III. EVALUATION ON SENSITIVITY OF SELECTED BIOMARKERS, CHOLINESTERASE ACTIVITY AND SIPHONING RATE OF *Mytilus edulis* TO TRICHLORFON

3.1. Abstract

World widely used organophosphorus pesticides have been recognized as anticholinesterase of target and non-target organisms. The current study demonstrated that organophosphorus pesticide, trichlorfon caused effects on both behavioral and cellular responses or biomarkers from blue mussels *Mytilus edulis*. The mussels were exposed to serial dilutions of the pesticides i.e., 0, 50, 100, 200, 500, and 1000 μg/l for 96 h. The notable behavior of mussels namely siphoning rate was inhibited at 200 μg/l concentration. However, the effects of the pesticide on the behavior no longer existed after the mussels were transferred to clean media. At cellular level, the experiment revealed that the pesticide induced effects on cholinesterase (ChE) activity of different organs at different concentrations. A significant inhibition of the ChE activity from gill occurred at the lowest concentration i.e., 50 μg/l, indicating that gill was the most sensitive organ. The moderate sensitive organs were foot and mantle, which elucidated the significant effects of 200 μg/l. The most insensitive organs were hemolymph, posterior adductor muscle (PAM) and digestive gland. The ChE activities from the three organs were inhibited by trichlorfon at the highest concentration, 1000 μg/l. After incubating the trichlorfon exposed mussels for seven days in clean media, the ChE activity from different organs of mussels was not cured completely. The ChE activity from hemolymph, gill, PAM and digestive gland recovered, while the persistence of inhibited ChE activity from foot and mantle were observed. The product moment Pearson correlation indicated the relationships between the siphoning rate and the ChE activity from three relevant organs which are mantle, gill and PAM under exposure of trichlorfon. Considering that the siphoning activity is the product of the three relevant organs movements, Backward Multiple Regression was applied to know which organ play a dominant role in the siphoning activity. The Backward Multiple Regression emphasized the Pearson procedure by indicating the dominant role of mantle in the siphoning activity. The implication of the use of the selected biomarkers on relevant organs in field and laboratory studies was discussed.

Keywords: Selected biomarker, siphoning rate, cholinesterase activity, trichlorfon, blue mussel.

3.2. Introduction

The extensive use of organophosphorous (OP) pesticides in agricultural and other anthropogenic activities causes increase of discharged pesticides wastes in environment. Proclivity of the OP pesticides application in agricultural activities due to the fact, that the pesticides are not relatively persistent in environment and effective for controlling and eradicating the pest. The considerable capability of the OP to control the pest is because of deliberate
synthesis of the pesticides to disrupt a neurotransmitter enzyme, viz. acetylcholinesterase (AChE) of the target animals. In fact, the enzyme has an ability to hydrolyse huge numbers of neurotransmitter compounds i.e. acetylcholine in a neuromuscular junction for very short time (Chang and Strichartz 2005). Consequently, in exposed animals the neurotoxic actions of the OP, which inhibit AChE activity lead to excessive accumulation of acetylcholine in postsynaptic cleft and hyperpolarisation of the postsynaptic membranes, which ultimately hinders and blocks the transmission of nerve impulse.

Although the occurrence of OP pesticides in the environment is thought to be unstable compared to organochlorine pesticides, the persistent effects of the pesticides in non-target organism and ecological system cannot be ruled out (Scholz and Hopkins 2006). Once OP pesticides enter the body of organism most of them are transformed in metabolites which in many cases are more toxic compounds than the parent compounds or induced directly to the target enzymes or organs (Belden and Lydy 2000). Consequently, the effects of most of the pesticides on AChE activity are considered as an irreversible action since the time of re-synthesis of the enzyme are naturally longer than the duration of dissociation of the OP-Complex (Gaglani and Bocquene 2000). Deteriorate effects of the pesticides become more prominent when dealkylation or what it called ageing occurs which involves cleavage of an alkyl group of the phosphoryl moiety and the formation of negative charge, which stabilizes it (Ray 1998). De novo synthesis of the enzyme is the only way to recover AChE activity in the synaptic cleft, which goes slower than dealkylation reaction.

In aquatic ecosystem, the mechanisms of the OP actions in aquatic organisms such as *Mytilus sp* in the enzymatic levels, particularly cholinesterase (ChE) activity, provide a comfortable tool as a biomarker for detection of the pesticide impacts in that ecosystem. Since the works of Grigor’eva and co-workers, 1968, on ChEs from cardiac muscle and hemolymph of *Mytilus edulis* (Moralev and Rozengrat 2004) and Wachtendonk and Neef (1979) on ChEs from hemolymph, the ChE activity from the mussels has been explored and employed as a biomarker to detect the effects of OP pesticides in laboratory and field study.
Different organs of the mussels have been used to evaluate the detrimental effects of the OP pesticides on the ChE activity, which showed that gill was more frequently used compared to others organ or whole tissue (Escartin and Porte 1997; Mc.Henery et al. 1997; Mora et al. 1999a; Mora et al. 1999b; Dizer et al. 2001; Kopecka et al. 2004). In addition, the correlation between the inhibitions of the ChE activity from gill and increasing usage of dichlorvos in marine culture was proved (Mc.Henery et al. 1997). However, compare to the tissue homogenate, hemolymph from *M. edulis* has higher AChE activity (Galloway et al. 2002), and can be used without sacrificing the animals. Hence, the authors claimed that the AChE activity from the mussel hemolymph provides a rapid, relatively cost-effective, reliable, and, non-destructive tool to assess the exposure of mussels to OP and carbamate pesticides. Despite the fact that this biomarker is able to elucidate the significant cellular impairment of OP impacts on the mussels, the ecological relevance of this biomarker is difficult to be predicted (Baird et al. 2007). Therefore, there is a requirement to combine the cellular biomarker such ChE activity with a biomarker at higher level of biological organization hereby the ecological relevance of the pesticide effects on the sentinel organism can be possibly estimated.

Behavioral biomarkers are relevant biological determinants for estimating the impact of OP pesticides, which may have a pertinent potency to reflect the pesticide effects ecologically (Peakall 1992; Sibley et al. 2000). Since the nervous system is an important biological system that almost underlying the physiological and mechanisms of behavior and the state of the effect mechanisms of pesticides is neurotoxin, the potential of the contaminants to disturb animal behavior is high (Grue et al. 2002). Eventually, disturbance of behavior performances of key species particularly due to the pesticide impact causes reduction of Darwinian fitness of the organisms, which would be manifested potentially to the loss of ecological functions through decreasing population viability (Baird et al. 2007).

Siphoning rate of bivalve is one of behavioral biomarker that has a close relationship to the mussel growth through energy acquisition (Lagadic et al. 1994). It has been used to recognize the effects of the OP pesticide in *M. edulis*
(McHenery et al. 1997; Donkin et al. 1997). Although Mc.Henery et al. (1997) could not detect the effect of the OP pesticide to which mussels filtered bacteria, Donkin et al. (1997) detected clearly the effect of dichlorvos to the siphoning rate of mussels using algae. Nevertheless, the correlations between the ChE activity from gill and the siphoning rate of *M. edulis* could not be observed (Donkin et al. 1997). In the Asiatic clam, *Corbicula fluminea*, the interaction between the ChE activity from the anterior and posterior adductor muscles and the siphoning rate were complicated by the closure of the valves when the clams were exposed to higher concentrations of chlorpyrifos (Cooper and Bidwell 2006). This experiment demonstrated that at lower concentrations the inhibition of the ChE activity was followed by the insignificant inhibition of the siphoning rate. In contrast, at higher concentrations the significant inhibition of the siphoning rate was associated with the intact ChE activity.

Those studies implied that the ChE activity from gill and the adductor muscles might not reflect directly to the siphoning rate and therefore the role of ChEs from gill and the adductor muscles on the siphoning rate regulation was probably rather insignificant. On that account, the use of other organs of mussels that were possibly involved in the siphoning rate regulation such as mantle to predict the pesticide effects in relation to ChE activity is indispensable. Furthermore, the serial dilutions of contaminant were used in the experiment should be arranged properly to avoid behavior disturbances of mussels on estimation of the contaminant effects particularly in the cellular levels.

Having taking into account what have been explained above, the recent study was addressed mainly to evaluate:

1. The effects of the OP pesticide, viz. trichlorfon on the ChE activities on different organs and the siphoning rate of *M. edulis*.
2. The recovery from the pesticide impact on the ChE activities and the siphoning rate after the animals were transferred to clean media.
3. The correlation between the ChE activities in selected organs and the siphoning rate of *M. edulis* after exposure to trichlorfon.
3.3. Material and Methods

Chemicals

Acetylthiocholine iodide, 5,5’-Dithio-bis(-2-Nitrobenzoic acid), \(\gamma\)-globuline, Zymosan (Z4250-1G) were obtained from Sigma, USA. Trichlorfon (PESTANAL\textsuperscript{®}, analytical standard (Riedel-de Haën), was purchased from Sigma-Aldrich, Germany. Bradford-reagent was purchased from BIO-RAD.

Sample Collection and Preparation

Mussels, *Mytilus edulis* (6-7 cm) were collected from a clean area of Sylt Island Germany and carried dry to the laboratory. Prior to the experiments the animals were acclimated to the laboratory condition for about two weeks by keeping them in artificial sea water (ASW) that were made of a commercial sea salt (Tropic Marine\textsuperscript{®}) in distilled water with 3 % salinity under temperature 5 ± 1 \(^\circ\)C. Afterward, mussels were placed into 4 l of ASW in glass aquarium for further pesticide exposure experiments.

*In Vivo* Exposure Experiment (I)

*In vivo* exposure experiment was conducted for 96 h in duplicate by using trichlorfon as a contaminant model. A stock solution was made by diluting trichlorfon with distilled water. Immediately, serial dilutions of trichlorfon were made by adding the trichlorfon stock solution into the glass aquarium containing 4 l of 3 % salinity of ASW until reaching the final concentrations, 0, 50, 100, 200, 500, and 1000 µg/l. The set up of these serial dilutions was referred to preliminary *in vitro* test by exposing free hemocytes hemolymph of mussels by trichlorfon which revealed IC\(_{50}\) and LOEL, 4188 and 2239 µg/l respectively. Furthermore, mussels were cleaned up from fouling animals before transferred to the experiments media. Each experiment media was loaded by 12 mussels. The dissolved oxygen in each aquarium was maintained not less than 80 % using an aerator. To assure the desired of the contaminant concentrations in each aquarium, the media and the contaminant concentrations were replaced everyday two hours after the mussels were fed by the commercial algae, Kroonaqa\textsuperscript{®} Aquatim which consist of *Nannochloropsis acculata*, *Isochrysis galbana*, and *Tetraselmis suecica*. 
After 96 h, the exposure experiment was terminated. Six mussels per treatment were transferred to 100 ml of plastic tubes for measuring the siphoning rate for 2 h. Immediately, the mussels were transferred back to the glass aquarium before continuing to the further analysis. Afterward, six mussels per aquarium were sacrificed and the intended organs namely, hemolymph, gill, foot, posterior adductor muscle (PAM), and digestive gland were collected and treated as outlined below.

**In Vivo Recovery Experiment (II)**

The recovery experiment in laboratory scale was conducted by placing the trichlorfon-exposed mussels (experiment I) into 4 l of clean-ASW media for seven days. Each aquarium acquired six mussels. To maintain the media, dissolved oxygen and food were performed as conducted in the experiment I. After seven days, the experiment was completed and some of the mussels from the exposure aquariums 0, 500, and 1000 µg/l were used in the siphoning rate measurement for about two hours. Immediately following the siphoning rate experiment, all the mussels were sacrificed and selected organs were collected and treated as described below.

**In Situ Recovery Experiment (III)**

This experiment was performed by transplanting the trichlorfon-exposed mussels into the clean original habitat in Sylt Island. Prior to the transplantation experiment, 12 mussels per aquarium in 4 l of ASW were exposed by 0 and 500 µg/L of trichlorfon for 96 h in duplicate. At the end of the exposure, six mussels per aquarium were sacrificed and the rest were sent dry to Sylt Island. In Sylt Island the mussels were transplanted in sea water using the net. After seven days, the mussels were removed from the sea water and sent back dry to the laboratory and sacrificed for dissecting and collecting the intended organs immediately.

**Siphoning Rate Measurement**

After the exposure and in vivo recovery experiment, the siphoning rate measurements were conducted. Siphoning rate was used as an indicator of food consumption, which were measured based on the decreasing number of zymosan particles from media as result of the mussels filtration. Six mussels from each
exposure concentration (1 animal per container) were transferred to 150 ml plastic containers, which contain 100 ml of ASW. The mussels were let to acclimatize for 10 minutes before the addition of the zymosan particles with the final concentration of 40 mg/l. Aerator was applied carefully to maintain the standard level of dissolved oxygen and to avoid sedimentation of the zymosan particles. Besides, the containers were covered by a plastic film to prevent splashing out the media. To control the sedimentation of the zymosan during the experiment in the siphoning rate calculation, three containers with 40 mg/l of the zymosan in 100 ml of ASW were run without mussels. The siphoning rate measurement were conducted by replacing 1 ml aliquot of media to 1.5 ml disposable cuvette just after adding the zymosan to the container and two hours after the mussels allowed to filtrate the suspension. Accordingly, optical density (OD) of the aliquot was read by using DR. LANGE Küvetten-Test® photometer at 605 nm. A standard curve was generated by measuring serial dilutions of the diluted zymosan in ASW from which the zymosan concentrations in each of the test containers could be calculated. Subsequently, the equation of Coughlan (1969) was applied to determine the siphoning rate.

where\[ M = \left[ \frac{M^7}{nt} \right] \log \left( \frac{Co}{Ct} \right) \]

where \( M \) is the volume of the test suspension, \( n \) the number of mussels used, \( t \) the duration of the test in hours, \( Co \) the initial concentration of the zymosan, \( Ct \) the concentration of the zymosan at time \( t \), \( m \) the siphoning rate (ml/animal/h).

**Organs Collection and Preparation**

Hemolymph of six mussels in each aquarium were sucked from posterior adductor muscle (PAM) using 1 ml syringe and 0.4 mm needle. Immediately, 1 ml of hemolymph was centrifuged at 10 000 \( x \) g for 10 minutes at 4 °C for separating the hemolymph from hemocytes. The free hemocytes hemolymph were then harvested, transferred into 1.5 ml eppendorf tube and kept under -80 °C prior to ChE activity determination. Intended organs of mussels, i.e. gill, foot, mantle, posterior adductor muscle (PAM), and digestive gland were dissected out, dam-dried and weighed. A Dounce homogenizer was used to homogenize 0.3 g of each tissue in 2 ml of potassium phosphate buffer (0.1 M/pH 8.0). The
homogenate obtained was centrifuged at 10,000 x g for 10 minutes at 4 °C. The supernatant was removed into a 1.5 eppendorf tube and kept under -80 °C before ChE activity measurement was conducted.

**Cholinesterase Activity Measurement**

The enzyme activity was measured following the modified Ellman method (Ellman *et al.* 1961), for a 96-well plate and microplate reading (Herbert, *et al.* 1995; Dizer *et al.* 2001). 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 °C with 200 µl of 0.75 mM 5,5'-Dithio-bis(-2-Nitrobenzoic acid) prior to starting the reaction by addition of 50 µl of 3 mM Acetylthiocholine iodide. Accordingly, the plate was read by using a photometer 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 organ, and the average activity was calculated.

Protein content measurement was carried out by diluting the organ 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 at room temperature for 20 minutes to allow color development. The absorbance was read at 620 nm using the spectrophotometer (Spectra Thermo TECAN). Finally, AChE activity is expressed as nmols of product developed per minute per mg of protein (nmol/min/mg protein).

**Statistical Analysis**

Parametric or non-parametric analysis of variant was used to determine the effects of the pesticide to the assays according to data distribution. Distribution and homogeneity of variant of data set were checked firstly. If the data set does not distribute normally and the variant is not homogenous, the data set were log-transformed. Parametric one-way analysis of variant was used on data set, which demonstrated a normal distribution and homogeneity of variant, both before and
after transformation. If the means were different significantly Bonferroni's Multiple Comparison test was applied to determine the different means between the treatments. Nevertheless, for the data set, which did not show a normal distribution and homogeneity of variant, a non-parametric Kruskall-Wallis test was used to determine the differences between the medians. If the differences were significant, the median values were compared by Dunn's Multiple Comparison test. P < 0.05 was considered as a statistically significant. The data set were analyzed by GraphPad Prism software program, which expressed mean and standard deviation.

The correlation between the siphoning rate of *M. edulis* and ChE activity from gill, mantle and posterior adductor muscle were examined using the product moment Pearson correlation using SPSS 11.0 program software. Furthermore, since the siphoning activity is a process by which the relevant organs such as gill, mantle and PAM work synergistically producing the activity, to recognize which the ChE activity of each organ have a significant correlation to the siphoning rate of the mussels backward elimination procedure was applied.

### 3.4. Results

#### Siphoning Rate

During the siphoning experiments, no mussels behave differently with the control which presented to have opening the valves. The siphoning rate of the mussels in the control demonstrated mean value of $65.08 \pm 6.39$ ml/animal/h. Following the pesticide-exposure experiment for 96 h the siphoning rate of mussels was inhibited significantly at concentration of 200 μg/l (Figure 7). The inhibition remained significantly at concentrations of 500 & 1000 μg/l. The percentages of inhibition of the siphoning rates from 200, 500, & 1000 μg/l of contaminated mussels compare to the siphoning rate of the control were 51, 62, & 68 % respectively.

After transferred to the clean media and incubated for seven days, the siphoning rate of mussels that were contaminated by 500 & 1000 μg/l of trichlorfon turned back to the level of control. The mussels showed the mean siphoning rates of $59.46 \pm 4.24$, $58.86 \pm 4.72$ and $62.36 \pm 0.68$ ml/animal/h from 0, 500 & 1000 μg/l treatments respectively.
Figure 7. Effects of trichlorfon on the siphoning rate of *M. edulis*. The siphoning rate of mussels exposed to trichlorfon for 96-h (striated area with ± standard deviation). The siphoning rate of post-trichlorfon exposed mussels which were incubated in clean water for 7 days (empty area with ± standard deviation). * indicate significant difference from control (0 μg/l) \((P < 0.05)\).

**Cholinesterase Activity**

**Hemolymph**

Hemolymph of mussels showed the highest ChE activity with mean value 104.70 ± 34.48 nm/min/mg protein. There was a stimulation of the ChE activity on mussels hemolymph (12 % compare to the control) which were exposed by the lowest concentration viz. 50 μg/l, but it was not significant. The effect of the used pesticide on the ChE activity from hemolymph of mussels occurred only significantly when mussels were exposed to 1000 μg/l (Figure 8). At this concentration, the ChE activity of mussel decreased 39 % compared to the control.

After incubation in the clean ASW for the next seven days hemolymph of mussels in the control showed a slight decrease of the ChE activity \((95.12 ± 25.60\ \text{nm/min/mg protein})\) compared to the previous control which were used for the 96 h exposure experiment. The recovery of the ChE activity from hemolymph of
mussels, which were exposed to 1000 μg/l of trichlorfon took place after the mussels were incubated in the clean ASW for seven days (Figure 8). The ChE activity of that mussel’s hemolymph showed a mean value of 73.17 ± 19.16 nmol/min/mg protein.

Figure 8. Effects of trichlorfon on the ChE activity from hemolymph of *M. edulis*. The ChE activity in hemolymph of mussels exposed to trichlorfon for 96-h (striated area with ± standard deviation). The ChE activity in hemolymph of post-trichlorfon exposed mussels which were incubated in clean water for 7 days (empty area with ± standard deviation). * indicates significant difference from control (0 μg/l) (*P* < 0.05).

Gill

Employing parametric one-way ANOVA on transformed data of the ChE activity from gill of mussel showed that significant differences of the ChE activity among the treatments were observed. Further calculation using Bonferroni's Multiple Comparison revealed that trichlorfon inhibited ChE activity significantly at concentration of 50 μg/l (3.44 ± 1.17 nmol/min/mg protein) (Figure 9). At this concentration, the pesticide reduced 29 % of the ChE activity from the control level (4.83 ± 1.36 nmol/min/mg protein). The inhibition of the pesticide on the ChE activity persisted significantly at all higher concentrations.
Figure 9. Effects of trichlorfon on the ChE activity from gill of *M. edulis*. The ChE activity in gill of mussels exposed to trichlorfon for 96-h (striated area with ± standard deviation). The ChE activity in gill of post-trichlorfon exposed mussels which were incubated in clean water for 7 days (empty area with ± standard deviation). * indicate significant difference from control (0 μg/l) (P < 0.05).

Decreasing activities of the ChE from all treatments were observed when mussels were transferred to the clean condition compare to the control of the 96 h exposure experiment (Figure 9). Furthermore, the differences of the ChE activities of the control and the treatments in the recovery experiment were not confirmed when the data were calculated using Kruskal-Wallis test.

**Foot**

The ChE activity from foot of mussel was induced significantly by trichlorofon at concentration of 200 μg/l (3.10 ± 0.56 nmol/min/mg protein) (Dunn’s Multiple Comparison test) (Figure 10). The reduction of the ChE activity at this concentration was 35 % from that level of the control (4.77 ± 1.19 nmol/min/mg protein). The inhibition of the pesticide persisted significantly when mussels were exposed to others higher concentrations i.e. 500 (2.89 ± 0.64 nmol/min/mg protein) and 1000 μg/l(2.10 ± 0.42 nmol/min/mg protein) (Dunn’s Multiple Comparison test).
Figure 10. Effects of trichlorfon on the ChE activity from foot of *M. edulis*. The ChE activity in foot of mussels exposed to trichlorfon for 96-h (striated area with ± standard deviation). The ChE activity in foot of post-trichlorfon exposed mussels which incubated in clean water for 7 days (empty area with ± standard deviation). * indicate significant difference from control (0 μg/l) (P < 0.05).

Transferring the pesticide-exposed mussels to clean condition resulted in relatively slight reduction of the ChE activities for all treatments. However, when comparing the mussels from the control (3.12 ± 0.79 nmol/min/mg protein) to others treatments at clean condition there were slight stimulations of the ChE activities from mussels at concentrations of 50 (3.40 ± 0.84 nmol/min/mg protein) and 100 μg/l (3.48 ± 1.10 nmol/min/mg protein). Furthermore, insignificant inhibitions of the ChE activities occurred at concentration of 200 (2.94 ± 0.63 nmol/min/mg protein) and 500 μg/l (3.11 ± 0.33 nmol/min/mg protein). The inhibition of the ChE activity appeared significantly at concentration of 1000 μg/l (2.11 ± 0.188 nmol/min/mg protein) (Dunn’s Multiple Comparison test) (Figure 10).

**Mantle**

Kruskal-Wallis and followed by Dunn’s Multiple Comparison demonstrated that a significant inhibition of the pesticide to ChE activity from mantle occurred at concentration of 200 μg/l (Figure 11). Comparision to the
control (3.82 nmol/min/mg protein) the ChE activity of mussels which were exposed to 200 μg/l of trichlorfon reduced at level of 26 %. Subsequently, at concentration of 500 μg/l the inhibition remained (26 %) and, seemed more distinct at concentration of 1000 μg/l (34 %).

**Figure 11.** Effects of trichlorfon on the ChE activity from mantle of *M. edulis*. The ChE activity in mantle of mussels exposed to trichlorfon for 96-h (striated area with ± standard deviation). The ChE activity in mantle of post-trichlorfon exposed mussels which were incubated in clean water for 7 days (empty area with ± standard deviation). * indicate significant difference from control (0 μg/l) (*P* < 0.05).

After the mussels were replaced to clean ASW the stimulation of the ChE activities occurred seemingly at concentrations of 50 (3.28 nmol/min/mg protein) and 100 μg/l (3.59 nmol/min/mg protein). Significantly the level of the ChE activities turned back to the level of control at concentrations of 200 (2.65 nmol/min/mg protein) and 500 μg/l (2.96 nmol/min/mg protein). In contrast, the effect of the pesticide to the ChE activity of mantle (1.76 nmol/min/mg protein) still appeared significantly (Dunn’s Multiple Comparison test) at concentration of 1000 μg/l when compared to the control (2.78 nmol/min/mg protein) (Figure 11).

**Posterior Adductor Muscle (PAM)**

The data set from the ChE activity of PAM were homogenous and distributed normally. Parametric ANOVA ascertained that the pesticide induced
significantly the ChE activity only at concentration of 1000 μg/l which produced the lesser ChE activity (1.82 nmol/min/mg protein) from PAM (Figure 12). The percentage of inhibition was 25 % compare to the control (2.43 nmol/min/mg protein).

![Figure 12. Effects of trichlorfon on the ChE activity from PAM of M. edulis. The ChE activity in PAM of mussels exposed to trichlorfon for 96-h (striated area with ± standard deviation). The ChE activity in posterior adductor muscle of post-trichlorfon exposed mussels which were incubated in clean water for 7 days (empty area with ± standard deviation). * indicates significant difference from control (0 μg/l) (P < 0.05).](image)

The ChE activity of PAM from the control of the clean incubated-mussels showed a lower level relatively compared to the control of the 96 h pesticide-exposure. Since the data also distributed normally and the variant was homogenous, parametric ANOVA was used to discriminate the differences among the treatments. The results revealed that there were no differences of ChE activities among the treatments.

**Digestive Gland**

The ChE activity from digestive gland of mussels in the control presented mean value 2.57 ± 0.55 nmol/min/mg protein. The ChE activity was only inhibited significantly (Bonferroni's Multiple Comparison test) when mussels...
were exposed to the highest concentration, 1000 \( \mu \text{g/l} \), resulting in 35 \% inhibition from the control (Figure 13).

The persistence of the pesticide inhibition on the ChE activity of digestive gland after incubating in the clean ASW was not observed statistically (Dunn’s Multiple Comparison test). However, stimulations of the ChE activities from the control (1.95 ± 0.48 nmol/min/mg protein) appeared insignificantly at concentrations of 50 (2.30 ± 0.49 nmol/min/mg protein) and 100 \( \mu \text{g/l} \) (2.33 ± 0.37 nmol/min/mg protein). Eventually, the level of the ChE activities turned back to the control level at concentration 200 and 500 \( \mu \text{g/l} \) and reduced slightly at concentration of 1000 \( \mu \text{g/l} \) (Figure 13).

![Figure 13. Effects of trichlorfon on the ChE activity from the digestive gland of M. edulis. The ChE activity in digestive gland of mussels exposed to trichlorfon for 96-h (striated area with ± standard deviation). The ChE activity in digestive gland of post-trichlorfon exposed mussels which incubated in clean water for 7 days (empty area with ± standard deviation). * indicates significant difference from control (0 \( \mu \text{g/l} \)) \( P < 0.05 \).](image-url)
Figure 14. Comparison between pre- and post-incubated mussels *M. edulis* after exposed to trichlorfon concentrations in term of the ChE activity from the six organs. The ChE activity in the six organs of mussels exposed to trichlorfon for 96-h (striated area with ± standard deviation). The ChE activity in the six organs of post-trichlorfon exposed mussels which were incubated in clean ASW for 7 days (empty area with ± standard deviation). * indicate significant difference from control (0 μg/l) (*P* < 0.05).
In Situ Recovery Experiment

In situ recovery experiment was conducted only by exposed mussels to 500 μg/l of trichlorfon and control. Consequently, data analysis was performed on the organs of mussels which showed the inhibition of the ChE activity at concentration ≤ 500 μg/l only. The organs were gill, foot and mantle. Repeated in vivo experiment for 96 h showed that the ChE activities of the three organs were significantly inhibited by 500 μg/l of trichlorfon. After transplanting the mussels to the original habitat in the coastal area of Island of Sylt the ChE activities of the organs turned back significantly to the activity level of the control (Figure 15).

Figure 15. Effects of trichlorfon on the ChE activity from three organs of M. edulis. The ChE activity in three organs of mussels exposed to trichlorfon for 96-h (striated area with ± standard deviation). The ChE activity in three organs of post-trichlorfon exposed mussels which were incubated in clean water of original habitat of mussels for 7 days (empty area with ± standard deviation). * indicate significant different from control (0 μg/l) (P < 0.05).

Correlation Between ChE Activity and Siphoning Rate

Correlation analysis was conducted on the ChE activities of mussel organs which play significant roles on siphoning activity of mussel and the siphoning data. The organs are gill, mantle and PAM (Gosling 2003). Coefficient
correlation (R) between the siphoning rate and the organs were 0.761, 0.656 and 0.510 for mantle, gill and PAM respectively (Figure 16, Figure 17, & Figure 18).

Moreover, when backward procedure was applied the regression equation was \( Y = -26.576 + 5.195 X_1 + 17.009 X_2 \), where \( X_1 \) and \( X_2 \) are gill and mantle respectively. The value of \( R^2 \) adjusted of the equation was 0.631. The backward multiple regression method eliminated the role of PAM in siphoning activity of the mussels since it showed the insignificant role statistically \( (p = 0.178) \).

Figure 16. Correlation between the siphoning rate and the ChE activity from the gill, \( p < 0.001 \).

Figure 17. Correlation between the siphoning rate and the ChE activity from the mantle, \( p < 0.001 \).
3.5. Discussion

Siphoning Rate

Siphoning rate of mussels was thought to be a useful biomarker since it has a close relationship to others endpoints and ecological indicators, and can be used without sacrificing the animals. However, the experiment using this biomarker should be conducted carefully since uncertain responses of other mussel behaviors such as a closing of the valves due to the technical problems instead of the tested contaminants during the experiment might appear. Furthermore, since changes in behavioral biomarkers in response to environmental stress are manifestation of superimposed physiological effects across a variety of organizational levels, from cellular to systematic levels, caution should be applied when interpreting the data (Anandraj et al. 2002). The current study demonstrated that trichlorfon clearly affected the siphoning rate at initial concentrations of 200 μg/l were which comparable to another biomarker i.e. the ChE activity in different organs. It is an important point since some authors were unable to detect the effect of some OP pesticides to mussels (Mc.Henery, et al. 1997; Canty et al. 2007). Mc.Henery et al. (1997) could not demonstrate the effect of serial dilutions of dichlorvos on M. edulis siphoning rate as they used unsuitable food (Donkin et al. 1997). Whereas undetectable effects of OP
pesticide azamethipos on siphoning rate of *M. edulis* (Canty et al. 2007) were probably due to the experimental design which did not have an adequate range of concentrations. They only exposed *M. edulis* to 100 µg/l of azamethipos and compared to negative and positive control (acetone) for 24 h.

Cooper and Bidwell (2006) conducted *in vivo* study on siphoning rate of freshwater clam, *C. fluminea* which were exposed to chlorpyrifos. They observed that there was no difference of the siphoning rate among the treatments when the clean media were used for the siphoning rate measurement. These results, however, contradicted with the visual observations of the clam, which were contaminated to the higher concentrations that showed to have their valves closed at all time (Cooper and Bidwell 2006). Furthermore, they performed different style of siphoning rate measurement by placing clams to the contaminated water directly and measuring the siphoning rate. The results revealed that at lower concentrations (0.05, 0.1, 0.5, 1 µg/l) the siphoning rate did not differ from the control. Whereas the significant difference of the siphoning rate occurred at concentration of 3.13 µg/l and other higher concentrations since the clams closed the valves during the siphoning rate measurement for 2 h. The authors argued that the closing of the valves of the clams at higher concentrations during the second siphoning rate measurement indicated how the clams protected themselves from the pesticide during the exposure experiment so that the siphoning rate decreased dramatically along with increased ChE activity at the control level. Notwithstanding, it should be kept in mind that the clam closed the valves for 2 h is reasonable, but the fact that the clams closed the valves for 96 h is questionable and should be re-observed with caution. Indeed, it is fallible to extrapolate the valves movement for 2 h to the 96 h experiment duration.

Closing the valves is an important strategy for bivalves to avoid severe effects of pollutants. However, there were indications that the valve movements (the closing and opening the valves) cannot simply be related to the siphoning rate when mussels are exposed in the range of contaminants (Widdows and Donkin 1992). Redpath and Davenport (1988) have observed that the reduction of pumping rate of *M. edulis* induced by copper did not relate to the valves movements since the shell valves remained open during experiment, even when
the pumping rate fell to zero. Closing of the valves was not observed in the current study particularly when the mussels from the exposed in vivo recovery experiments were replaced to the clean water for the siphoning rate measurement. It was not the case of “brief recovery” as interpreted by Cooper and Bidwell (2006) because the significant inhibition of the siphoning rate was recorded at concentrations of 200 μg/l. Nevertheless, we did not carry out an observation on the valves movements of mussels during the exposure and in vivo recovery experiments.

Many authors have observed contaminants induced inhibitions of siphoning rate of M. edulis. Very toxic pesticide TBT (tributyltin) has been detected to inhibit siphoning rate of M. edulis (Widdows and Page 1993). The siphoning rate was also sensitive to sediments that were spiked by lindane at 150 μg/kg, but not for 1600 μg/kg of hexachlorobenzene (Hermsen et al. 1994). Toro et al (2003) observed that there was high degree of negative correlation of siphoning rate of Choromytilus chorus which collected from field study and PAHs concentrations in tissues of the animals. The siphoning rate decreased as PAHs concentrations increased. In the laboratory scale, Donkin et al. (1997) also observed the effect of some pesticides such as dichlorvos on M. edulis siphoning rate. They found that dichlorvos reduced 40 % of siphoning rate of M. edulis at concentration 300 μg/l for 72 h exposure, but a further increase in concentration to 1000 μg/l did not produce any additional effect on the siphoning rate. The recent study indicated that at concentration of 200 μg/l, trichlorfon reduced 51 % of the siphoning rate and an escalation of the exposure concentrations caused additional depressions on the siphoning rate. The siphoning rate was also inhibited when M. edulis were exposed to carbaryl (Donkin et al. 1997).

After the incubation of the exposed mussels for seven days in clean water, the siphoning rate of the mussels that were exposed by 500 and 1000 μg/l of trichlorfon turned back to the level of the control. The mean range of the siphoning rates was 59.46 ± 4.24 to 62.36 ± 0.68 ml/animal/h. The recovery of the siphoning rate of M. edulis may associate with the recovery of the ChE activities from the related organs such as gill, mantle and PAM (see discussion below). Gregory et al. (2002) observed that the recovery of siphoning rate from
*Perna perna* after exposing to Hg for 24 days took place after eight days transferring to clean water along with the ciliate recovery of gill.

**Cholinesterase (ChE) Activity From Different Organs**

Although there are considerable diversity biochemical properties of ChEs in aquatic organisms (Escartin and Porte 1997), ChEs in mussels are generally divided into two main classes, acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BuChE; EC 3.1.1.8). The distribution and the properties of ChEs in mussels are organ-dependent (Bocquene *et al.* 1990; Brown *et al.* 2004). The current study referred to the enzyme as ChEs since they employed acetylthiocholine as non-specific substrate of the enzyme. Therefore, the term of ChE that was used in this study referred to the sum of AChE and BuChE as frequently used as a diagnostic tool for ecotoxicological risk assessment studies in aquatic ecosystem (Torre *et al.* 2002).

The alteration of ChE activity is a well-known cellular response of marine mussels which particularly induced by OP and carbamate pesticides. Subsequently, this enzyme activity from the mussels was considered as a respectable tool for detecting the effects of the pesticides (Herbert *et al.* 1995). Mussel organs such as hemolymph, gill, mantle, PAM, digestive gland and gonad have been employed as target organs for measuring the alteration of ChE activity induced by contaminants. The preference of the used organs usually depends on some factors such as the goal of the study and the availability of the organs that were used for analysis. The ChE activity from hemolymph of mussel is considered to be rapid, inexpensive and reliable means for measuring the biological impact of pesticide mainly if sacrificing the animals is avoided (Moreira *et al.* 2001; Galloway *et al.* 2002). Nevertheless, the link effect of the alteration of the ChE activity from hemolymph induced by contaminants to higher biological organization such as feeding behavior is less relevant compared to the innervated organs like gill, mantle and PAM.

The current study evaluated the effects of trichlorfon to ChE activity from various organs from *M. edulis*, viz., hemolymph, gill, foot, mantle, PAM and digestive gland. The highest ChE activity was found in hemolymph and to be more than 20 fold as compared to others organs. These activities from different
organs of *M. edulis* were comparable to those which were observed by Herbert *et al.* (1995).

The results displayed that different organs of *M. edulis* had different sensitivity to trichlorfon. The ChEs from gill was the most sensitive to reveal the effects of trichlorfon followed by foot, mantle, the hemolymph, digestive gland and PAM. The lowest observable effect concentration (LOEC) of gill took place at concentration of 50 $\mu$g/l. While the LOEC of the ChE activity from foot and mantle were similar, the inhibition level of the ChE activity from foot (35 %) at concentration of 200 $\mu$g/l is higher than that from mantle (26 %). The significant inhibition of trichlorfon to the ChE activity from the hemolymph, digestive gland and PAM were initiated at 1000 $\mu$g/l, even though the ChE activity from the hemolymph experienced greater inhibition (39 %) than those from digestive gland (36 %) and PAM (25%). These results demonstrated that the threshold of trichlorfon to inhibit the ChE activity from *M. edulis* was organ dependent. Accordingly, the sensitivity of *M. edulis* organs to trichlorfon in term of the ChE activity can be divided into three clusters, which are high (gill), moderate (foot and mantle) and low sensitivity (the hemolymph, digestive gland and PAM).

Most of studies on ChE activity from mussels were oriented to identify the sensitivity of pesticides by using single organ. There were some ChEs studies used multiple organs of mussels in the laboratory scale. Herbert *et al.* (1995) conducted *in vitro* test to evaluate the sensitivity of ChEs from different organs of *M. edulis* which were hemolymph, gill, PAM, digestive gland and gonad. The experiment revealed that gill and the hemolymph were the most sensitive which were followed by digestive gland, PAM and the gonad. Comparison between solid tissue homogenates which were gill, mantle, PAM and whole body showed that gill is the most sensitive organ when the tissue homogenates were exposed to aldicarb. Escartin and Porte (1997) also found that the ChE activity from *M. galloprovincialis* gill tissue homogenate was more susceptible to inhibition by fenitrothion, fenitrooxon and carbofuran compared that from digestive gland. In addition, a recent study conducted by Canty *et al.* (2007) recorded that the ChE activity from gill of *M. edulis* was more sensitive than that from hemolymph when exposed to azamethiphos.
Comparison of the sensitivity of the ChE activity from *M. edulis* organs to the pesticides from different studies should be carried out carefully. It is due to the different sensitivities of the studies do not merely reflect the sensitivities of the organ *per se*, but mostly they are resulted from different methodology and the tested pesticides.

**Recovery of ChE activity From Different Organs**

Recovery mechanisms in sentinel organisms after exposed to the environmental stress are point of interest in the use of biomarker in biomonitoring campaigns. To be useful tool in biomonitoring, biomarker must reveal the environmental stress over time so that the knowledge about induction, adaptation and recovery of the stress are required and considered prior to monitoring (Wu et al. 2005).

The present study applied two strategies to recognize the recovery of the ChE activity from *M. edulis* after being inhibited by trichlorfon in artificial and natural conditions. The results demonstrated that before transferring to the clean media, the percentage of inhibition of the ChE activity in gill, foot, and mantle from the trichlorfon exposed mussels, which were used in laboratory scale recovery were 55.94, 39.31 and 25.65 % respectively. Furthermore, for the mussels that were used in recovery experiment and transplanted in natural sea water, the percentage of inhibition induced by trichlorfon on the ChE activity in gill, foot, and mantle were 56.51, 58.84 and 36.89 % compared to the control respectively. After incubation for seven days, in both laboratory and natural conditions the ChE activity from the three organs turned back to level of control statistically. This indicated that both artificial and natural conditions served as a suitable media for mussels to recover from ChE activity inhibition. Besides, the result also indicated that the artificial media used in the laboratory scale was sufficient to mimic natural sea water for serving the mussels to recover from the induction of 500 μg/l of trichlorfon for 96 h.

In laboratory scale, mussels were exposed to serial concentrations of trichlorfon. After being replaced and incubated in clean media for seven days, the ChE activity from tested organs increased to the level of control. This recovery occurred on mussels were exposed to the pesticide in range concentrations from 50
to 500 μg/l. Nonetheless, the exposed mussels to 1000 μg/l of trichlorfon did not reveal increase the ChE activity from all studied organs. After transferring the animals to the clean media only the ChE activity from gill, hemolymph, digestive gland and PAM that turned back to the level of control, but the evidence were not observed in foot and mantle. These evidence suggested that recovery mechanisms on trichlorfon inhibited the ChE activity from mussels were organs specific. In other words, the complete recovery of the ChE activity in whole organs of mussels after being exposed by 1000 μg/l of trichlorfon was not confirmed. Gill illustrated both as a sensitive and quick recovery organ regarding pesticide effects. Mc.Henery et al. (1997) also observed that fast induction and recovery of the ChE activity from gill of M. edulis occurred when the animals were exposed to serial concentrations of dichlorvos (10, 100 and 1000 μg/l) and replaced in clean media for seven days. In contrast, the current study demonstrated that foot and mantle as moderate sensitive organs, which can retain pesticides effects for such period. The lowest sensitive organs i.e. the hemolymph, digestive gland and PAM elucidated fast recovery response. On that account, it is suggested that the use of foot and mantle from M. edulis in study on induction of neurotoxic contaminants on the ChE activity and the recovery mechanism is recommended.

The application of these organs viz. foot and mantle as target organs for measuring the ChE activity from mussels could be considered as counterparts of common employment organ such as gill to elucidate more comprehensive understanding on neurotoxic xenobiotic effects in mussels when the ChE activity would be applied as biomarker. As demonstrated in the study that the sensitivity and the recovery response of the ChE activity in mussels differed from organ to organ, combining all types of inductions and recoveries of target organs is needed to reduce potential false positive or negative on assessing the impacts of the pesticides on mussels.

**Correlation Between Cholinesterase (ChE) and Siphoning Rate**

Although ChE activity of aquatic biota is considered as a convenient biomarker to reveal considerable effects of OP and carbamate pesticides for underlying ecological risk based aquatic management, the critical aspect for integrating the biomarker in ecological risk assessment is to translate the
biomarker into an ecological perspective (Peakall et al. 2002). In fact there are some evidence indicating that inhibition of ChE activity from aquatic biota correlated well with acute endpoint i.e., mortality so that the ecological relevance of the lethal level of ChE activity inhibition can be estimated (Sibley et al. 2000). However, at relatively low levels exposure of anticholinesterase the level of ChE activity inhibition does not cause moribund effects, which ultimately led to the death of animals. Hence, sublethal endpoint such as behavioral changes can be a good mediator endpoint for translating ChE activity inhibition into the ecological relevance of sublethal exposures (Brewer et al. 2001; Sandahl et al. 2005). It is due to dysfunction of nervous system, which can be recognized in inhibition of ChE activity is considered as a rational basis of high biological organizations like behavioral alterations (Peakall et al. 2002).

Aquatic teleost have been studied frequently to show the correlation between induced behavioral responses and their possible rational basis in cellular levels such as ChE activity. For instance, Post and Leasure (1974) observed a correlation between inhibition of ChE activity in brain and impairment of swimming performance induced by malathion from brook trout (Salvelinus frontalis), rainbow trout (Oncorhynchus mykiss), and coho salmon (O.kisutch). Inhibition of brain ChE activity correlated well with reduction of swimming ability of rainbow trout larva (O. mykiss) after exposed by carbaryl (Beauvais et al. 2001) and malathion (Brewer et al. 2001). In addition, Sandahl et al. (2005) demonstrated significant correlations between reduction of ChE activity of brain from coho salmon larva with both impairment of spontaneous swimming speed and feeding rate after exposed to chlorpyrifos.

Compared to the relationship of ChE activity and behavioral data from fishes, the data from bivalves are scanty. In fact, there were some studies assessing the impacts of pesticides to the ChE activity and mussel behavior, but the association between impairment on the ChE activity and behavior from mussels were not regarded (Rickwood and Galloway, 2004; Cooper and Bidwell, 2006; Canty et al. 2007). On the other hand, due to complexity of bivalve behavior concerning the ability of the animals to avoid contaminant exposure by closing the valves, a measurement of bivalve siphoning rate induced by
contaminants is fraught difficulty (Cooper and Bidwell 2006). Another problem with bivalve behavior, which was expressed as the siphoning rate in correlation with the lower level biological organization endpoint is selecting proper organs underlying the siphoning rate mechanisms in the study. Donkin *et al.* (1997) did find the effect of dichlorvos on the ChE activity from mussel gill and on the siphoning rate, but the form of the ChE inhibition curve was not the same as that of siphoning rate. Eventually, the authors were tempted to imply that the role of ChEs in gill to regulate siphoning rate of bivalve was likely uncertain (Donkin *et al.* 1997).

In spite of the fact that the role of ACh in regulating gill activity for transferring the food particles is still debatable, Aiello (1990) concluded that ACh is major neurotransmitter along with serotonin and dopamine in gill activity. In the current study, the relationship between the ChE activity from gill and the siphoning rate of mussels was observed. Using product moment Pearson the correlation was recorded between the ChE activity from PAM and mantle with the siphoning rate. These indicated that the three organs engaged in the siphoning activity of *M. edulis*. In addition, by comparing the correlation coefficient of the ChE activity from the three organs and the siphoning rate, the ChE activity from the mantle was calculated to be the highest degree of the correlation which was followed by gill and PAM. Hence, the data suggested that the ChEs of mantle play more significant role in regulation of the siphoning activity of mussels compared to those from gill and PAM. Both gill and PAM seemed to play a secondary role in siphoning activity of mussels as observed by Newell *et al.* (2001). The authors observed that the ability of mussels to adjust the siphoning rate depend on the musculature of mantle and independent from controll by PAM.

Backward multiple regression procedure showed that there were two models of the correlation between the ChE activities from innervated organs i.e. gill, mantle and PAM. The first model revealed that the regression equation was 

\[ Y = -36.366 + 4.363X_1 + 15.927X_2 + 7.255X_3, \]

where \( X_1, X_2 \) and \( X_3 \) are gill, mantle and PAM, respectively. In the first model it was recognized that PAM played insignificant function in the siphoning rate of the mussels (\( p = 0.178 \)). Subsequently, the backward procedure eliminated PAM from the multiple
regression model. Furthermore, the second model depicted that the regression equation was \( Y = -26.576 + 5.195 X_1 + 17.009 X_2 \), where \( X_1 \) and \( X_2 \) are gill and mantle. The \( R^2 \) adjusted of the two models are 0.641 and 0.631, respectively. The \( R^2 \) adjusted elucidated that the organs only played significant roles around 60% and the rest might be others factors such as water and food particles movements. The backward procedure also emphasized that mantle possessed the highest significant role, while PAM had a minimal function in the mussel’s siphoning rate.

In fact the involvement of PAM in the valves movement cannot be ruled out completely, but the lowest degree of coefficient correlation from ChE activity in PAM and the siphoning rate might be due to a catch phenomenon occurring during the siphoning activity. The catch phenomenon is the ability to maintain tension at considerably reduced ATP cost compared to phasic tension development (De Zwaan and Mathieu 1992). This phenomenon is the strategy that is used by mussel to reduce the energy expenditure (De Zwaan and Mathieu 1992) and therefore minimizes the role of PAM in the siphoning activity.

The link between the ChE activity from the three organs and the behavioral change that was revealed in this study indicated that this biomarker could be used as an index of effects instead of an index of exposures. It means that inhibition of the ChE activity in relevant organs is transformed to reduction of the siphoning activity that has high possibility to reduce growth and others Darwinian fitnesses. As a consequence, the information are very valuable to establish the consequences of the ChE activity induction particularly from marine mussels that were caused by the pesticides as an ecological perspective.

In the context of revealing the ecological relevance of lower level biomarkers from mussel via siphoning activity, the use of the ChE activity from mantle is more suggested than that from gill and PAM when the measurement of the ChE activity from more than one organ in such circumstance is not possible. However, involving the measurement of the ChE activity from the three organs provides better results and understanding for recognizing the health status of mussels since the three organs act synergistically in the regulation of siphoning activity of *M. edulis* (Jorgensen *et al.* 1988) which indicated in the present results.
In addition, employing more than one relevant organ of *M. edulis* for determining the pesticides effects using the ChE activity can generate more comprehensive understanding on how each organ of mussels react to the pesticides that enter their body. Finally, the strategy that uses battery of relevant organs not only refines the application of the selected biomarkers in laboratory trials and field scales, but also enhances its potential competency to reveal the consequence of pesticides exposures in ecological stages.

3.6. References


