of the *Cu/ZnSOD* gene, and inhibited the antioxidant abilities of scallops exposed to OA. Similarly, Zhang *et al.* [41] revealed that SOD activity in one-month-old mice was significant lower than that in the control group at 24 h after intraperitoneal injection of OA, similar to our results. Results of the present study suggest stimulated ROS production exceeded the neutralising capabilities of the antioxidant system and reduced the GSH level.

In addition, heat shock proteins (HSPs) play a significant role in preventing irreversible protein denaturation, promoting either repair or destruction of damaged proteins. The expression of the *HSP90* gene notably increased after exposure to OA in a dose and time-dependent manner, suggesting that HSPs (*HSP90* gene) protected the bay scallop against higher concentrations of OA as the exposure time increased. These results are consistent with those reported by Manfrin *et al.* [43] who showed that the expression of the *HSP90* gene in the mussel *Mytilus galloprovincialis* was significantly induced by OA exposure. Wang *et al.* [27] also reported that expression of the *HSP90* gene was significantly induced after 24 h exposure to environmental ammonia-N. Although OA exposure stimulated the antioxidant systems of scallops, significant increases in MDA, ROS level, and LDH activity in the hemolymph of scallops were observed. This reflected the limited abilities of antioxidant systems in scallops to fully remove these harmful superoxide radicals, resulting in oxidative damage to macromolecules.

Metallothionein (MT) is a group of molecules involved in responses to oxidative stress, especially from toxic metals [31]. MT induction has also been found to respond to tissue injury, infection, and inflammation, therefore, MT is

probably important in the immune system of scallops [23, 44]. In our study, expression of the *MT* gene was significantly suppressed in the 50 and 100 nM OA-treated groups up to 6 hpe, and then markedly increased until 48 hpe; however, the result was opposite in the highest concentration of OA treated group. A previous study also revealed [29] that expression of the *MT* gene was up regulated by 80 mg/L of a chemical contaminant palmitoleic acid (PA) in a short-space of time at 12 hpe, and was suppressed up to 48 hpe. This phenomenon may be attributed to the high concentration of OA modulating *MT* gene expression in a short exposure period and inducing *MT* mRNA transcription to briefly counter the tissue injury or oxidative stress, but finally strongly inhibiting expression. However, the lower concentrations took more time to modulate *MT* gene expression, and it could be that the *MT* mRNA transcription was inhibited with the increasing exposure time. In general, these results reflect the weakened ability of scallops treated with OA to respond to oxidative stress, tissue injury, infection, and inflammation.

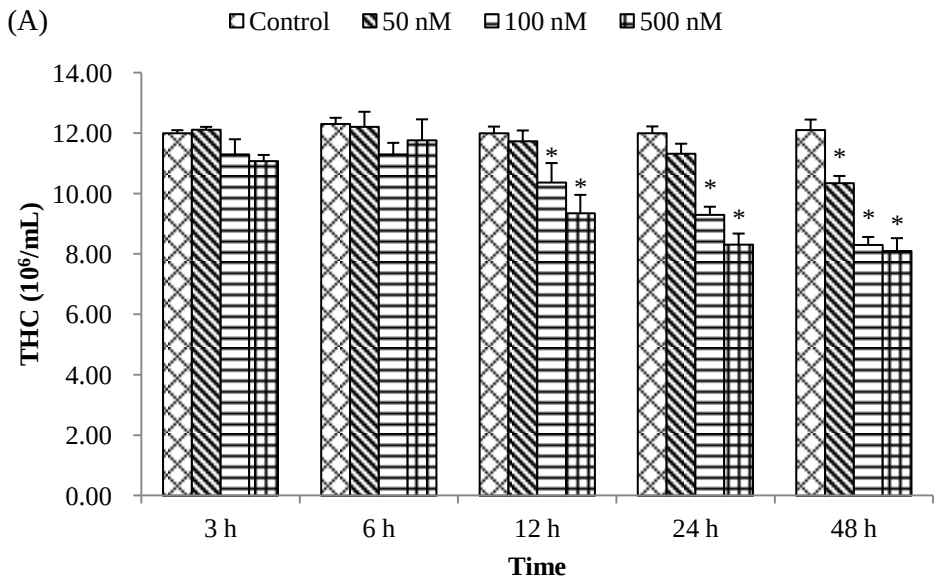
C-type lectins act as a first line of defense against pathogens; they recognize and bind to terminal sugars on glycoproteins and glycolipids, and play significant roles in non-self recognition and the clearance of foreign particles, either as cell surface receptors for microbial carbohydrates or as soluble proteins existing in scallop tissue fluids [43]. We found that scallops exposed to the lowest concentration of OA attenuated the expected expression of *CLT-6* mRNA at 3–6 hpe, with the higher concentrations of OA producing significantly higher *CLT-6* expression at each time interval. These results are consistent with the previous investigation in which PA exposure modulated the expected expression of the *CLT-6* gene, suggesting that the antibacterial or antivirus ability of scallops was

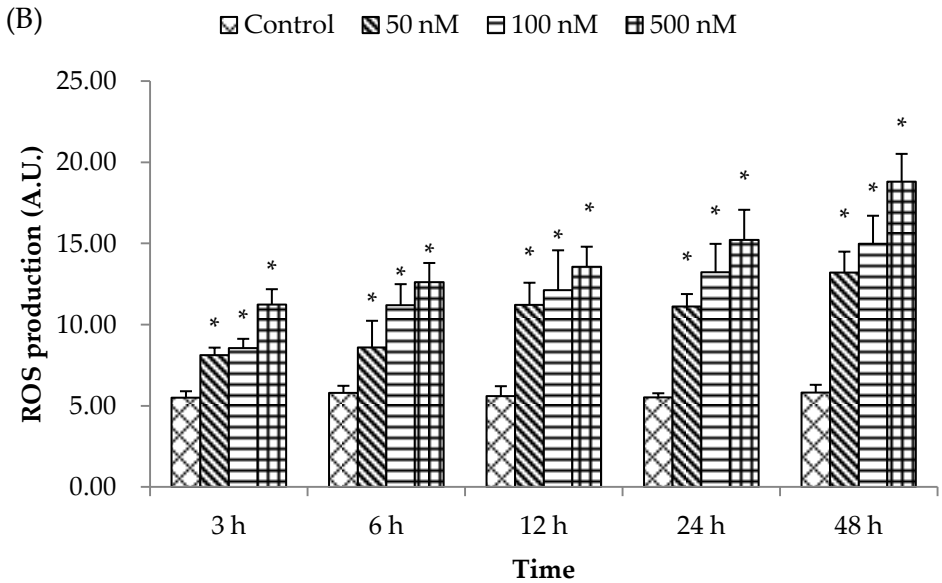
negatively impacted by OA exposure [29]. Fibrinogen-related protein (FREP) is another pattern recognition receptor in innate immune system responses, especially in the clearance of non-self, such as microorganisms and late apoptotic cells through the lectin pathway [22]. In this study, expression of the *FREP* gene was inhibited in all of the treatment groups at 3–48 hpe, except 100 and 500 nM at 6 hpe. These results indicated that OA exposure inhibited *CLT-6* and *FREP* gene expression, and then restrained the ability of scallops to recognize and clear non-self particles, such as microorganisms and late apoptotic cells through the lectin pathway.

In conclusion, this study shows that the bay scallop, *A. irradians* was affected immunologically by OA exposure. Altogether, these results showed significant changes in several of the immune-system-related parameters that we monitored (THC, ROS, MDA, LDH, NO, and GSH) indicating that the toxin OA can slowly diminish the immune system of bay scallop, especially when they are exposed to high concentrations of toxins or a long exposure period. Furthermore, significant changes in expression of immune response related genes (*CLT-6*, *FREP*, *HSP90*, *Cu/ZnSOD*, and *MT*) suggests cellular stresses. This study revealed OA-induced immunological and physiological effects on a species of bivalve – the bay scallop – and helps to establish the species of bivalves that are immunologically more sensitive to algal toxins. This could serve to better control potential infections, inflammation, or other oxidative damage that may further affect cultivated scallops already immuno-depleted by the algal toxin OA.

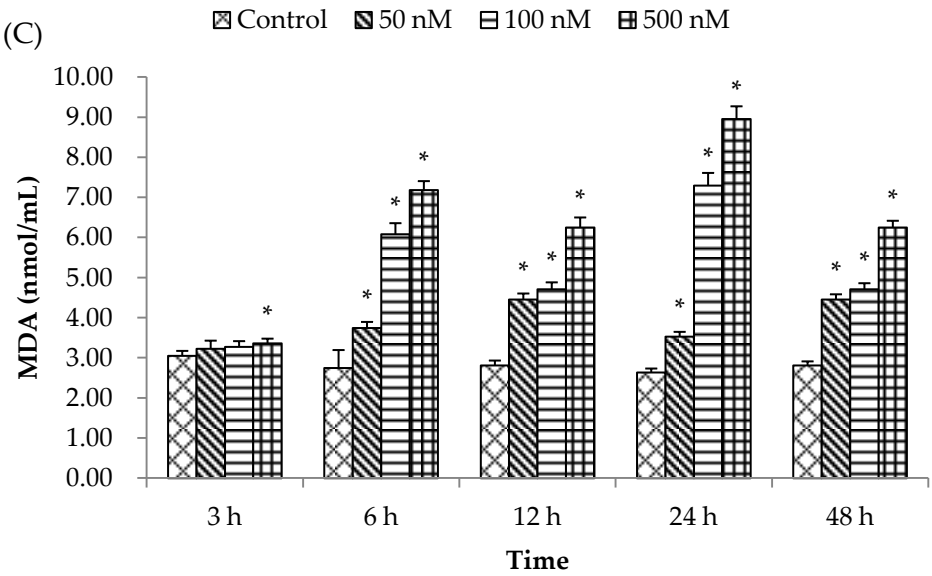
Table 3.1 Primers used for the analysis of mRNA expression by qRT-PCR.

Genes	Primer sequence	Accession no.
β-actin	F: 5′CAAACAGCAGCCTCCTCGTCA 3′ R: 5′CTGGGCACCTGAACCTTTCGTT 3′	AY335441
CTL-6	F: 5′CAGTTGCTACAGGGTTCG 3′ R: 5′GGGCGTTATCTGGCTCAT 3′	GQ202279
FREP	F: 5′CGTCGCAAATGCTGAAGATG 3′ R: 5′TAAGTTGTGGTCGGTCCTGAGA 3′	EU399719
HSP90	F: 5′TCAGTATGGTTGGTCCGCTAA 3′ R: 5′CGGTTGCCTTTTCCTTCAGA 3′	EF532406
MT	F: 5′AACTTGCTGTAGTGGGAATG 3′ R: 5′AGGCTGGAAACTGCTGTGGT 3′	EU734181
Cu/ZnSOD	F: 5′GTATTGAAAGGTGATTCGGAGG 3 ′ R: 5′ATGCACATGAAAGCCATGTAGG 3 ′	EU563958

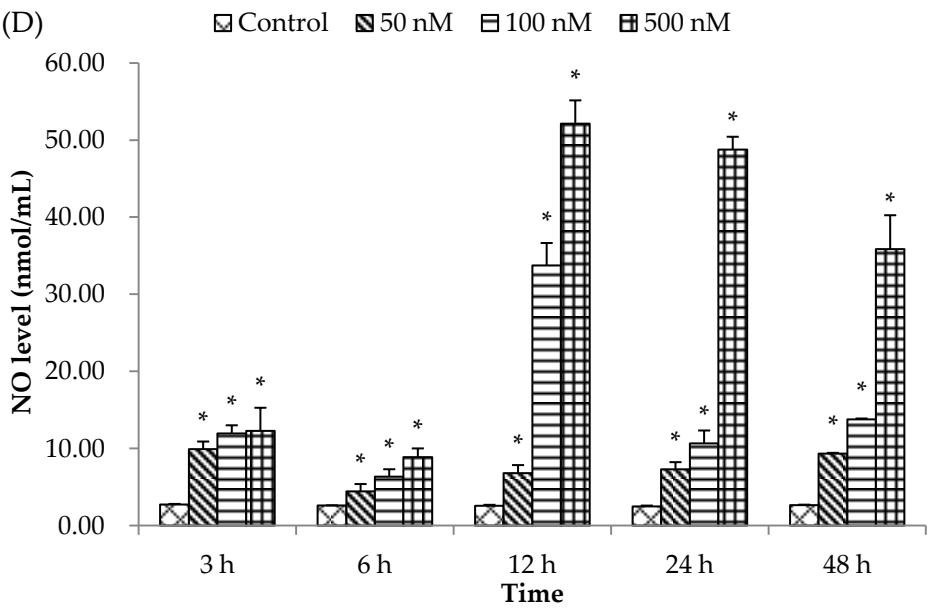

(A)
Control
50 nM
100 nM
500 nM
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
THC (10⁶/mL)
3 h
6 h
12 h
24 h
48 h
Time


(B)
Control
50 nM
100 nM
500 nM
25.00
20.00
15.00
10.00
5.00
0.00
ROS production (A.U.)
3 h
6 h
12 h
24 h
48 h
Time

(C)


Control
50 nM
100 nM
500 nM
MDA (nmol/mL)
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
3 h
6 h
12 h
24 h
48 h
Time

(D)


Control
50 nM
100 nM
500 nM
NO level (nmol/mL)
60.00
50.00
40.00
30.00
20.00
10.00
0.00
3 h
6 h
12 h
24 h
48 h
Time

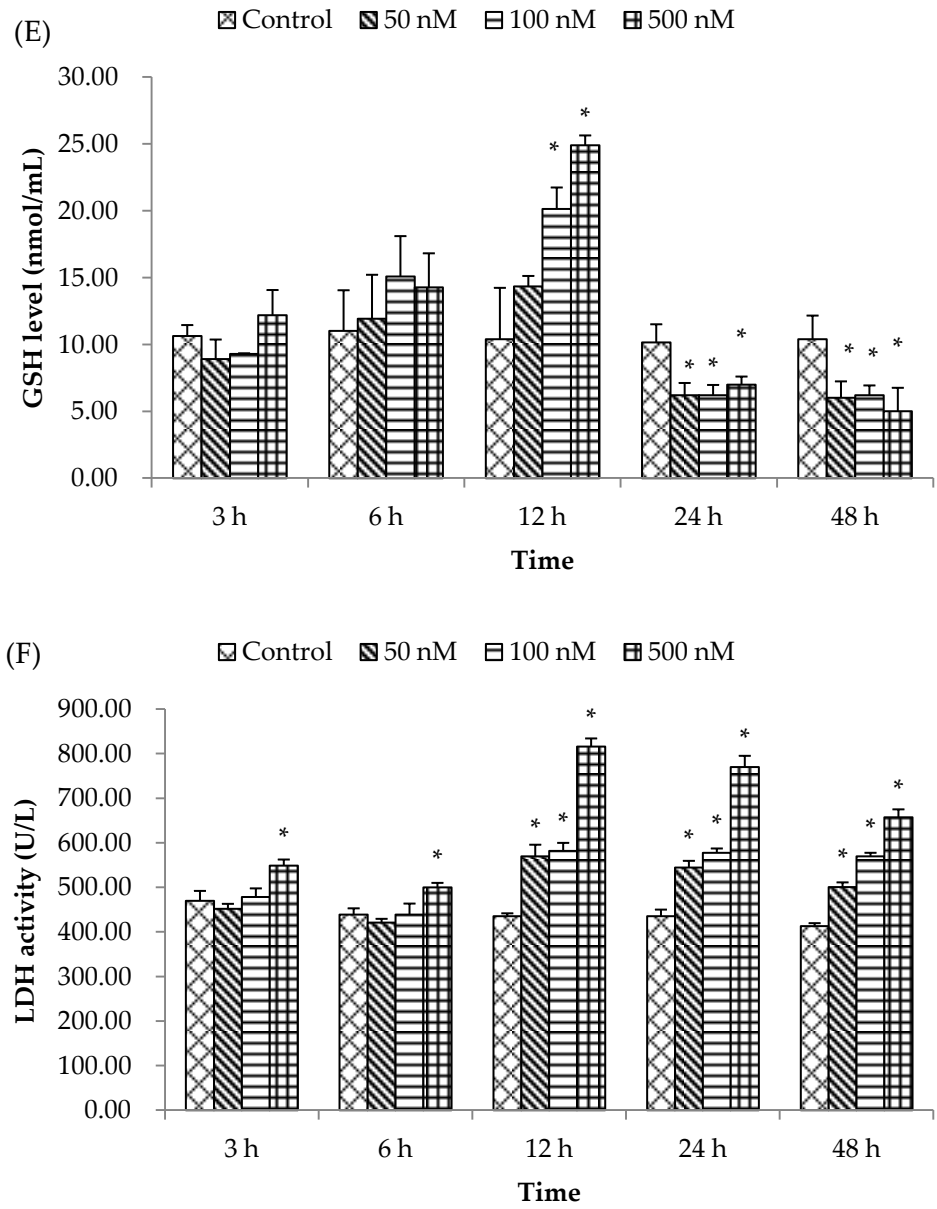
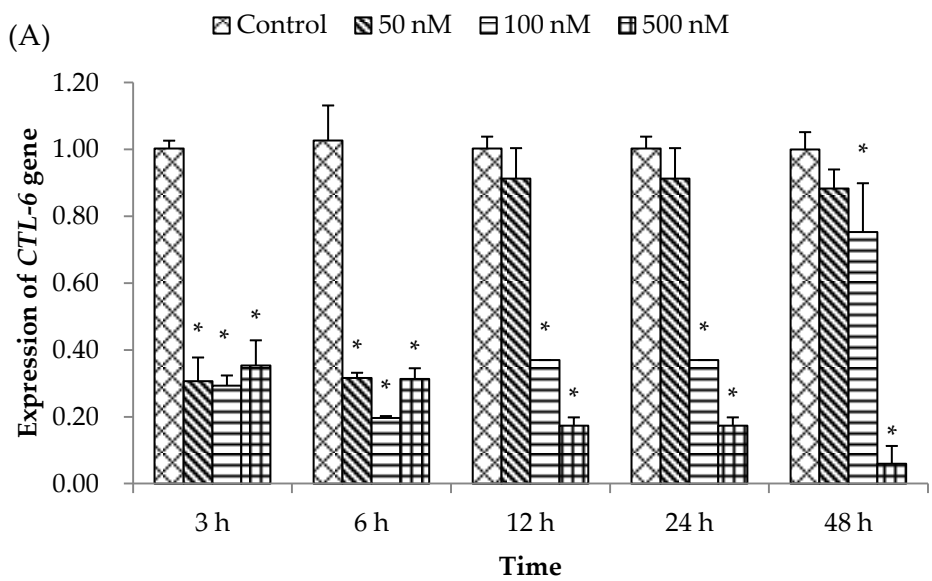
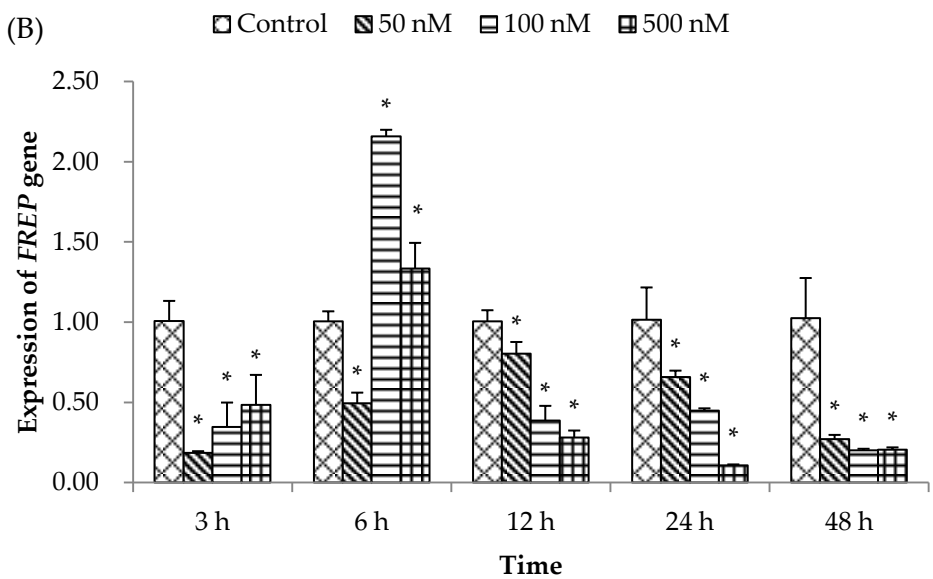


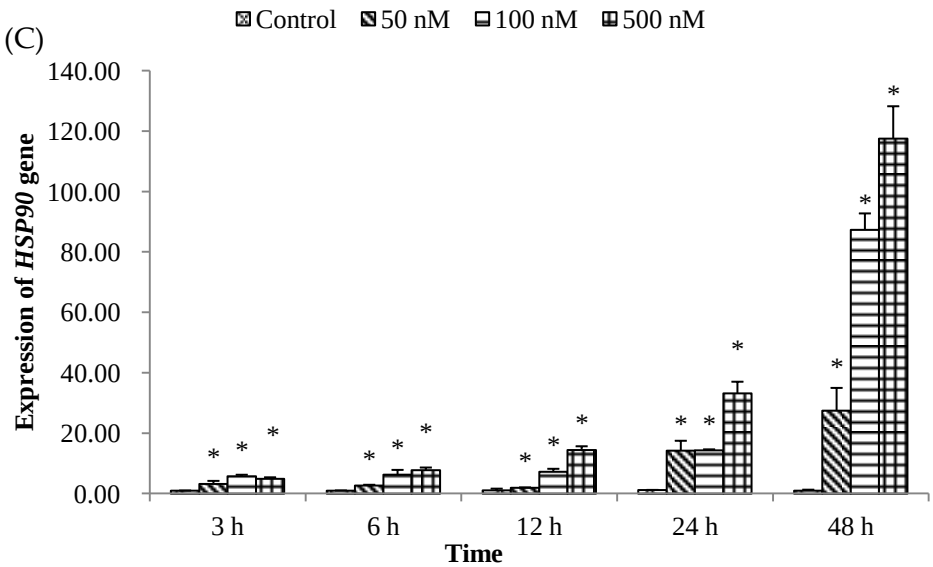
Figure 3.1. Effects of okadaic acid (OA) on non-specific immune responses of the bay scallop *Argopecten irradians* at different time intervals after exposure to three concentrations (50, 100, and 500 nM) of OA. (A) total hemocyte counts (THC); (B) reactive oxygen species (ROS); (C) malondialdehyde (MDA); (D) nitrite oxide (NO) level; (E) glutathione (GSH) level; (F) lactate dehydrogenase (LDH) activity.

Data represent mean ± SD values ($n = 3$) at the same time interval with "*" denoting significant differences ($P<0.05$).

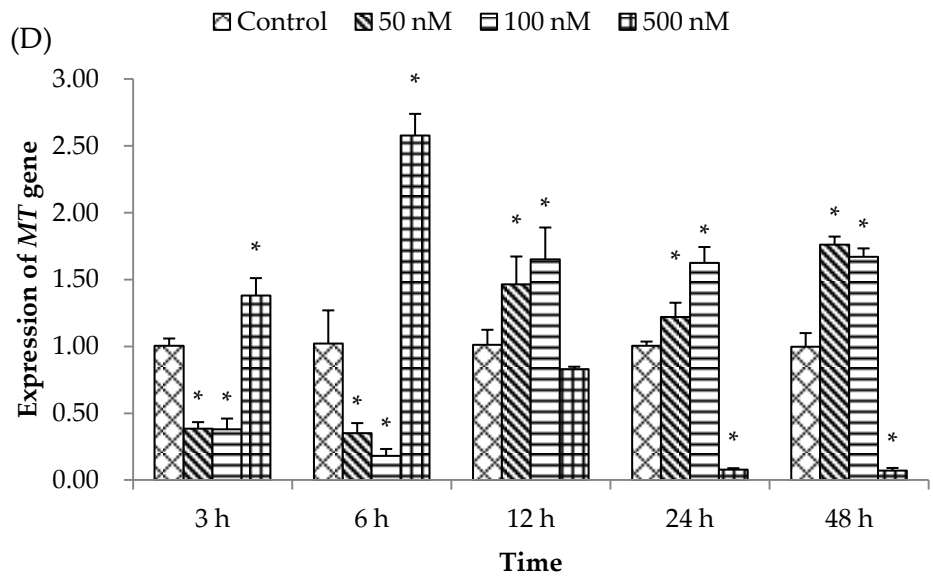

(A)
Control
50 nM
100 nM
500 nM
Expression of CTL-6 gene
1.20
1.00
0.80
0.60
0.40
0.20
0.00
3 h
6 h
12 h
24 h
48 h
Time


(B)
Control
50 nM
100 nM
500 nM
Expression of FREP gene
2.50
2.00
1.50
1.00
0.50
0.00
3 h
6 h
12 h
24 h
48 h
Time

(C)


Control
50 nM
100 nM
500 nM
Expression of HSP90 gene
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
3 h
6 h
12 h
24 h
48 h
Time

(D)


Control
50 nM
100 nM
500 nM
Expression of MT gene
3.00
2.50
2.00
1.50
1.00
0.50
0.00
3 h
6 h
12 h
24 h
48 h
Time

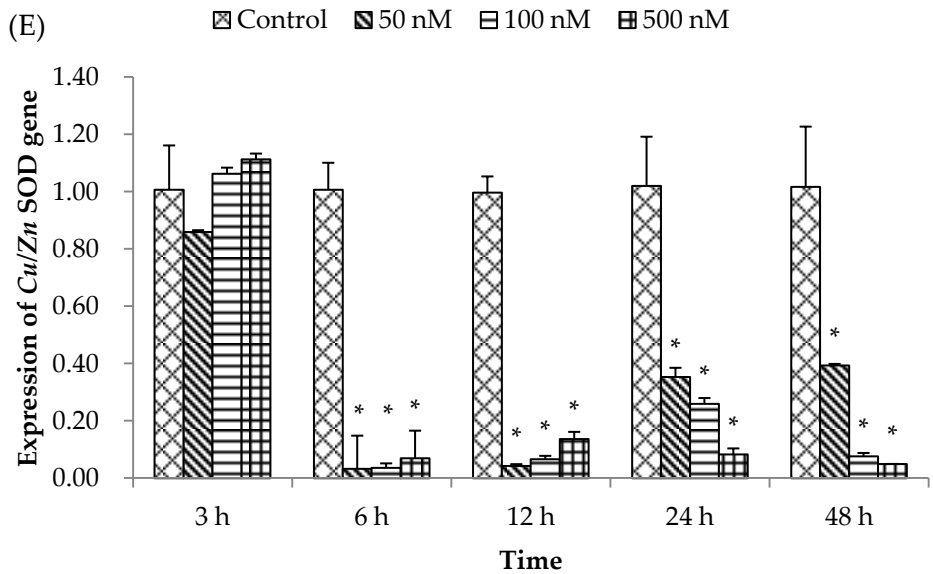


Figure 3.2. Effects of okadaic acid (OA) on non-specific immune responses in the bay scallop *Argopecten irradians* at different time intervals following exposure to three concentrations (50, 100, and 500 nM) of OA. (A) *CTL-6* gene; (B) *FREP* gene; (C) *HSP90* gene; (D) *MT* gene; (E) *Cu/ZnSOD* gene. Data represent mean ± SD values ($n = 3$) at the same time intervals with "*" denoting significant differences ($P<0.05$).

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Chapter IV

Antioxidant and Non-specific Immune Responses of Bay Scallop (*Argopecten irradians*) Exposed to Marine Toxin Okadaic Acid

Abstract

Okadaic acid (OA) is produced by dinoflagellates during harmful algal blooms and is a diarrhetic shellfish-poisoning (DSP) toxin. This toxin is particularly problematic for bivalves that are cultured for human consumption. This study aimed to reveal the effects of exposure to OA on the non-specific immune responses of bay scallop, *Argopecten irradians*. Various immunological parameters (superoxide dismutase (SOD), acid phosphatase (ACP), alkaline phosphatase (ALP), lysozyme activities, and total protein level) were assessed in the hemolymph of bay scallops at 3, 6, 12, 24, and 48 h post-exposure (hpe) to different concentrations (50, 100, and 500 nM) of OA. Moreover, the expression of immune system-related genes (*MnSOD*, *PrxV*, *PGRP*, and *BD*) was also measured. Results showed that SOD and ACP activities were decreased between 12–48 hpe. The ALP, lysozyme activities, and total protein levels were also modulated after exposure to different concentrations of OA. The expression of immune-system-related genes was also assessed at different time points during the exposure period. Overall, our results suggest that the exposure to OA had negative effects on the antioxidant and non-specific immune responses, and even disrupted the metabolism of bay scallops, making them more vulnerable to environmental stress-

inducing agents; they provide a better understanding of the response status of bivalves against DSP toxins.

Keywords: Okadaic Acid, Harmful Algal Blooms, Bay Scallop, *Argopecten irradians*, Non-specific Immune Response, Antioxidant Response.

4.1. Introduction

Harmful algal blooms (HABs), result from a combination of physical, chemical, and biological mechanisms and their interactions with other components of the food web that are mostly poorly understood. HABs are well known to cause ecological and economic losses in the coastal areas [1]. HABs also negatively impact the public health and threaten the aquaculture industry [2], as they can cause mass mortality of cultivated animals due to the toxins they produce [3]. Bivalves are particularly affected during the HAB events as they accumulate high levels of algal toxins in their tissues because of their sessile and filter-feeding habits [4]. Previous studies have revealed the impact of algal toxins on oysters, mussels, or clam shells, following exposure to toxic algae [4]. Moreover, some studies have also reported the effects of phycotoxins by feeding or exposing mussels and oysters to harmful algae, which secrete various phycotoxins and other metabolites.

Shellfish toxins, which are the main marine phycotoxins, can ultimately be consumed by humans in the food chain, and cause a variety of gastrointestinal and neurological illnesses, including paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), amnesic shellfish poisoning (ASP), neurotoxic shellfish poisoning (NSP), and azaspiracid shellfish poisoning (AZP) [4, 5]. The DSP toxins are induced by a series of microalgal toxins, including okadaic acid (OA), dinophysistoxins, pectenotoxins, and yessotoxins [6].These are produced by some microalgae of the genera *Dinophysis* and *Prorocentrum*, such as *Prorocentrum lima*, *Prorocentrum concavum*, *Prorocentrum maculosum*, *Dinophysis acuminata*, *Prorocentrum rhathymum*, and *Dinophysis fortii* [4, 5]. These toxins can accumulate in the fatty tissue of the bivalves [7]. Among them, OA and its derivatives are the best representatives of the DSP toxins [8], which have the ability to inhibit protein serine/threonine

phosphatases due to their interactions with the protein phosphatase 1- and protein phosphatase 2A-catalytic domains of these enzymes [6], leading to metabolic process deregulation and hyperphosphorylation of many cellular proteins [9]. It is well known that microalgae *Prorocentrum spp*. often participate in symbiotic relationships with different benthic marine invertebrates and macrophytic algae. This is the reason for easy penetration of OA into planktivorous mollusks as well as into other marine animals through the food chain [6]. Previous studies have reported that exposure to OA or *P. lima* causes damage to the hemocyte function and the viability of carpet shell clams (*Ruditapes decussatus*) [4, 10]. Huang *et al.* [5] also revealed that *P. lima*, a dinoflagellate producing OA, induced cytoskeleton disorganization, oxidative stress, and dysfunction of metabolism in mussels. With regard to the effects of marine toxins on bivalves, most of the studies have focused on clams or mussels [9-11]. Indeed, much less information is available in scallops, some of which are widely farmed by the aquaculture industry for food and have important economic value. Scallops accumulate toxins in their tissues to a greater extent as they have a low metabolic rate [12]. Hence, scallops are also vulnerable to the marine phycotoxins. The bay scallop (*Argopecten irradians*) was introduced into China from America and has been cultured in the coastal provinces of China for more than 30 years. Bay scallop farming is also presently suffering from HABs [13]. Previously we investigated the toxicity of different concentrations of OA on bay scallopand we demonstrated that various physiological parameters, including total hemocyte counts (THC), and contents of reactive oxygen species (ROS), malondialdehyde (MDA), glutathione (GSH), lactate dehydrogenase (LDH), and nitric oxide (NO) in the hemolymph of scallops were affected by different concentrations of OA (50, 100, and 500 nM), 3, 6, 12, 24, and 48 h post-exposure (hpe). Overall, our previous study demonstrated that the exposure to

OA was toxic for the physiological function, increased the oxygenic stress, and disrupted the metabolism of bay scallops. However, the effect of OA on the immune status or modulation of the immune response of bay scallop, which is related to defense mechanism against various pathogens, and morbidity or mortality, needs to be understood clearly.

Bivalves, including scallops only have limited mechanism for controlling and reducing the impact of pathogens, and lack a specific immune response and immunological memory. Therefore, they rely totally on their non-specific immune system to overcome diseases [14]. As scallops do not produce antibodies, vaccination cannot be used to protect them against pathogens. The non-specific immune system of scallops depends on circulating cells and a large variety of molecular effectors. Invertebrate cellular defense depends mainly on the hemocytes. The different molecules that are biologically active in the hemolymph of the bivalve mollusks are generally classified into two categories: serologically active molecules (opsonins, lysins, agglutinins, antimicrobial factors, lysozymes) and the enzymes of lysosomal origin (aminopeptidases, β-glucuronidases, acid phosphatase, alkaline phosphatase, α-mannosidase, esterases, and peroxidases) [14]. Alkaline phosphatase (ALP), which is a short-term biomarker for stress, is a polyfunctional phosphomonoester hydrolase [15]. In addition, some antimicrobial compounds involved in the humoral immune responses, such as the lysosomal enzyme acid phosphatase (ACP), also participate in the degradation of foreign proteins, carbohydrates and lipids [16]. Several antioxidant enzymes, such as superoxide dismutase (SOD) are considered to be the first and most important line of defense against ROS and protect the tissues from oxidative damage. Therefore, ALP, ACP, and SOD are essential components in the understanding of the immune response of scallops [17, 18].

The aim of this study was to assess the impact of OA exposure on the non-specific immune responses of the scallop *A. irradians*. Scallops were exposed to different concentrations of OA (50, 100, and 500 nM) for 48 h. At 3, 6, 12, 24, and 48 hpe, several non-specific immune parameters (like acid phosphatase, alkaline phosphatase, lysozyme, and superoxide dismutase activities) chosen as immunomarkers were monitored. In addition, we examined the transcription levels of several immune system-related genes (*PrxV*, *PGRP*, *BD*, and *MnSOD*). To our knowledge, this is the first study evaluatingthe effect of purified OA on the non-specific immune and antioxidant responses and the expression of immune and antioxidant -related genes in bay scallop by *in vitro* exposure in order to ascertain its effects on the general immune and physiological status, and to obtain new insights regarding the response mechanism of bivalves against DSP toxins.

4.2. Materials and methods

4.2.1. *Okadaic acid*

Okadaic acid (92–100% HPLC purified), was obtained from Sigma–Aldrich Co. LLC (Sigma, USA) and stored at 4 ℃ in a refrigerator until use.

4.2.2. *Animals*

Bay scallops (mean weight: 46.02 ± 2.67g), *A. irradians*, averaging 60–70 mm in shell length, were collected from the Noryangjin fisheries wholesale market (Seoul, South Korea) and maintained in lantern nets suspended in 800-L tanks containing filtered and aerated sea water to acclimatize them to the laboratory conditions (temperature: 10 ± 1 ℃; salinity: 30 ± 0.1‰) for 2 weeks. Half of the seawater was changed every day. The scallops were fed commercial shellfish diet (Instant Algae®

Shellfish Diet; Reed Mariculture Inc., Campbell, CA, USA) at a rate of approximately 1.2×10^{10} algae cells per scallop per day.

Three hundred and sixty bay scallops were randomly divided into a control (without OA) and three treatment groups (with OA). Each group consisted of 30 scallops with three replicates. The OA was dissolved in 1 mL of dimethyl sulfoxide (DMSO) to prepare the OA solution [10], and it was ensured that the final concentration of OA in the three OA treatment groups was 50, 100, and 500 nM, respectively. The control group was treated with an equal volume of DMSO. The three OA concentrations were selected based on previous studies reporting the cytotoxic and genotoxic effects of OA on different cell lines, including HeLa, Caco-2, U-937, Clone 9, and IEC-6 [8, 10, 19, 20]. Three scallops from each replicate treatment group were randomly collected at 3, 6, 12, 24, and 48 hpe. Two milliliter of hemolymph was collected from each adductor muscle using a 1-mL sterile syringe fitted with a 22-gauge needle within 1 min of removing a scallop from the tank. Individual scallops were sampled once to avoid repeated drawing of blood and/or handling stress. A 100-μL sample of hemolymph from each replicate treatment group was used for RNA extraction. The remaining hemolymph from each replicate treatment group was centrifuged at $750 \times g$ for 3 min to collect the cell-free hemolymph, which was then stored at –80 ℃, until testing for humoral immune parameters [4].

4.2.3. *Assay for the non-specific immune parameters*

4.2.3.1. Measurement of superoxide dismutase activity

Superoxide dismutase (SOD) activity was determined according to the method described by Ōyanagui [21] using SOD kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China), following the manufacturer's instructions. The optical density

(OD) was measured at 550 nm. One unit of SOD was defined as the amount required to decrease the rate of xanthine reduction by 50% in a 1-mL reaction system. The specific SOD activity was expressed as SOD units per mL of hemolymph.

4.2.3.2. Measurement of acid phosphatase activity

Acid phosphatase (ACP) activity in the hemolymphwas spectrophotometrically measured with an acid phosphatase detection kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) using disodium phenyl phosphate as a substrate. One unit of ACP activity was defined as the amount of enzyme in 100 mL of hemolymph necessary to produce 1 mg of nitrophenol in 30 min at 37 ℃.

4.2.3.3. Measurement of alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was determined using a chemical detection kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). One unit of ALP activity corresponded to 1 mg of phenol liberated per 100 mL hemolymph.

4.2.3.4. Measurement of lysozyme activity

Lysozyme activity was measured using a lysozyme kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) following the manufacturer's instructions. One unit of lysozyme activity was defined as the amount of lysozyme that caused a decrease in absorbance by 0.001 Unit per min at 530 nm.

4.2.3.5. Measurement of the total protein concentration

The total protein concentration in the scallop hemolymph was determined using kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China), following the

manufacturer's instructions.

4.2.4. *RNA Extraction and Reverse Transcription*

Total RNA was extracted from the hemolymph by using TRIzol Reagent (CWBio, Beijing, China). The quality and purity of RNA were assessed spectrophotometrically by determining the ratio of absorbance at 260 and 280 nm. Thereafter, the genomic DNA contamination was removed from the RNA preparation using DNase I (Promega, Madison, WI, USA). The cDNA was then synthesized using the PrimeScript™ RT Reagent Kit (TaKaRa Bio, Ostu, Japan) following the manufacturer's instructions, and was stored at –80 ℃ .

4.2.5. *Real-time quantitative PCR analyses of gene expression*

The expression of genes involved in the immune response (*PrxV*, *PGRP*, *BD*, and *MnSOD*) was monitored using real-time quantitative PCR (qPCR) (Qiagen, Hilden, Germany). All qPCRs were performed using SYBR Premix Ex Taq™ Perfect Real-Time Kits (TaKaRa Bio, Ostu, Japan) on a QiagenRotor-Gene Q RT-PCR Detection System (Qiagen, Hilden, Germany). The gene expression was normalized using the housekeeping gene *β-actin*. The sequences of the PCR primers used for qPCR are listed in Table 1 [22-25]. The reaction mixture included 10 μL SYBR Premix Ex Taq™, 1 μL of the forward and reverse primers (10 mM), and 1 μL cDNA. Ultra-pure water was then added to the reaction to make up the final volume to 20 μL. The reaction conditions were as follows: 95 ℃ for 10 min followed by 40 cycles of 95 ℃ for 45 s, 56 ℃ for 45 s, and 72 ℃ for 30 s. After the amplification phase, a melting curve analysis was conducted to account for the possibility of non-specific amplification or primer dimer formation [26]. A standard curve was created from serial dilutions of the

sample cDNA and was drawn by plotting the natural log of the threshold cycle (Ct) against the number of molecules. The standard curve of each gene was run in duplicate and triplicate to obtain a reliable measure of the amplification efficiency. The correlation coefficients (R^2) for all the standard curves were > 0.99 and the amplification efficiencies were between 90 and 110%. The relative expression ratios of the target genes in the treatment groups versus those in the control group were calculated according to the following formula: Fold changes = $2^{-\Delta\Delta}$Ct, where $^{\Delta\Delta}$Ct = [Ct (treatment group) – Ct (treatment *β-actin*)] – [Ct (control group) – (control *β-actin*)] [27]. In all the cases, the PCR was carried out in triplicate.

4.2.6. *Statistical analyses*

Normality and homogeneity of variance were tested using the Kolmogorov–Smirnov and Cochran's tests, respectively. All the percentage data were arcsine-transformed and subjected to one-way ANOVA. The values are expressed as the arithmetic mean ± standard deviation (SD). The differences were determined using the LSD test in the SPSS statistical software version 19.0 (IBM Corp., Armonk, NY, USA) with *P*-values < *0.05* indicating a statistical significance.

4.3. Results

4.3.1. *Non-specific immune responses*

4.3.1.1. SOD activity

The SOD activity decreased (*P<0.05*) in the groups treated with 100–500 nM of OA after 3 h of exposure (Figure 4.1 A). Thereafter, the activity was significantly increased in all the treatment groups, 6 hpe. However, the activity was subsequently reduced (*P<0.05*) in all the OA-treated groups, 12–48 hpe.

4.3.1.2. ACP activity

The A*C*P activity (Figure 4.1 B) did not show a significant change upon exposure to the three concentrations of OA from 3 to 6 hpe compared to that in the control, except in the 500-nM OA-treated group, in which there was a significant decrease at 6 hpe. Thereafter, a significant decrease ($P<0.05$) in the A*C*P activities was observed at 12–48 hpe in all the OA treatment groups compared to that in the control.

4.3.1.3. ALP activity

The ALP activity (Figure 4.1 C) was affected by the OA exposure compared to the control group, and was significantly increased at the high concentration of OA (500 nM) at 12–24 hpe, after which the results were inversed at 48 hpe, as well as at the medium concentration of OA (100 nM) at 48 hpe. However, no significant change was identified at the low concentration of OA (50 nM) at any time point.

4.3.1.4. Lysozyme activity

The lysozyme activity (Figure 4.1 D) was significantly lower in the group treated with low concentration of OA at 3–6 hpe compared to that in the control group; however, it increased at 12 hpe. In the group treated with 100 nM of OA, an increased lysozyme activity was only recorded at 6 and 12 hpe. Moreover, a significant increase ($P<0.05$) in the lysozyme activity following the treatment with 500 nM OA was observed only at 3–12 hpe.

4.3.1.5 Total protein content

The total protein content (Figure 4.1 E) showed no significant difference in the

group treated with the low concentration of OA compared to that in the control group at each time point; however, it was higher ($P<0.05$) in the groups treated with the medium and high concentrations of OA at 12 hpe.

4.3.2. *Expression of immune system-related genes*

4.3.2.1. Manganese superoxide dismutase (MnSOD) gene

The *MnSOD* expression in all the OA-treated groups was significantly down-regulated ($P<0.05$) at the initial 3 hpe; the expression was transiently up-regulated ($P<0.05$) at 6 hpe, after which it significantly decreased again at 12–48 hpe in the OA-treated groups compared to that in the control (Figure 4.2 A).

4.3.2.2. Peroxiredoxin atypical 2-Cys (PrxV) gene

PrxV expression was down-regulated ($P<0.05$) during the initial hour in the three OA treatment groups; however, it was higher ($P<0.05$) in all the OA treatment groups at 6–48 hpe than in the control group, with the highest expression values observed at 24 hpe (Figure 4.2 B).

4.3.2.3. Peptidoglycan recognition protein (PGRP) gene

The expression of *PGRP* mRNA in the hemolymph of bay scallop treated with OA is shown in Figure 4.2 C. The expression of the *PGRP* in the 50 and 100 nM OA treatment groups was higher ($P<0.05$) at 6–48 hpe than in the control group, with the highest expression values obtained at 12 hpe. Moreover, the expression was up-regulated ($P<0.05$) in the group treated with 500 nM OA at 3–24 hpe, with the highest expression level obtained at 12 hpe; at 48 hpe the expression was restored to the same level as in the control.

4.3.2.4. Big defensin (*BD*) *gene*

The expression of *BD* in the hemolymph of bay scallop treated with OA is presented in Figure 4.2 D. The *BD* gene expression in all the OA-treated groups was significantly up-regulated ($P<0.05$) compared to that in the control. The expression in the group treated with low concentration of OA gradually decreased from 3 to 48 hpe; however, the expression in the groups treated with medium and high concentrations of OA was increased from 3 to 48 hpe.

4.4. Discussion

Okadaic acid (OA) is a dinoflagellate toxin, that accumulats in shellfish and causes diarrhetic shellfish poisoning (DSP) in humans [28]. It is a potent and non-selective inhibitor of serine/threonine phosphatases and has been shown to be cytotoxic in a variety of cell lines [29]. Our previous study revealed that exposure to OA could affect the physical responses, induce oxygenic stress, and disrupte the metabolism in bay scallops [4]. To further evaluate whether OA could cause immunosuppression in bay scallop, making them more vulnerable to the stressful agents in the environment, in this study, we evaluated a number of non-specific immune responses (e.g. SOD, ACP, ALP, and lysozyme activities and the total protein content) and determined the expression of the immune system-related genes (*MnSOD*, *PrxV*, *PGRP*, and *BD*) in the hemolymph of bay scallops exposed to different concentrations of OA (50, 100, and 500 nM).

The total protein content in the hemolymph has also been used to evaluate the health status in bivalves [9]. It has been suggested to be affected under stress situations, such as in physiological changes or in the presence of xenobiotics [3]. Consistent with

these findings, an increment in the total protein level in the hemolymph of bay scallop was observed during the OA exposure. These results were similar to those of Simões *et al*. [3], in which the total protein level in the mussel, *Perna perna*, was shown to increase significantly during the blooms of the toxic alga, *Dinophysis acuminata*, that produces OA. The induction of total protein content was also described in the mussel, *P. perna*, and a clam, *Anomalocardia brasiliana*, exposed to *D. acuminata* [9]. The increases in the total protein content might indicate histolysis triggered by the OA exposure.

ALP is involved in the degradation and breakdown of invading non-self molecules, which have been reported in numerous bivalve species [12]. In this study, the ALP activity was elevated by exposure to high concentration of OA at 12–24 hpe. This result is consistent with that of a previous study [18], which showed that 20 and 40 mg/L of palmitoleic acid (PA) significantly induced the ALP activity in bay scallop from 3 to 12 hpe. Similar results were also reported in previous investigations, in which the ALP activity in zhikong scallop, *Chlamys farreri*, was observed to increase in response to the exposure to a number of environmental agents, such as temperature fluctuation, hypoxemia, virus infection, and Cu^{2+}. However, the ALP activity decreased significantly with time until 48 hpe upon treatment with 100 and 500 nM OA, a result consistent with those of a previous study where exposure to medium and high concentrations (40 and 80 mg/L, respectively) of PA, caused a decrease in the ALP activity at 24–48 hpe. The results presented here indicate that the ALP activity is sensitive to changes in the concentration and the duration of OA exposure. Low concentration of OA may not induce obvious changes in the ALP activity, whereas higher concentrations may induce it or may also cause the release of ALP into the extracellular medium during or after phagocytosis by hemocytes [14]. However, long-

term exposure may inhibit the ALP activity after 48 hpe.

Acid phosphatase (ACP) is an important hydrolytic enzyme in phagocytic lysosomes [30]. The ACP activity in the hemolymph of bay scallop decreased significantly after exposure to 500 nM OA from 6 hpe; however, from 12 to 48 hpe the ACP activity in all the treatment groups was dramatically lower than that in the control group. This result indicates that higher concentrations of OA or exposure for longer durations might easily inhibit the ACP activity, because OA is an inhibitor of phosphatase activity, which inhibits enzyme secretion by interfering with the later processes in stimulus–secretion coupling [31, 32]. Stronger ACP activity could enable the phagocyte to destroy and clear the pathogens more effectively, conferring an increased resistance to scallops against long-term pathogen invasion [16]. On the contrary, relatively weaker ACP activity might suggest the inhibitory effect of OA exposure on the non-specific immune responses of bay scallop.

Lysosomal enzymes are usually important parameters for evaluating the immunotoxicity of environmental stimuli or pollution to bivalves [33]. These enzymes are present in numerous animals and several body fluids [14]. At the initial stage of OA exposure, the lysozyme activity in the low concentration treatment group was inhibited, which is consistent with the results of previous research demonstrating the suppression of the lysozyme activity in the hemolymph of bay scallop upon exposure to PA. However, the lysozyme activity in the treatment groups exposed to higher concentrations of OA was induced at 3–12 hpe, whereas the increase in the low concentration treatment group was observed at 12 hpe, reflecting the fact that higher concentration of OA or longer duration of exposure can modulate some lysosomal enzymes to help bay scallop respond to the environmental stimuli or pollution until adaptation to such conditions.

The rapid adaptation of a species to sudden changes in the environmental oxygen content depends on its ability to increase its capacity for the production of antioxidants [34]. SOD catalyzes the disproportionation of the lipid peroxidation initiator and the transformation of superoxide radicals to H_2O_2 and O_2^- to prevent lipid peroxidation [6]. SOD has been used as a biomarker for monitoring environmental pollution [35]. Although the SOD activity was induced in a short time at 6 hpe, it was decreased from 12–48 hpe and the levels of MDA and LDH were consequently increased, as reported in our previous study [4], where bay scallops were treated with different concentrations of OA. This result indicates that the hemolymph might suffer from serious oxygen pressure caused by OA exposure for long time. Similarly, Zhang *et al.* [36] revealed that the SOD activity in one-month-old mice was significantly lower than that in the control group at 24 h after intraperitoneal injection of OA. Based on the metal ion cofactor in the active site, SODs are classified into iron SOD (FeSOD), manganese SOD (MnSOD), nickel SOD, and copper-zinc SOD (Cu/ZnSOD) [37]. Although the expression of *MnSOD* in the hemolymph of bay scallop was up-regulated at 6 hpe, it was significantly decreased at 3, 12, 24, and 48 hpe. Similar results were observed in our previous investigation [4], in which the expression of *Cu/ZnSOD* was observed to be down-regulated from 6 to 48 hpe after exposure to different concentrations of OA. Moreover, these results were also consistent with the SOD activity determined in the current study, which increased only at 6 hpe; however, the activity was suppressed in the groups treated with medium and high concentrations of OA at 3 hpe and all the OA treatment groups from 12 to 48 hpe. In the light of our previous study, the present results indicate that OA modulates the SOD gene expression in the hemolymph of bay scallop..

Under normal conditions, the intracellular levels of ROS are strictly maintained

and controlled by peroxiredoxins (Prxs) and other enzymes. Prxs constitute a family of ubiquitously expressed proteins that play a protective antioxidant role by scavenging H_2O_2 [19]. Prxs from mammals are divided into three categories: 2-Cys (PrxIeIV), atypical 2-Cys (PrxV), and 1-Cys (PrxVI) [20]. PrxV prevents ROS mediated damage and ensures that cells respond appropriately to the increased levels of oxidative stress through H_2O_2-mediated signaling pathways [19]. Our results revealed that *PrxV* expression was down-regulated initially and was up-regulated at 6–48 hpe. These results were similar to those of a previous investigation demonstrating that *PrxV* expression in the hemolymph of bay scallop was suppressed initially after PA treatment, and was subsequently induced in the PA treatment groups at 6–24 hpe [16]. This phenomenon suggests that OA might be able to suppress *PrxV* expression immediately following a short exposure as a stress response; however, the increase of *PrxV* expression in the hemolymph of bay scallop suggests that PrxV is crucial for the protection of the organism from oxygen stress caused by the increased ROS levels, as reported in our previous study [16].

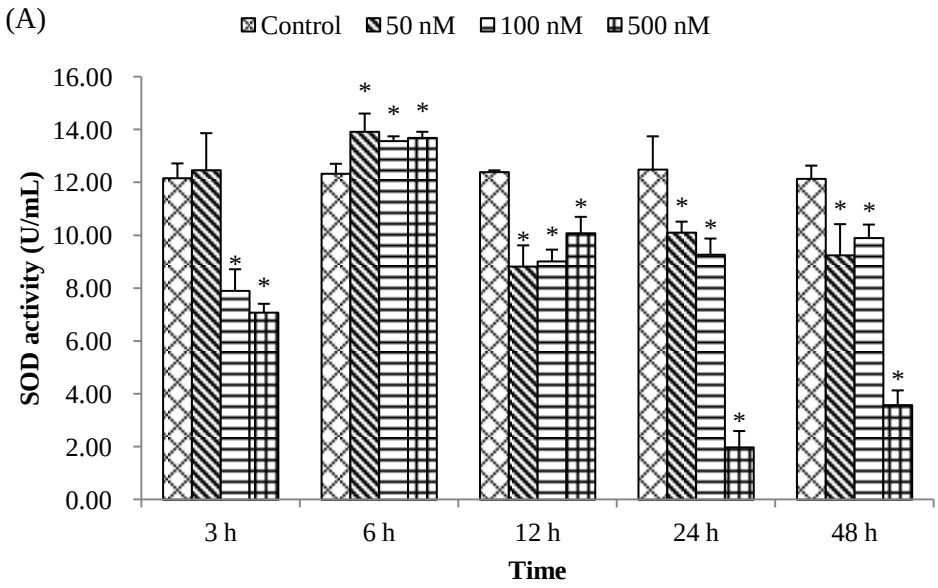
Peptidoglycan recognition protein (PGRP) is a kind of pattern-recognition receptors recognizing and binding the pathogen-associated molecular patterns of microorganisms, such as lipopolysaccharide (LPS), peptidoglycan (PGN) and related molecules, and some PGRPs can even recognize and bind other molecules. [23]. Antimicrobial peptides (AMPs) are often small cationic molecules widely distributed in all the organisms, and are thought to be a common feature of non-specific immunity in animals. Big defensin (BD) is an AMP with remarkable microbicidal activity against gram-positive and gram-negative bacteria, and fungi in scallops [24]. The expression of the *PGRP* gene in the hemolymph of bay scallop was significantly increased after exposure to different concentrations of OA, indicating that PGRP was a constitutive

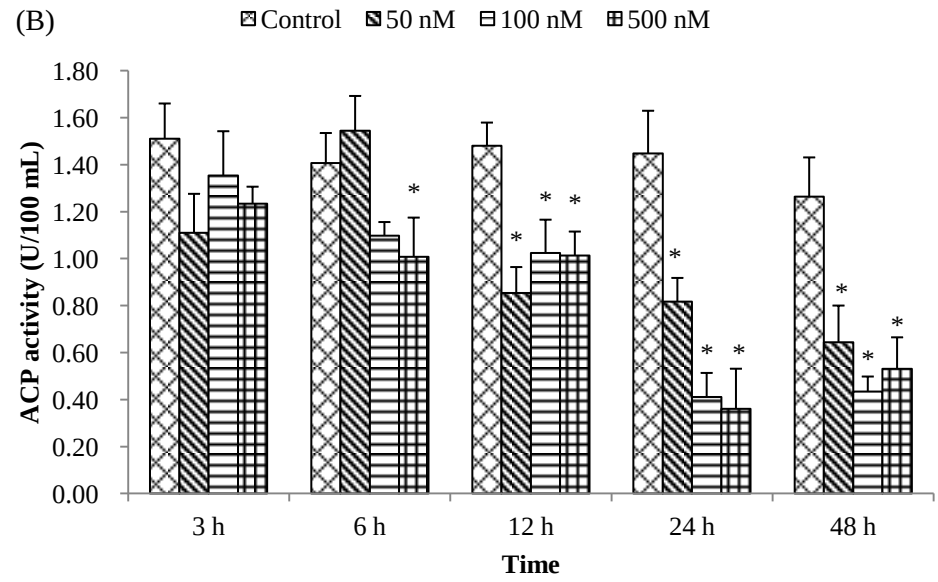
and inducible acute-phase protein that might be induced by OA. The expression of the *BD* gene was up-regulated during exposure to OA. It was speculated that during this period, BD-producing hemocytes were considerably recruited and mobilized to synthesize the *BD* mRNA. It was also inferred that OA exposure caused alteration in the immune responses of bay scallop. However, to understand the OA-driven immune gene regulations, transcriptome analysis is currently underway.

In general, the results of the present investigation demonstrated that exposure to different concentrations of OA modulates the various immune and antioxidant-related parameters (SOD, ACP, ALP, lysozyme activities, and the total protein level) and gene expression (*MnSOD*, *PrxV*, *PGRP*, and *BD*). Altogether, these changes in general physiological status of bay scallop indicate that the exposure to the toxin OA could induce negative effects on the important defense and antioxidant mechanisms, which could mitigate damage in scallops experiencing oxidative stress. Therefore, our findings might provide preliminary information or new insights for the identification of an early response or adaptation of the bay scallops against marine biotoxins. However, further work is required to assess its effects on the immune factor response at cellular and DNA level, or with regard to the cell function.

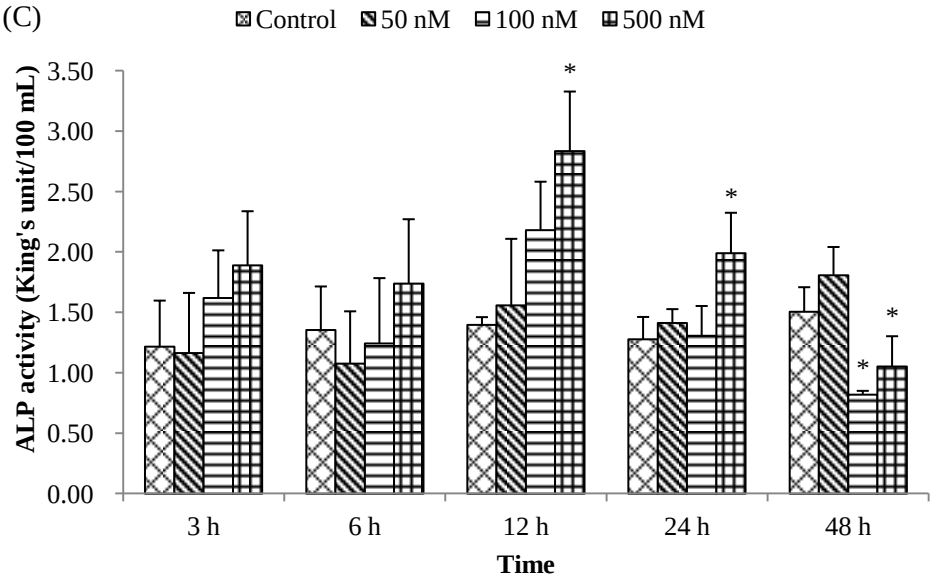
Table 4.1 Primers used for the analysis of mRNA expression by qRT-PCR.

Genes	Primer sequence	Accession no.
β-actin	F: 5′-CAAACAGCAGCCTCCTCGTCA-3′ R: 5′-CTGGGCACCTGAACCTTTCGTT-3′	AY335441
MnSOD	F: 5′-AATAGGGATTTTGGCTCGTTTG-3′ R: 5′-TGGTTGAAGTGGGTCCTGGTTA-3′	EU137676
PrxV	F: 5′-AATCAAGGAGCGGCTGGCA3′ R: 5′-TCAACTTCTCAATCTTCCCGTCAT-3′	HM461987
PGRP	F: 5′GGGCAAGTGTATGAGGGAAGAG-3′ R: 5′-TCCGATGAAGGAGACAGCGTAG-3′	AY437875
BD	F: 5′-CGTGCCATACCCATTGCTTA-3′ R: 5′-ATGATTGTCGTTGCTCCTTGAT-3′	DQ334340

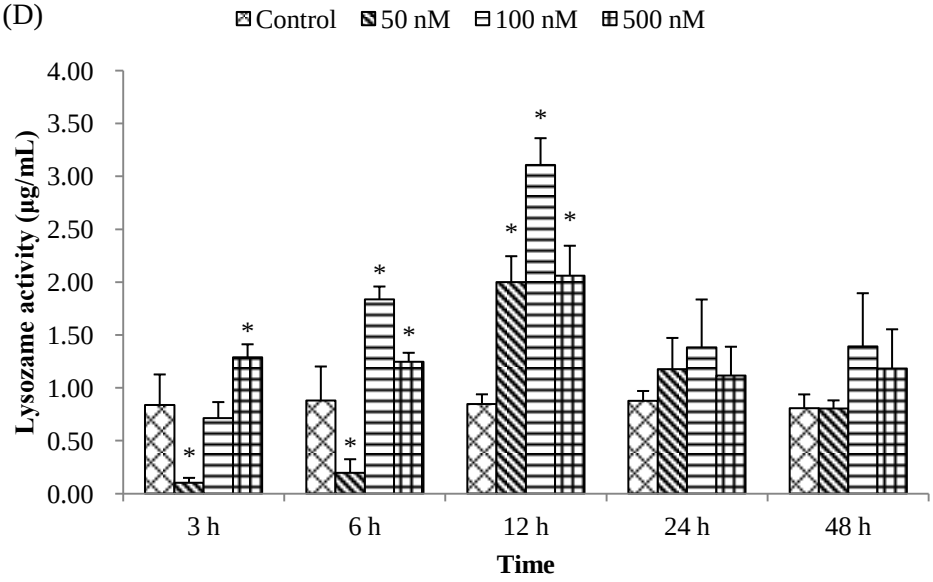

(A)
Control
50 nM
100 nM
500 nM
SOD activity (U/mL)
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
3 h
6 h
12 h
24 h
48 h
Time


(B)
Control
50 nM
100 nM
500 nM
ACP activity (U/100 mL)
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
3 h
6 h
12 h
24 h
48 h
Time

(C)


Control
50 nM
100 nM
500 nM
ALP activity (King's unit/100 mL)
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
3 h
6 h
12 h
24 h
48 h
Time

(D)


Control
50 nM
100 nM
500 nM
Lysozame activity (μg/mL)
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
3 h
6 h
12 h
24 h
48 h
Time

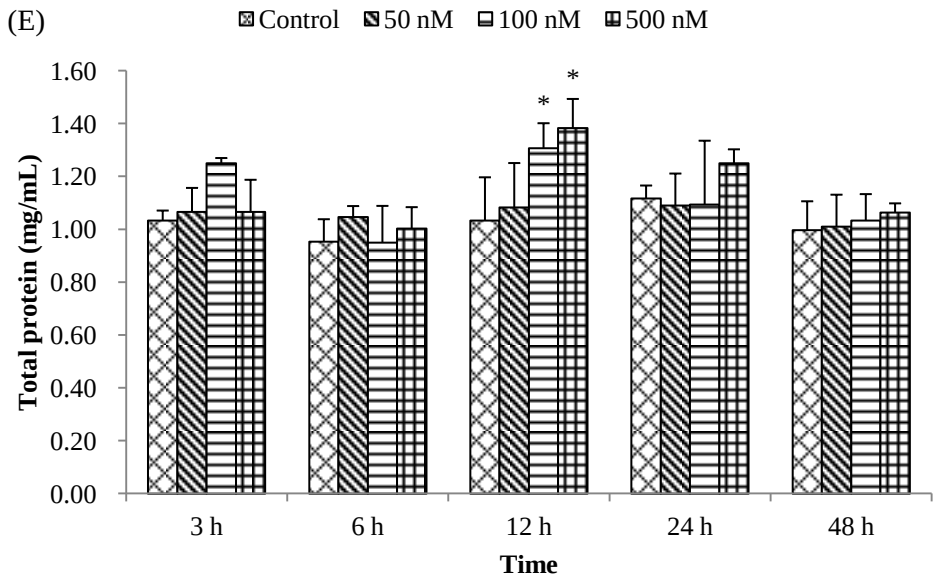
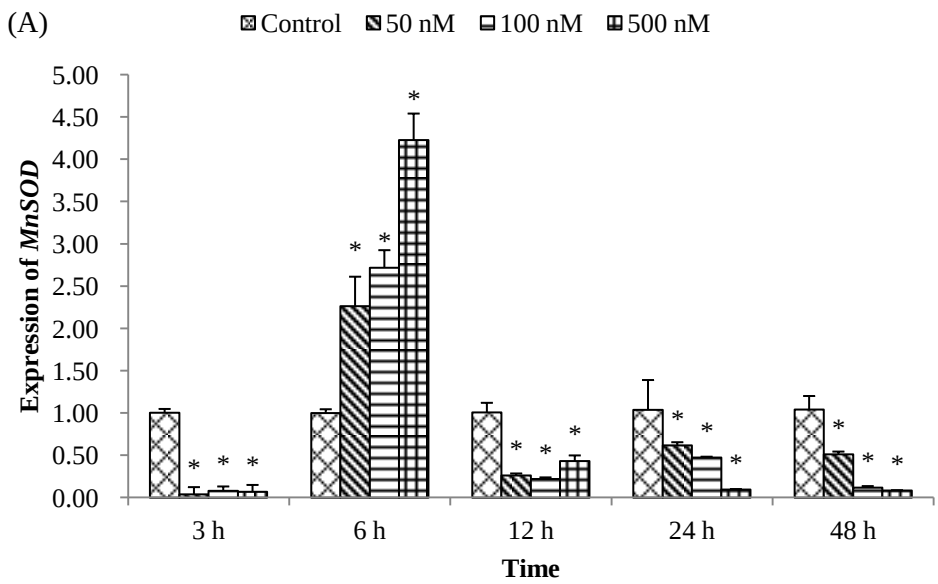
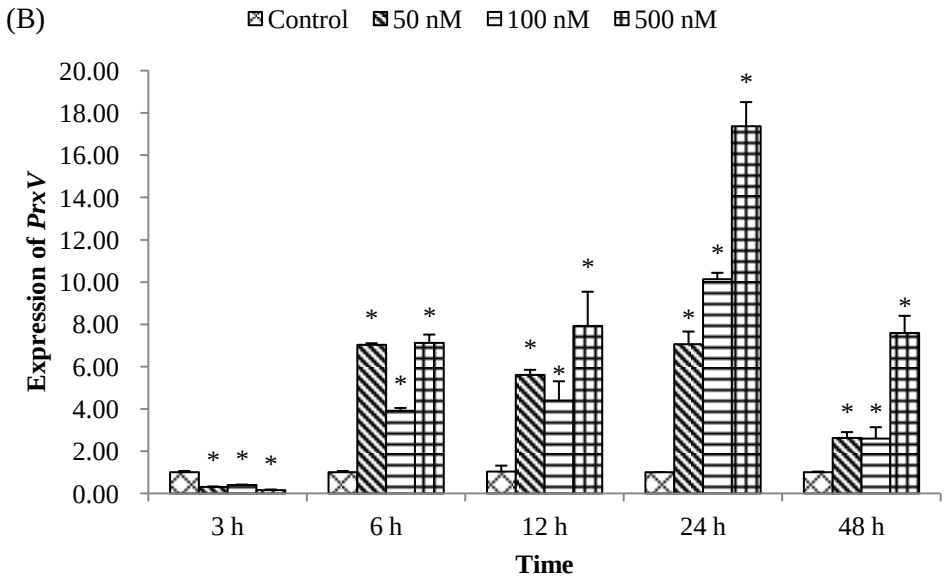


Figure 4.1. Effect of okadaic acid (OA) on the non-specific immune responses in the bay scallop *Argopecten irradians* at different time points after exposure to three concentrations (50, 100, and 500 nM) of OA. (A) superoxide dismutase (SOD) activity; (B) acid phosphatase (ACP) activity; (C) alkaline phosphatase (ALP) activity; (D) lysozyme activity; (E) total protein content; Data represent mean ± SD values ($n = 3$) at the same sampling time with "*" denoting significant differences ($P<0.05$).


(A)
Control
50 nM
100 nM
500 nM
Expression of MnSOD
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
3 h
6 h
12 h
24 h
48 h
Time


(B)
Control
50 nM
100 nM
500 nM
Expression of PrxV
20.00
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
3 h
6 h
12 h
24 h
48 h
Time

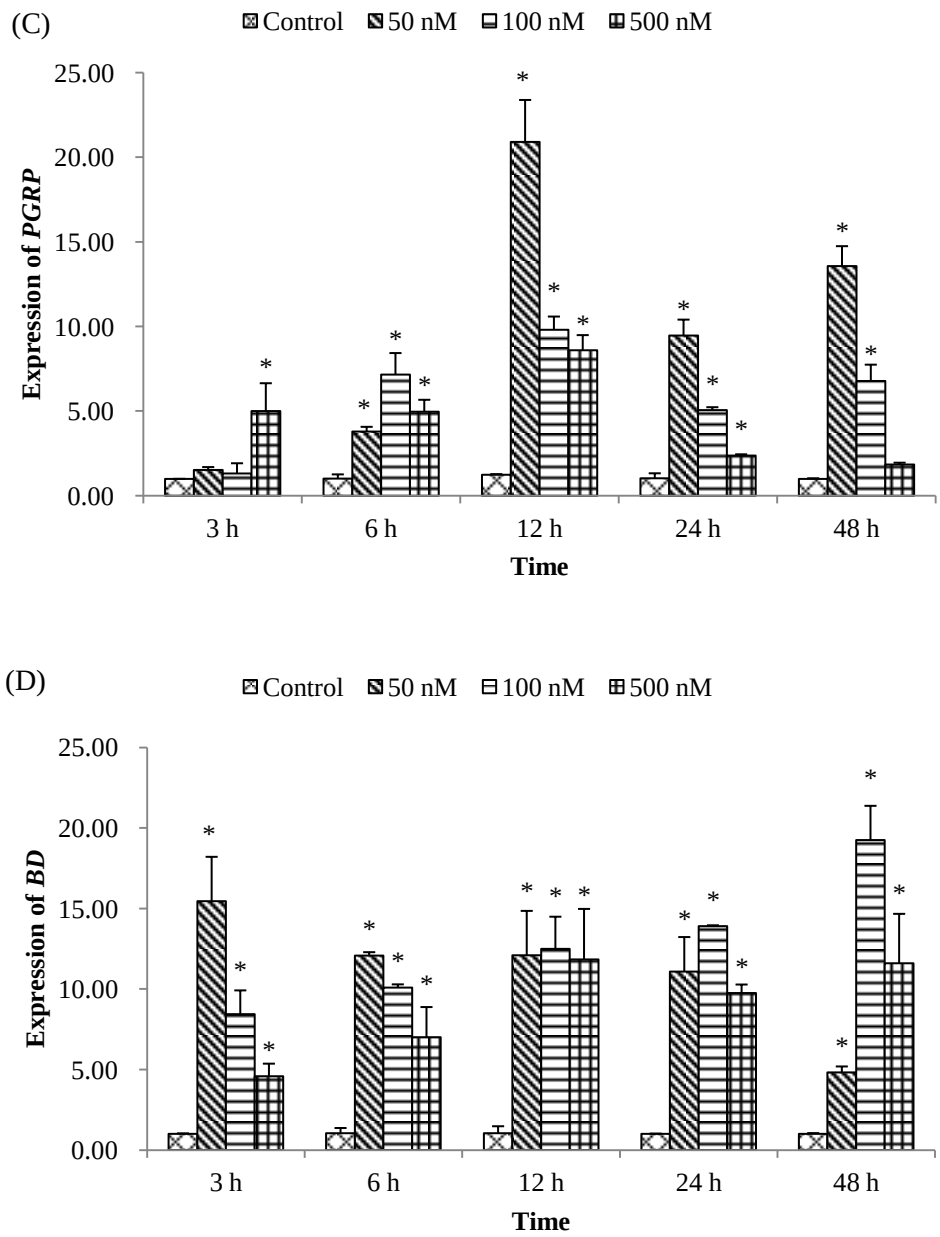


Figure 4.2. Effect of okadaic acid (OA) on the immune-related genes in the bay scallop *Argopecten irradians* at different time points after exposure to three concentrations (50, 100, and 500 nM) of OA. (A) *MnSOD* gene; (B) *PrxV* gene; (C) *PGRP* gene; (D) *BD* gene; Data represent mean ± SD values (*n = 3*) at the same

sampling time with "*" denoting significant differences ($P<0.05$).

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General conclusion

The present study revealed an alteration in immune responses caused by exposure to investigated concentrations of algicide PA (20, 40, and 80 mg/L) and toxin OA (10, 50 and 100 nM). Although, it is not clear whether overall immunocompetence of the organism was compromised, exposure to different concentrations of PA and OA can significantly affect several immune parameters and immune related genes of scallop. Therefore, these findings implicate a potential risk of popularizing and applying of PA as an algicide management in scallop production. Also, the results of the present study may could arouse researchers' attention to investigate the impacts of using some other algicides to control the outbreak of algal blooms in the marine environment. Currently, further studies are underway to explore the mechanism of action of PA. As the mollusc immune system consists of a multifaceted defence system, suppression of one aspect of immune function may be compensated for by another defence mechanism.

In addition, the results of the present investigation demonstrated that exposure to different concentrations of OA modulates the various immune and gene expression. Altogether, these results revealed significant changes in general physiological status of bay scallop several of the immune-system-related parameters and expression of gene that were monitored, indicating which indicate that the exposure to the toxin OA could induce negative effects on the important defense, which could mitigate damage in scallops experiencing oxidative stress. can slowly diminish the immune system of bay scallop., especially when they are exposed to high concentrations of toxins or are exposed for a long period.

Furthermore, significant changes in the expression of antioxidant and immune response-related genes suggest the presence of cellular stress.

Therefore, our findings might provide preliminary information or new insights for the identification of an early response or adaptation of the bay scallops against variouse of exgenous substances.

국문초록

이매패류에 있어 외인성 화합물(Palmitoleic Acid와 Okadaic Acid)에 의한 생리면역학적 특성 규명

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살조제 성분인 Palmitoleic acid (PA)는 유해성 와편모조류인 *Alexandrium tamarense* 제거를 위해 사용되지만 가리비에 미치는 영향은 현재까지 밝혀지지 않았다. Okadaic acid (OA)는 유해 적조현상 시 발생하는 와편모조류에 의해 생성되며 설사를 유발하는 패류 독소로 알려져 있다. 본 연구는 유해 적조현상과 관련된 환경 물질인 살조제 PA와 조류 독소 OA가 가리비에 미치는 영향을 평가하여 향후 가리비 양식산업의 지속적인 발전을 통한 생산량 향상을 위해 실시 되었다. 따라서 본 연구에서는 다양한 면역학적 매개 변수들인 총 혈구 계수 (THC), 활성 산소 (ROS), malondialdehyde (MDA), glutathione (GSH), lactate

dehydrogenase (LDH), nitric oxide (NO), superoxide dismutase (SOD), acid phosphatase (ACP), alkarine phosphaase (ALP), lysozyme activity 그리고 총 단백질 조성을 다양한 농도의 PA (20, 40, 80 mg/L)와 OA (50, 100, 500 nM)에 가리비를 노출 시킨 후 3, 6, 12, 24, 48시간 간격으로 혈림프를 추출하여 확인하였다. 또한 면역과 관련된 유전자인 *CLT-6, FREP, HSP90, PGRP, MT, Cu/ZnSOD, MnSOD, PrxV* 그리고 BW의 발현을 세가지 다른 농도의 PA와 OA에 노출 시킨 후 3, 6, 12, 24, 48시간 간격으로 조사하였다.

실험 결과 lysozyme activity의 경우 PA를 처리한 가리비는 대조군에 비해 12, 24, 48 hpe에서 감소하는 것으로 확인되었다. SOD activity의 경우 PA를 처리한 모든 그룹에서 높게 확인되었으나, 급격한 증가는 20 mg/L로 처리한 그룹의 12, 24, 48 hpe에서만 확인되었다. ACP activity의 경우 대조군에 비해 80 mg/ml의 PA를 처리한 그룹의 6-48 hpe에서 향상된 결과를 나타내었다. 총 단백질량 정도는 PA를 처리한 모든 그룹에서 향상된 것으로 확인 되었으며, 특히 40 mg/L의 3 hpe와 80 mg/L로 처리된 그룹의 6, 12, 24, 48 hpe에서 뚜렷하게 나타났다. THC의 경우 PA를 처리한

그룹에서 감소를 나타냈지만 ALP는 PA를 처리한 모든 그룹의 3 hpe에서 현저히 증가하였다가 다시 현저하게 감소하였다. LDH와 NO의 경우 중간 농도와 고농도에서 현저히 향상 되었다. 특히 GSH는 PA를 처리한 모든 그룹에서 각각의 시간 간격에서 증가하는 양상을 나타내었다. 본 연구는 또한 다른 농도의 PA를 처리한 후에 면역 체계 반응에 관여하는 유전자 발현에 대한 가변적인 영향이 관찰되었음을 확인하였다. 또한 PA가 가리비의 내분비계나 면역 반응들을 방해할 수 있다는 것을 입증하였다. 따라서 본 연구는 해양 환경에서 적조 현상의 발생을 조절하기 위해 살조제로써 PA를 사용하는 것에 대한 잠재적인 위험성이 있음을 강조하였다.

게다가, 본 연구에서는 OA가 가리비의 면역 반응에 미치는 영향을 확인하였다. 연구 결과 SOD와 ACP 활성은 24-48 hpe 사이에 감소되는 것으로 확인되었다. ALP, lysozyme activity 그리고 총 단백질 정도 또한 다른 농도의 OA에 노출시킨 후 조절되었다. ROS, MDA, NO level 그리고 LDH activity의 경우 다른 농도의 OA에 노출 시킨 후 향상되었다; 그러나 THC와 GSH의 경우 24-48 hpe 사이에 감소되는 것으로 확인

되었다. 면역체계와 관련된 유전자의 발현 또한 노출 시간 간격 동안 상이한 시점에서 평가되었다. 따라서 이러한 결과들은 DSP 독소에 대한 이매패류의 반응 상태에 대해 더 나은 이해를 제공한다.

종합적으로, 본 연구의 결과는 살조제인 PA와 조류 독소인 OA에 대한 노출이 가리비의항산화 및 비특이적 면역 반응에 부정적인 영향을 미친다는 것을 시사하며, 환경 스트레스를 유발하는 요인들에 더욱 취약하게 만들어 해만 가리비의 신진대사를 방해하는 것으로 확인되었다.

Key words: 무척추동물, 면역 반응, 해만 가리비, 살조제, 조류 독소, Palmitoleic Acid, Okadaic Acid

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