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The Use of Selected Biomarkers, Phagocytic and Cholinesterase Activity to Detect the Effects of Dimethoate on Marine Mussel (*Mytilus edulis*)

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Effects of organophosphorous pesticide, dimethoate on blue mussels, *Mytilus edulis* using selected biomarkers have been studied. Mussels were exposed to serial dilutions of dimethoate, 7.88, 15.75, 31.35, and 63.00 µg/l including positive and negative controls for 14 days. The suppression effects of dimethoate on phagocytic activity significantly occurred at two lowest concentrations of dimethoate (7.88 and 15.75 µg/l), but stimulation effects significantly emerged at the following highest concentrations (31.35 and 63.00 µg/l). The declining tendency of the cholinesterase (ChE) activity (23% lower than the control) appeared when mussels exposed to 7.88 and 15.75 µg/l dimethoate. Moreover, the significant inhibition of the ChE activity occurred at 31.35 µg/l dimethoate exposure. This study suggested that the phagocytic and the ChE activity are useful biomarkers for assessing the affects of organophosporous pesticide, dimethoate on neuro-immune system of blue mussels, *M. edulis*.

Key words: dimethoate, cholinesterase, phagocytic, blue mussels

INTRODUCTION

Organophosphorus (OP) pesticides are extensively used in broad applications to replace persistence organochlorine pesticide due to the fast degradation rate and hence less persistence in any environmental compartment (Lartiges & Garrigues 1995; Floesser-Mueller & Schwack 2001). In spite of the fact that these compounds are much more unstable than organochlorine in the environment, their persistence toxicity on biota may leads to damage ecosystem (Gaglani & Bocquene 2000). Accordingly, the biological response characterization of biota that exposed to pesticide is an important step toward the evaluation of the risks. It is due to most of modern OPs compounds are deliberately synthesized to inhibit an important enzyme of nervous system, i.e. acetylcholinesterase (AChE) of target organisms (Galloway & Handy 2003). This enzyme plays a significant role on preventing an accumulation of a neurotransmitter compound, acetylcholine (ACh) at cholinergic synapses by hydrolyzing the compound. Consequently, the inactivation of AChE leads to the accumulation of ACh at the synaptic cleft, which ultimately blocks the transmission of nerve impulses (Lund *et al.* 2000). The inhibition effect of these pesticides on AChE was considered an irreversible effect, because the time needed to synthesis *de novo* of this enzyme is longer than the time of dissociation of the OP-AChE complex (Gaglani & Bocquene 2000; Hyne & Maher 2003). Likewise, the wastes of routine wide-spectrum of OP applications may cause adverse effects on non-target organisms significantly, which are raging from terrestrial to aquatic organisms (Fulton & Key 2001).

OP compounds not only inhibit cholinesterase (ChE) activity, but also interfere immune system of organisms (Banerjee *et al*. 1998; Galloway & Handy 2003). These insecticides are reactive and labile that can directly damage cell membranes, protein and DNA (Videira *et al*. 2001; Pena-Llopis 2005). They can also reduce vertebrate ability to make either humoral immune or cytotic T lymphocyte responses (Voccia *et al*. 1999). OP insecticides were used to control mosquito in coastal area have been detected inducing phagacytosis activity of lobster in laboratory resulting in decreasing of lobster immune capability against virus (De Guise *et al*. 2004). Moreover, Anees (1978) showed that OP pesticides like dimethoate were able to reduce erythrocyte densities and hemoglobin and color index of freshwater fish (*Channa punctatus*) indicating that the pesticide brought about an effect similar to the production of anemia. Hatching rate of characid fish (*Prochilodus lineatus*) eggs and the hatched larvae mobility were disrupted by low concentrations of pesticide containing 40% of dimethoate (Campagna *et al*. 2006).

Dimethoate is an organophosphorus pesticide that is known as an AChE inhibitor. Despite the main use of the pesticide is in the up land, the occurrence of this pesticide was detected in the shore of Mediterranean Sea up to the level of 39.9 µg/l (Hernandes *et al*. 1993). There are many experimental studies have been conducted to test the toxicity

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of dimethoate using freshwater organisms. In contrast, very few studies have been conducted to recognize the toxicity of dimethoate on marine organisms. Some acute toxicity tests using marine mussels (*Mytilus edulis*) (Serrano *et al*. 1999) and estuarine organism mysid (*Americamysis bahia*) (Roast *et al*. 1999) failed to show appreciable effects of dimethoate using the lethality as an endpoint. Nevertheless, inhibitions of ChE activity have been detected on freshwater fishes, *Poecilia reticulata* (Frasco & Guilhermino 2002) and common carp (*Cyprinus carpio*) (De Mel & Pathiratne 2005) exposed to sublethal concentrations in the chronic test. Besides, Perret *et al*. (1996) reported that dimethoate caused the inhibition of ChE activity of freshwater zebra mussel (*Dresissena polymorpha* Pallas).

Biomarkers and marine mussels have been employed as useful tools for risk assessment of chemical compounds that are discharged in marine ecosystem (Cajaraville *et al*. 2000; Livingstone *et al*. 2000; Dizer *et al*. 2001a) as these mussels have a strong capacity for bioconcentration of xenobiotic (Amiard *et al*. 2000). In fact, *M. edulis* has been well studied as a sentinel organism to assess the effects of some OP pesticide pollutants using ChE activity assay (Galloway *et al*. 2002; Rickwood & Galloway 2004; Brown *et al*. 2004) and to detect the potential immune suppression of some heavy metals and other pollutants in marine ecosystem using phagocytic activity (Pipe *et al*. 1999; Galloway & Depledge 2000). Notwithstanding, there is a scarcity of scientific data of dimethoate effects on *M. edulis* neuro-immune system to provide a basic knowledge of risk assessment of this pesticide in marine ecosystem. Hence, the studies to assess the effects of dimethoate on neuro-immune response of *M. edulis* using ChE and phagocytic activity assay as biomarkers are of interest. The objective of this current study was then to test the chronic effects of dimethoate on neuro-immune system of marine mussel, *M. edulis* using ChE and phagocytic activity assay.

MATERIALS AND METHODS

Chemical and Animal Preparation. The chemicals used in this study were purchased from Sigma (Germany), unless otherwise stated.

Marine mussels, *M. edulis* were collected from Sylt Island, Germany. The length of the mussels was 6-7 cm. The animals were acclimated to the laboratory temperature of 5 $\pm$ 1 ^{o}C and kept for two weeks in artificial seawater (ASW) (Tropic Marine® in distilled water) with salinity 3% prior to the experiment. Thereafter, the mussels were transferred to 4 l of ASW in glass aquarium following dimethoate (PESTANAL®, analytical standard (Riedel-de Haën)) exposure.

***In Vivo* Test.** The *in vivo* study was conducted for 14 days by changing ASW every 3 days at room temperature of 5 $\pm$ 1 ^{o}C. Adjustment of the AWS pH (pH 7) was performed prior to the medium replacement to ensure the stability of the used pesticide. Eight mussels were placed into 4 l of ASW and dosed with dissolved dimethoate in methanol to final concentrations of 0.00, 7.88, 15.75, 31.50, and 63.00 µg/l, including positive control. The setup of the serial dilutions of dimethoate was referred to the concentration of which revealed an inhibition effect on ChE activity of aquatic vertebrate, *Poecilia reticulata* (Frasco & Guilhermino 2002). The serial nominal concentrations covered also a realistic occurrence of the pesticide in the seawaters (Hernandes *et al*. 1993). Furthermore, a renewal of the contaminant was performed along with the renewal of the media. Mussels were fed per day by using 1 ml of commercial algae Kroonaqa® Aquatim consisting of *Nannochloropsis acculata*, *Isochrysis galbana*, and *Tetraselmis suecica*. The experiment was carried out in duplicate.

Cholinesterase Assay. The enzymatic activity was measured following the Ellman method (Ellman *et al*. 1961), but modified for a 96-well plate and microplate reading (Herbert *et al*. 1995; Dizer *et al*. 2002). Mussels were dissected out and gill tissue (0.32 $\pm$ 0.039 g) was homogenized in a Dounce homogenizer with 2 ml of potassium phosphate buffer (0.1 M/pH 8.0). The homogenate was centrifuged for 10 minutes at 10,000 x g and the supernatant was harvested and stored at -80 ^{o}C before analysis of ChE activity and protein content. The supernatant was diluted in 1:2 of potassium phosphate buffer (0.1 M/pH 8.0) following the enzyme measurement.

The enzyme measurement was carried out by placing 50 µl of the diluted sample into each well of the microplate. A blank was made by putting 50 µl of potassium phosphate buffer into a blank section of the microplate wells. The plate was incubated for 5 minutes in 25 ^{o}C with 200 µl of 0.75 mM 5,5'-Dithio-bis-(-2-Nitrobenzoic acid) prior to the reaction started by an addition of 50 µl of 3 mM Acethylthiocholine iodide. Accordingly, the plate was read by using a spectrophotometer for microtiter plate (Spectra Thermo TECAN) in an interval of 30 s for 5 min at 405 nm. Four independent measurements of ChE activity were carried out for each individual of *M. edulis*, and the average activity was calculated.

A protein content measurement was carried out by diluting the gill extract 1:10 with distilled water. It was measured previously by placing 10 µl of the diluted extract and 10 µl of serial dilutions of γ-globuline protein standard into separate well sections of the microplate. A blank was made by placing 10 µl of distilled water into a blank section of the microplate. After the addition of 5% Bradford-reagent solution (200 µl) into the microplate wells, the samples were left in room temperature for 20 minutes to allow color development. Furthermore, the absorbance was red at 620 nm using the spectrophotometer (Spectra Thermo TECAN).

Finally, AChE activity is expressed as nmoles of product developed per minute per mg of protein (nmol/min/mg protein).

Phagocytosis Assay. Phagocytic activity of hemocytes was determined by a microplate-based fluorescence measurement method (Hansen 1992; Anderson & Mora 1995). Briefly, 1 ml of mussel hemolymph was withdrawn from each posterior adductor muscle of mussels using 1 ml syringe and 0.4 mm needle. Subsequently, 100 µl of hemolymph was dropped into 96-microplate well. Five replicates of wells were used to analysis phagocytic activity and three replicates were used for protein analysis. The density of hemocytes from each mussel was calculated by using hemocytometer under a light transmission microscope. After the incubation of the plate for 30 minutes to allow hemocytes deposition at the bottom of the microplate well, 25 µl of Fluoresceinisothiocyanate (FITC)-labeled yeast was added into each phagocytic activity section of microplate wells. A standard was made by adding 100 µl of phosphate buffer saline (PBS) and 25 µl of standard section of FITC- labeled yeast into microplate wells. One column (8 wells) was used as a blank section by adding 125 µl of PBS. The plate was incubated for 90 minutes in 21 ^{o}C at dark condition. At the end of the incubation, 25 µl of 0.6 mg/ml trypan blue dissolved in PBS was added to each well of the microplate for quenching the fluorescence background of unphagocytosed cells. The plate was incubated for 20 minutes prior to the removing of all supernatants. The fluorescence was red at excitation of 485 nm and an emission of 535 nm using a fluoro meter for microplate (Dynatech, Fluorolite 1000).

A protein measurement was carried out using hemocytes only. Prior to the measurement, hemocytes were lysed with 50 µl of 0.1 N NaOH. After incubating the lysed hemocytes for 10 minutes in a shaking chamber, 10 µl of lysed hemocytes and protein standard were added to 96-microplate wells. Accordingly, 200 µl of 5% Bradford-reagent solution was added into the plate and incubated for 10 minutes to allow color development. The fluorescence of protein was measured at 620 nm using the spectrophotometer (Spectra Thermo TECAN). Finally, phagocytic activity was expressed as Relative Fluorescence Units (RFU) and calculated as a Phagocytic Index: RFU/mg hemocyte protein.

Statistical Analysis. Since both the phagocytic and the ChE activity data did not follow normal distribution, non-parametric test i.e. Kruskall-Wallis was used to differentiate the effect of administered dimethoate on the phagocyotosis and the ChE activity. Dunn's Multiple Comparison was used to recognize the differences among the treatments (Newman 1995).

RESULTS

Phagocytic Activity. The current study showed that both hemocyte numbers and phagocytic activity of the blue mussels before treatment were not significantly different (Figure 1 & 2) which provided a uniform state of the experiment. The exposure of dimethoate for 14 days to the animals depicted that the alteration of hemocytes numbers occurred. Circulating hemocyte density of mussel significantly increased ($P < 0.05$) on animals exposed to 31.50 and 63.00 µg/l of dimethoate (Figure 3), but there was no significant difference of circulating hemocyte numbers between them. There was a visible stimulation of circulating hemocytes numbers at 7.88 and 15.75 µg/l of dimethoate. However, because of high individual variations the statistical analysis could not detect any stimulation.

The dosed dimethoate to the mussels resulted in decreasing of phagocytic activity significantly at the concentrations of 7.88 µg/l and 15.75 µg/l of dimethoate (Figure 4). On the other side, the stimulation of the activity was significantly occurred at 31.50 µg/l of dimethoate ($P < 0.05$) compare to previous levels and persisted significantly at the same level at 63.00 µg/l of dimethoate (Figure 4).

Cholinesterase Activity. ChE assay was performed on the mussel gills at the end of the experiment. The results showed that dimethoate caused a significant effect on ChE activity at concentrations of 31.50 and 63.00 µg/l (Figure 5). Although,

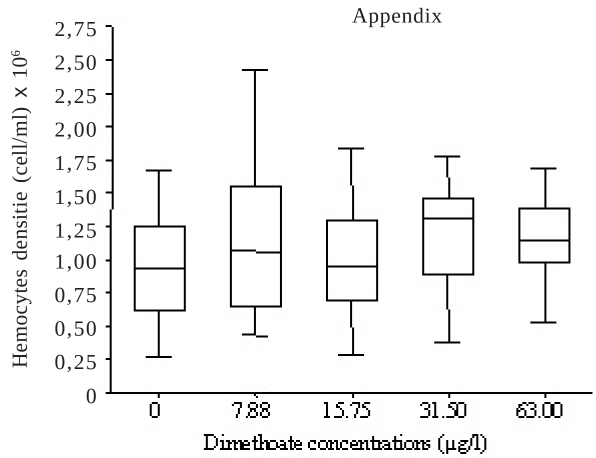

Figure 1. Circulating hemocyte density of *M. edulis* before the treatments. Data were expressed as median (25 and 75% quartile, 5 and 95% confidence interval).

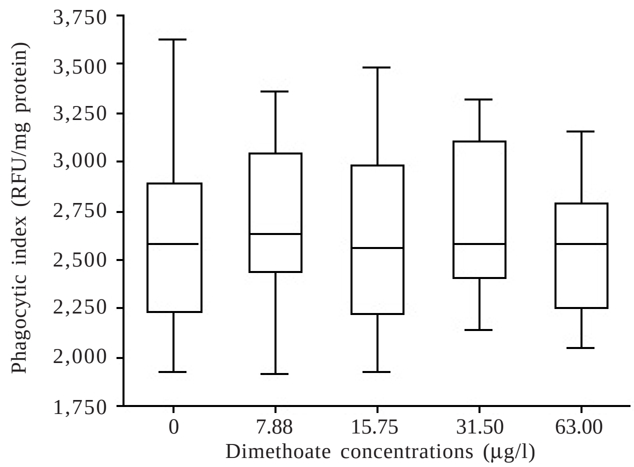

Figure 2. Phagocytic activity of *M. edulis* hemocyte before the treatment. Data were expressed as median (25 and 75% quartile, 5 and 95% confidence interval).

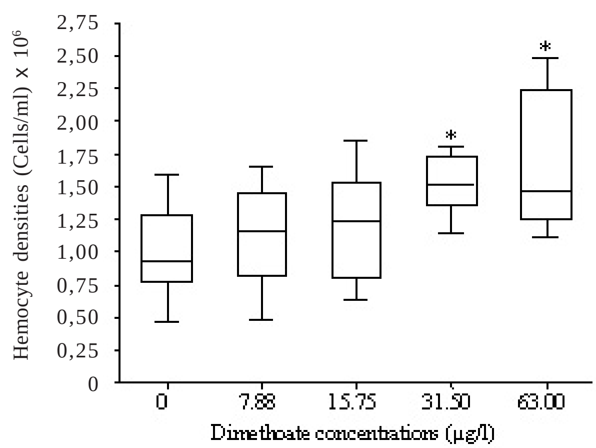

Figure 3. Circulating hemocyte density of *M. edulis* after 14 days of dimethoate exposure. Data were expressed as median (25 and 75% quartile, 5 and 95% confidence interval). *indicated the different number of hemocyte from the treatments and from those observed in the control ($P < 0.05$).

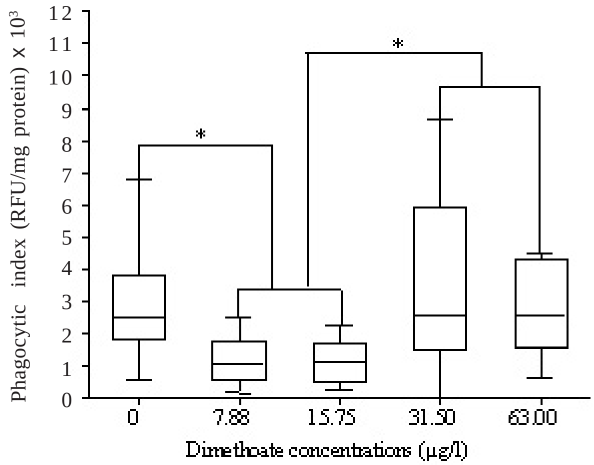

Figure 4. Phagocytic activity of *M. edulis* hemocyte after 14 days of dimethoate exposure. Data were expressed as median (25 and 75% quartile, 5 and 95% confidence interval). *indicated the different phagocytic activity of mussels among the treatments ($P < 0.05$).

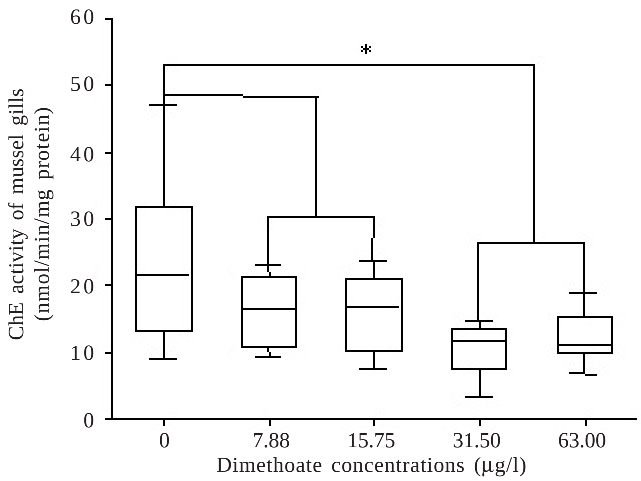

Figure 5. ChE activity of *M. edulis* gill after 14 days of dimethoate exposure. Data were expressed as median (25 and 75% quartile, 5 and 95% confidence interval). *indicated the different enzyme activity of the treatments compare to the control ($P < 0.05$).

there were apparent reductions of the ChE activity about 23% of the control from the mussels that exposed to dimethoate at both concentrations of 7.88 and 15.75 µg/l, but due to high variability between individuals, these were not significant. On the other hand, significant suppression of the ChE activity ($P < 0.05$) occurred at concentrations of 31.50 and 63.00 µg/l compared to the control. Moreover, the statistical analysis showed that the different suppression of the ChE activity between the two treatments was not evidenced (Figure 5).

DISCUSSION

Phagocytic Activity. This study was unable to elucidate clearly dose-dependent phagocytic activity of *M. edulis* hemocytes following 14 days dimethoate exposure. Nevertheless, the circulating hemocyte numbers at concentrations just above the control (7.88 and 15.75 µg/l) demonstrated a tendency of elevation, yet the statistical analysis justified an undifferentiated numbers of hemocytes between them. This indicated that dimethoate at low level did not clearly alter the circulating hemocytes numbers. However, hemocytes density was significantly stimulated at 31.50 µg/l of dimethoate, but the following dimethoate treatment (63.00 µg/l) did not cause an elevation of circulating hemocytes numbers compared to the previous treatment (31.50 µg/l). The alteration of hemocyte numbers as responses to stressors such as chemical compounds is still debatable (Sokolova *et al*. 2004) even a tendency of stimulation under stress condition was a common response (Pipe *et al*. 1999). Some researchers have reported that hemocyte numbers of bivalve elevated as results of exposures to environmental stressors (Coles *et al*. 1994a; Coles *et at*. 1994b; Pipe *et al*. 1999; Dizer *et al*. 2001b; St-Jean *et al*. 2002), whereas others have shown that the stressors declined the hemocytes numbers (Suresh & Mohandas 1990; Dizer *et al*. 2001a; Auffret *et al*. 2002). Undefined response of hemocyte numbers to environmental stressors may implied that the numbers of circulating hemocyte do not fundamentally reflect the total size of the hemocyte population in mussel body which may alter over short time as result of dynamic association/ dissociation between hemocytes and bivalve tissues (Ford *et al*. 1993). This current study was in accordance with the common tendency of affected hemocyte numbers under environmental stressors, which depicted the elevation of mussel hemocyte numbers when exposed to dimethoate.

Hormetic-like effects of dimethoate existed seemingly at the concentrations just above the control i.e. 7.88 and 15.75 µg/l, resulted in decreasing of phagocytic activity. These indicated that dimethoate suppression on the mussels hemocytes occurred at those concentrations. On the other hand, stimulated phagocytosis activity reaching the control level was observed at higher concentrations. The hormetic pattern of phagocytic activity response following dimethoate exposure agreed to the pattern of which was observed by Nicholson (2003) on green mussels, *Perna viridis*, hemocytes following copper exposures. The phagocytic activity decreased at lower concentrations and increased in the next higher concentrations. It could be related to the numbers of circulating hemocytes which showed lower levels at lower contaminants than those at higher contaminants. Moreover, as mentioned above that the first two low levels of dimethoate exposures had the circulating numbers of hemocytes, which were similar to the control statistically, but the phagocytic activity at those concentrations demonstrated lower level than that at the control. It might be as a result of different type of hemocytes composing the population of circulating hemocytes. Probably, the population of circulating hemocytes at two lower levels of contaminants consisted of unphagocytic and/or death cells predominantly due to dimethoate suppression, whereas the control was dominated by phagocytic cells that are responsible for the phagocytic capability. Accordingly, the phagocytic activity of mussels in the control was higher than that at the lower levels (7.88 and 15.75 µg/l), although the hemocyte numbers of two treatments were at the same level statistically. On the other hand, significant stimulation of hemocyte numbers at the two highest contaminants (31.50 and 63.00 µg/l) has been not followed by a distinct stimulation of the phagocytic activity compared to the control. Again, it might be due to the unphagocytic and/or death cells composed predominantly hemocyte population at the two highest dimethoate concentrations, although the hemocyte numbers of the last two treatments were at higher level statistically than that of the control. Consequently, this study revealed that there might be the hormetic-like effects of dimethoate on hemolymph of *M. edulis*, which suppressed the phagocytic activity in the two lower level exposures, but stimulated it at the two highest levels. The U-shape hormetic-like effects of chlorfenvinphos has been observed on ChE activity of hemolymph from *M. edulis* when the studied animals exposed to the pesticide for 96 h (Rickwood & Galloway 2004).

In fact, there are functional differences between mussel hemocyte types (Cheng 1984; Dyrynda *et al.* 1997; Pipe *et al*. 1999). The granulocytes are phagocyti cells containing abundant hydrolytic enzymes, whereas the hyalinocytes have limited phagocytic ability and lower levels of hydrolytic enzymes (Carballal *et al.* 1997). Unfortunately, the microtiter technique for detecting phagocytic activity, which used in this recent study, did not involve a differentiation of hemocyte types so that the exact correlation between proportion of hemocyte types and the phagocytic activity could not be conducted.

Cholinesterase Activity. Cholinesterase (ChE) is a generic term used for a family of released enzymes that hydrolyze neurotransmitter compound, acetylcholine (ACh), to terminate nerve impulse transmission. Organophosphorous and carmabate pesticides are known as potential inhibitors of ChE activity, which lead to acetylcholine accumulation in the synaptic cleft. The accumulation of ACh causes nerve exhaustion and consequently a failure of the nervous system. Hereafter, when organisms are exposed to the two types of pesticides at the critical level of concentrations and time of exposures, they will undergo a range of deleterious effects, which may result in paralysis or death. Therefore, it has been hypothesized that inhibition of ChE activity could be potentially used as an indicator of environmental stress (Bocquene *et al*. 1990) and this activity is a good example of use of a biomarker of effect arising from the presence of pesticides (Galgani & Bocquene 2000).

By using serial dilutions of dimethoate concentrations from 7.88 to 63.00 µg/l, this current study demonstrated that the effects of dimethoate on the ChE activity of blue mussels were dose-dependent. The results depicted that the declined ChE activity occurred at concentrations just above the control. The decrease was 23% compare to the control on mussel that exposed to dimethoate at two concentrations, 7.88 and 15.75 µg/l of dimethoate. However, these suppressions were not significant difference to the control due to high variability responses among individuals of mussels (Figure 5). In laboratory condition, it is broadly accepted that a > 20% decrease in ChE activity indicates exposure to OP pesticides in different species (Ludke *et al.* 1975; Bayers & Sikoski 1994). Coppage (1972) also suggested that inhibition of the ChE activity from brain of fish in the range of 20 to 70% indicating organophosphorous exposures. Moreover, Horsberg *et al*. (1989) reported that the dead salmon concerning trichlorfon and dichlorvos exposures showed inhibitions of 80% in the ChE activity. By considering the criteria and the statistical consideration, it might be suggested that dimethoate at low concentrations (7.88 and 15.75 µg/l) has already showed a potential inhibition of the ChE activity in the mussel gills.

The significant inhibition of the ChE activity clearly occurred when mussel exposed to 31.50 µg/l of dimethoate, decreasing 47% of the ChE activity compared to the control. The percentage of the inhibition tended to be increase (48%) when mussels were exposed to 63.00 µg/l of dimethoate. Therefore, these results suggested that the threshold of dimethoate inhibition to the ChE activity in mussel gills was below the concentration of 31.50 µg/l.

The significant inhibition of ChE activity exposed to the two highest concentrations of dimethoate provides an appreciable explanation to avoid immature justifications concerning the stimulation of the phagocytic activity at the two highest concentrations. The dimethoate stimulated the phagocytic activity occurred at the two highest concentrations tempted to suggest that those were the sign of the recovery of the mussel immune system in light of phagocytic activity after exposured to the highest concentrations. Instead of following dose-dependent response curve, the pattern of dimethoate effect on the phagocytic activity which depicted the decreas of the phagocytic activity at lowest concentrations and the stimulation at the highest concentrations agreed with U-shaped hormetic dose-response pattern which proposed by Teeguarden *et al*. (1998) and Calabrese and Baldwin (2001). In terms of U-Shaped hormesis paradigm, the biphasic pattern figured out an over-compensation response of mussel hemocytes to overcome the severe damages caused by over-exposures of dimethoate at the two highest concentrations through enhancing the phagocytic activity. The severe damages were evidenced by the significant inhibitions of the ChE activity at the two highest concentrations. Consequently, by taking into account the inhibition of the ChE activity as a disruption of mussels health, which can reflect to other health parameters including phagocytic activity, it could be suggested that the enhancing of phagocyitosis activity indicated continuing deleterious effects of the mussels health rather than the improving of the mussels fitness. Accordingly, the significant decrease of the phagocytic activity caused by dimethoate at the two lowest concentrations could be proposed as initial damages of dimethoate disruption followed by severe damages when mussels were exposed to the higher concentrations. The hypothesis of the initial damages was probably also strengthened by the fact that the inhibition of the ChE activity of the mussel gills about 23% compare to the control when mussels were exposed to dimethoate at the lowest level concentration. Therefore, it could be suggested that the threshold of dimethoate effects on the phagocyotis and the ChE activity of the mussels was probably taken place at concentration below 31.50 µg/l.

Finally, the results showed that the selected biomarkers were useful tools for detecting the effects of dimethoate on neuro-immune of blue mussels in the laboratory scale as far as the hormetic dose-response paradigm was considered along with the dose-dependent response paradigm. This paradigm provide a worthy outlook to move forward scientifically from a traditional dose-dependent to others realistic phenomena which appear in the laboratory experiments commonly (Calabrese & Baldwin 2003).

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