SELECTED BIOMARKERS; IN VIVO AND IN SITU APPLICATIONS

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Bogor, 8th of September 2008

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ABSTRAK

KHUSNUL YAQIN. Biomarker; Aplikasi In Vivo dan In Situ. Dibimbing oleh BIBIANA WIDIYATI LAY, ETTY RIANI, dan ZAINAL ALIM MASUD.


Kata kunci : biomarker, kolinesterase, fagositosis, laju penyaringan, dimethoate trichlorfon.
SUMMARY

KHUSNUL YAQIN. Selected Biomarkers; In Vivo and In Situ Applications. Under direction of BIBIANA WIDIYATI LAY, ETTY RIANI, and ZAINAL ALIM MASUD.

Selected biomarkers, cholinesterase (ChE) and phagocytic activities from blue mussels, *Mytilus edulis* have been used to detect the effects of neuro-immunotoxicity of organophosphate (OP) pesticide, dimethoate. The serial dilutions of dimethoate concentrations which were 0.00, 7.88, 15.75, 31.50 and 63.00 µg/l, showed dose-dependent effects on the ChE activity of blue mussels, *Mytilus edulis* after being exposed for 14 days. Statistical analysis showed that the significant effects of the pesticide on the ChE activity occurred at concentrations 31.50 and 63.00 µg/l (p <0.05). In contrast, the dose-dependent effects of dimethoate were not observed in the phagocytic activity of dimethoate-exposed mussels. The suppression effects of dimethoate on phagocytic activity occurred significantly (p < 0.05) at two concentrations of dimethoate (7.88 and 15.75 µg/l), but stimulation effects significantly (p < 0.05) emerged at the following concentrations (31.35 and 63.00 µg/l). The reduction which occurred at the lowest concentrations along with the stimulation at the highest concentrations implied the occurrence of a U-shape hormetic response of the phagocytic activity under dimethoate exposure. The results suggested that two selected biomarkers, ChE and phagocytic activities were respectable tools to detect the effects of the pesticides when the dose-dependent and U-shape hormetic responses were taken into account in the laboratory scale study.

World widely used organophosphorus pesticides have been recognized as anticholinesterase of target and non-target organisms. One of the challenges in biomarkers study is to transform low levels of biological integrity responses such as enzymatic responses to closely related higher levels of biomarkers. The disruptions of nervous system are potentially manifested in behavioral levels such as feeding activity. 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 in terms of ChE activity. 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 activities from hemolymph, gill, PAM and digestive gland recovered, while the persistence of inhibited ChE activities from foot and mantle were observed. The
Pearson procedure was applied to recognize the correlation degree of the ChE activity from each relevant organ and siphoning rate. Coefficient correlation (R) between the siphoning rate and the organs were 0.761, 0.656 and 0.510 for mantle, gill and PAM respectively. The result indicated that mantle is the most correlated organ to the siphoning activity which was followed by gill and PAM. 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 regression equation was $Y = -26.576 + 5.195X_1 + 17.009X_2$, where $X_1$ and $X_2$ are gill and mantle respectively. The value of adjusted $R^2$ of the equation was 0.63. It was indicated that the role of the two organs i.e., mantle and gill in the siphoning activity was about 60%. The Backward Multiple Regression method eliminated the role of PAM in siphoning activity of the mussels since it showed insignificant role statistically ($p = 0.178$).

Tropical green mussels (*Perna viridis*) play an important economic and ecological role in the coastal areas of Indonesia. *P. viridis* has been used as an eco-sentinel organism for marine biomonitoring program in Asia regions. To magnify its competency as an eco-sentinel organism in biomonitoring characterization of cholinesterases (ChEs) of *P. viridis* from a selected coastal area of Indonesia has been conducted. In addition, the characterization of ChEs of *P. viridis* is a requirement which has to be conducted prior to using ChE activity as biomarker. Dissected organs which were gill, foot, mantle and posterior adductor muscle (PAM) were examined for substrate specificity and inhibitors sensitivity using selective and non-selective substrates and inhibitors. The results indicated that highest level of the ChE activity was observed in gill and followed by foot, mantle and PAM. The substrate specificity approach using various alkylthiocholines indicated that the ChEs of *P. viridis* constitute an acetylcholinesterase (AChE) and a butyrylcholinesterase (BuChE). Likewise, the inhibitors sensitivity approach using eserine, BW284C51, and iso-OMPA sustained the substrate approach which recorded typical AChE and atypical BuChE. Hence, the study revealed that ChEs of *P. viridis* gill contain a typical AChE and an atypical BuChE. The results also suggested that the *P. viridis*’s gill is the most suitable organ for employing the ChE activity as a biomarker in marine monitoring.

Application of selected biomarkers, Cholinesterase (ChE) and phagocytic activities have been conducted with the exposed green mussel *Perna viridis* in Indonesian coastal waters. An operative effect-based monitoring on two polluted sites and one reference area were investigated for aquaculture enterprises and human health aspects. Statistical analysis showed that green mussels collected from an expected clean site in Pangkajene Kepulauan (Pangkep), South Sulawesi possessed the highest level of the ChE activity compared to those that were collected from two sites of Jakarta Bay, i.e. Cilincing and Kamal Muara ($p < 0.05$). It was interesting that the ChE activity of *P. viridis* was able to distinguish statistically the different effect levels of pollutants that existed in Jakarta Bay. The ChE activity of the mussels from Kamal Muara was significantly higher than that of the mussels originated from Cilincing ($p < 0.05$). Moreover, the enzymatic results were emphasized by the phagocytic response of the mussels. The
phagocytic activity of green mussels from the polluted sites demonstrated a significant higher activity than that of green mussels from the pristine site, Pangkep. However, there were no significant differences of phagocytic activity between the polluted sites. This might indicate that the existing pollutants in Jakarta Bay were more neurotoxic rather than immunotoxic substances. The results showed clearly that both selected biomarkers were potential valuable tools for effect-based monitoring and pollution impacts in coastal zones of Indonesia. The hot spot biomonitoring contributes to a tailor-made toolbox focussing on risk-based coastal zone management in Indonesia.

Key words: biomarker, cholinesterase, phagocytosis, siphoning rate, dimethoate trichlorfon.
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FOREWORD

I would first like to express my sincere thanks and gratitude to my supervisor committee (Prof. Dr. drh. Bibiana Widiyati Lay, M.Sc, Dr. Ir. Etty Riani, MS, Dr. Zainal Alim Masud, DEA) for their guidance in accomplishing the studies. I would like to extend my deep and warm thanks and gratitude to Prof. Peter-Diedrich Hansen for facilitating me to use the ecotoxicology laboratory in Institute for Ecological Research and Technology, Department of Ecotoxicology Technische Universitaet Berlin, Germany which made the studies possible. He also trained me to dig out scientific experiences in ecotoxicology. I wish also to thank Dr. Eckehard Unruh, Birgit Fischer, Dr. Gerd Huscheck, Martin Kern from Department of Ecotoxicology Technische Universitaet Berlin for the constructive advices and Birgit Hüssel from Alfred Wagner Institute for the organization of blue mussel, *Mytilus edulis*.

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Bogor, 8th of September 2008

Khusnul Yaqin
BIOGRAPHY

The author was born in Gresik 26 July 1968. He spent his academic experiences from elementary to senior high school in that city. Afterward, he migrated to Makassar for pursuing his study in Department of Fishery, Hasanuddin University from 1987 to 1992. He was registered as a lecturer in that department in 1994 until now. In 2001, he continued his study on master degree in Department of Marine Ecology, University of Aarhus, Denmark which was supported by DANIDA. In this department, he started to work on marine ecotoxicology subjects under direction of Dr. Vibeke Simonsen and Dr. Janeck Scott-Forsmand. He completed the study in 22 January 2003. Accordingly, he pursued his scientific experiences in ecotoxicology by registering on doctoral degree in Environmental Science Study Program of Graduate School of Bogor Agricultural University in 2003. His doctoral work was supported by DAAD in sandwich scheme scholarship. The scholarship set up him to synthesize tropical and temperate academic atmospheres by taking a class experience in Bogor Agricultural University for one year and laboratory works in Department of Ecotoxicology, Technische Universitaet Berlin for three years. In addition, in the Department of Ecotoxicology he was involved as a leading scientist in an international laboratory inter-calibration excersise on a new Microbial Assay Technique for Risk Assessment.

During doctoral study he has published some papers:


To promote the use of biomarkers in marine biomonitoring in Indonesia he disseminated his scientific works by involving in international scientific meeting such as 9th meeting of the Ecosystem Health Network (EHN) under the Germany - Canada Bilateral Agreement on Science & Technology – Potsdam, 15th of November 2005, SETAC-Europe (SETAC = Society of Environmental...
Toxicology and Chemistry) meeting in The Hague, Netherlands, 7th -11th of May 2006, and in Porto, Portugal, 20th – 24th of May 2007. His popular papers were also published in Indonesian newspaper and magazine such as KOMPAS (Ada kerang abnormal di Teluk Jakarta, 8th of September 2004) and MAJALAH ADIL (Gangguan kolinergis kerang hijau di Teluk Jakarta, August 2006).
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I. GENERAL INTRODUCTION

1.1. Background

The term of biomarker has been obtaining intriguing attention, although it has been defined in various meaning thereby the clear definition is rather vague (Schlenk 1999). The National Academy of Science in the USA has defined the term of biomarker as a xenobiotically-induced variation in cellular or biochemical components or processes, structures, or functions that is measurable in a biological system or sample (NRC 1987). Moreover, Walker et al. (2001) has defined biomarker as any biological response to an environmental chemical at the individual level or below demonstrating a departure from the normal status. Thus, biochemical, physiological, histological, morphological and behavioral measurements are to be considered as biomarkers. Rather similar meaning of the biomarker definition has been proposed by Depledge (1993) emphasized on the matter of measurement and the purpose of biomarkers in screening test and monitoring in fields. The definition is biochemical, cellular, physiological or behavioural variations that can be measured in tissue or body fluid samples or at the level of whole organisms to provide evidence of exposure and/or effects from one or more contaminants. The last two definitions have attempted to restrict the term of biomarker that merely addressed to biological responses at individual level. The limitation of biomarker definition had been widened and modified to the level of population, community and ecosystem as illustrated by Adams (Adams 1990). Accordingly, Walker et al. (2001) had termed the responses at higher organizational levels – population, community and ecosystem – as a bioindicator. On the other hand McCarty and Munkittrick (1996) broadened the terminology of bioindicator by including biochemical, physiological, or ecological structures or processes which have correlations or causal links to biological effect measured at one or more levels of biological organization. Eventually, the current dissertation uses the working biomarker definition as what proposed by Walker et al. (2001) to pave the way for the discussions of biomarkers appropriately and to avoid overlapping with the terminology that has been used as bioindicator.

Many biomarkers have been proposed as a sensitive early warning tool for modern environmental assessment in biomonitoring campaigns, which are ranging
from specific to unspecific responses to delineate and record the effect of xenobiotic compounds to living organisms (Beliaeff and Burgeot 2002; Sherry 2003; Hagger et al. 2006). As early warning tool, the biomarkers are also efficacious in allowing the initiation of bioremediation strategies before irreversible deleterious damage of ecological consequence are taking place (Cajaraville et al. 2000). The specific biomarkers could be possible replacing chemical analysis of the surrounding environment due to their sensitivity, while non-specific biomarkers provide a generalized indication that a living organism may be suffering from stress induced by the presence of xenobiotic compounds (Connell et al. 1999). The present dissertation is aimed to provide evidence and explore the use of three selected biomarkers by conducting *in vivo* and *in situ* studies. Evident-based concept of the biomarkers originated from blue mussels *Mytilus edulis* were applied to green mussels, *Perna viridis*. This effort was addressed to give a rational basis of the use of *P. viridis* as eco-sentinel organism in effect-based biomonitoring campaign using biomarkers in Indonesian coastal areas since the selected biomarkers such as phagocytic and cholinesterase activities has not been studied yet in the region. Therefore, the studies that discussed in this dissertation will support Indonesian government or environmental managers to manage their marine ecosystem.

The three selected biomarkers are considered as a specific biomarker i.e. cholinesterase (ChE) activity and non-specific biomarkers such as phagocytic activity and siphoning rate in terms of *in vivo* and hot spot *in situ* applications. Nevertheless, it should be kept in mind that ChE activity can be inhibited by others contaminants other than organophosphorous and carbamate pesticides such as heavy metals (Guilhermino *et al.* 1998; Tabche *et al.* 1997; Elumalai *et al.* 2007), PAH (Tabche *et al.* 1997; Akcha *et al.* 2000; Moreira *et al.* 2004), and detergent (Tabche *et al.* 1997) so that the use of that enzyme as biomarker could be extended (Walker *et al.* 2001).

In respect to biological integrity, the three selected biomarkers can be classified into three different levels which are biochemical (ChE activity), physiological/immunological (phagocytic activity) and behavioral levels (siphoning rate). They also have a potential to be extrapolated to higher level of
biological responses such growth and reproduction which may have a relevancy on ecological levels. As consequence, the use of the three different selected biomarkers either in laboratory or field scales may open an opportunity to show how toxicants interfere to biological integrity from biochemical, cellular to behavioral levels that may envisage consequences on ecological levels. Determination of ecological status of such zones which involve biomarkers from different levels of biological integrity as rational basis along with the chemical analysis approach will be meaningful efforts for supporting environment managers and governments in protecting, remediating and managing the environment concerning the anthropogenic activities and the deleterious impacts of wastages.

1.2. Logical Framework

Pesticides are chemical compounds that have broad-spectrum applications in anthropogenic activities. They are used from domestic, agricultural, sport, public health to industrial sectors (Sobiech and Henry 2003). In a developing country like Indonesia the production of these chemical compounds may increase along with increasing of economic activities since the capability of the pesticides to prevent, control, eradicate and destroy any pest animal in pre and post harvest of agricultural products, in industrial sectors and to kill disease-causing organism in public health. Table 1 shows elevation of pesticide productions in Indonesia from 1999-2004. The data may reflect not only elevation of pesticides supply, but also increase of the potential use of pesticide in anthropogenic activities in Indonesia. Table 2 shows the use of pesticides in aquaculture campaigns from 2001-2006 in Indonesia.


<table>
<thead>
<tr>
<th>Year</th>
<th>Pesticide production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kilogram</td>
</tr>
<tr>
<td>2001</td>
<td>1,542,455</td>
</tr>
<tr>
<td>2002</td>
<td>11,981,352</td>
</tr>
<tr>
<td>2003</td>
<td>12,208,281</td>
</tr>
<tr>
<td>2004</td>
<td>13,889,837</td>
</tr>
</tbody>
</table>
Table 2. Usage of pesticides in brackishwater and freshwater ponds (2001-2006) (DKP 2006).

<table>
<thead>
<tr>
<th>Year</th>
<th>Brackishwater pond (Kg)</th>
<th>Freshwater pond (Kg)</th>
<th>Total (Kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2001</td>
<td>749,539</td>
<td>150,688</td>
<td>900,227</td>
</tr>
<tr>
<td>2002</td>
<td>30,542,938</td>
<td>1,421,447</td>
<td>31,964,385</td>
</tr>
<tr>
<td>2003</td>
<td>371,171</td>
<td>105,986</td>
<td>477,157</td>
</tr>
<tr>
<td>2004</td>
<td>855,307</td>
<td>109,216</td>
<td>964,523</td>
</tr>
<tr>
<td>2005</td>
<td>1,237,743</td>
<td>150,951</td>
<td>1,388,694</td>
</tr>
<tr>
<td>2006</td>
<td>370,023</td>
<td>677,411</td>
<td>1,047,434</td>
</tr>
</tbody>
</table>

The use of pesticides not only increases quality and quantity of food productions and other products that give benefit to humankind, but also escalates pesticide waste and pesticide-adverse effects on the environment. It is due to the fact that only small portion of pesticides (less than 5 %) can reach targeted organisms during the application, while the remaining are wasted, and contaminate the environment and its compartment (Porte and Albaiges 2002). In addition, many non-agrochemicals such as cleaning products, antibacterial soaps, lawn, garden, and swimming pools chemicals are pesticides that are washed down the drain and can become part of the waste stream which reach surface waters directly via surface runoff (Sobiech and Henry 2003). Therefore, to improve health and nutrition in developing countries Black et al. (2003) suggested using more effective pesticides such as OP pesticides and emphasizing the urgent need for adequate risk assessment of the deleterious effects of the pesticides to the environment and its compartments.

Environmental quality assessment which were based merely on a chemical based analysis is considered to be an insufficient approach to delineate deleterious effects of environmental stressors on biological integrity of organisms or population. It is because the chemical based analysis can only describe the level of discharged xenobiotic compounds in the environment without illustrating the effects of the xenobiotic compounds in living organisms. In addition, the chemical monitoring can detect the chemical compounds when they are persistent. It cannot detect the degradable chemical compounds such as organophosphate...
pesticides which have short biological half-lives, even though, they have long-term effects in biological system of living organism (Peakall and Walker 1994). Moreover, the chemical-based approach is extremely expensive particularly for developing countries, applicable to only a small proportion of the pollutants in the environment, provides a little biologically meaningful information, and therefore fail to notice the complexity of the studied ecosystems (Butterworth 1995). As a consequence, the chemical based analysis solely cannot answer the most critical aspect of assessment for society which is to determine how much deleterious impact of contaminants to the environment that can be prepared to tolerate and handle (Peakall and Walker 1994; Sheery 2003).

Concerning the degradable pesticides such as organophosphate pesticides, the problem of assessing deleterious environmental impacts of the pesticides which were based on chemical analysis approach is complicated by rapid degradation and bitransformation of the pesticides in aquatic environments or living organisms (Walker et al. 2001). Once the pesticides get into living organisms they will be transformed and metabolized to be metabolite compounds which differ from their parent compounds that may increase or decrease their toxicities. These complications bring about the interpretations of potential affects of the pesticides in aquatic environments and living organisms even more ambiguous (Sobiech and Henry 2003).

To evaluate the quality of environments, the level of contaminant resulting from the chemical based monitoring are referred to the environmental quality criteria which is generated from laboratory test based mostly on mortality endpoint. Although the mortality-based acute test is popular in ecotoxicological assessments, the test still generates some problems such as recognizing long-term deleterious effects of chemical compounds concentrations which does not cause the death of tested organisms (Connell et al 1994). The test only illustrates the concentration of contaminants to bring about mortality at duration of experiments. Hence, based on the mortality test the contaminants that get into the tissue of tested organisms slowly will be justified as low toxicity contaminants since the short duration of the test does not allow the contaminants reaching the toxicity level to kill the tested organisms (Landis and Yu 1999). Moreover, the test only
delineates the magnitude of the tested doses which cause the death of organisms. In fact, the tested organisms undergo biological destructive damages from molecular to behavioral levels induced by contaminants before they are dead. It means that the pre-mortality destructive damages induced by contaminants may reduce the Darwinian fitness such as metabolism process, fecundity, reproduction, and growth rate (Depledge 1993) which may be manifested in population and ecosystem levels cannot be detected by the mortality approach. Therefore, the mortality-based acute test is considered as an insufficient tool to detect and illustrate risks that will occur in the environment due to the environmental stressors in early state.

Figure 1. The role of biomarkers in ecological risk assessment (ERA) (Modified from Hansen 2007).
The biomarker concept is considered as a breakthrough concept to complete the conventional approach for evaluating the environmental quality. The concept offers opportunities to picturize holistic interactions between pollutants and pollutant-induced biological damages of sentinel organisms from molecular, cellular to behavioral levels. It is acknowledged that a healthy organism exposed to increasing pollutant loads will suffer a continuum deterioration in health which shows reversible to irreversible conditions that culminate on the death of organisms (Depledge and Fossi 1994). Biomarkers have capability to recognize in which point of the continuum pollutant-exposed organisms are located so that they offer potentially an early warning system for environmental deterioration induced by pollutants (Depledge and Fossi 1994). Last but not least, biomarkers can also detect prevented adverse effects of xenobiotic compounds on living organisms (Wu et al. 2005). Therefore, biomarkers can be used conceptually as valuable tools in detecting adverse effects of xenobiotic compounds both in laboratory and field scales (Figure 1).

1.3. Problem Formulation

Biomarkers can be used either in early step of the ERA or in the further risk characterization both in field and laboratory scales. The applicability of biomarkers in both laboratory and field scales should fulfill the requirements as rapid, sensitive, easy and cost-effective tools. ChE and phagocytic activities which are miniaturized in micro-plate application are two biomarkers that fulfill the requirements (Dizer et al. 2001; Blaise et al. 2002). Therefore, the experiment that used the two biomarkers to detect the neuro-immune disruption induced by OP pesticides using blue mussels is needed to be studied as a strategy of exposure in laboratory scale for assessing the pesticide effects.

In fact, the use of biomarkers for detecting effects of pollutants can be derived along continuum of biological integrity from molecular to behavioral levels. In general, the lower levels of biological compartments such as molecular and cellular levels show sensitivity to environmental stressors, but they have poor linkage to the ecological levels. In contrast, higher level of biological integrity such as behavioral level has relatively significant relationship to the ecological levels (Hansen 2007). One source of uncertainties in ERA using lower level of
biomarkers such as cholinesterase and phagocytic activities is the lack of ecological perspective. To solve the problem establishment of quantitative relationship between measurement of biomarker along the biological integrity is necessary (Sibley et al. 2000). Hence, in terms of OP pesticide impacts combining ChE activity that is a main target of the OP pesticide toxicity and siphoning rate as surrogate of behavioral level from blue mussels is necessary to be studied. The study allows us to recognize a transformation of ChE activity inhibition which is induced by the pesticides to the behavioral level. Accordingly, this study will facilitate the interpretation of cellular damage induced by contaminant to ecological perspective through the behavioral level.

As an early warning system, biomarkers can be inserted to early step of ERA to recognize and characterize the hazardous of environmental stressors. In the context of early recognition of the effect of existing pollutants in the environment biomarkers should be applied to assess rapidly hot spots of pollution, thereby stimulating and supporting more detailed risk assessment. To be implemented in Indonesian waters the selected biomarkers which are phagocytotic and ChE activities should be applied in indigenous mussels i.e. green mussels, *P. viridis*.

Based on the role of biomarkers in ERA of OP pesticides four studies on the selected biomarkers from two well-accepted eco-sentinel organisms namely green mussel (*Perna viridis*) and blue mussel (*Mytilus edulis*) for tropical and temperate regions were conducted. The following are the structures of the studies.

1. In vivo test of dimethoate using ChE and phagocytic activity from blue mussel, *M. edulis*, as biomarkers.
2. In vivo test of trichlorfon using ChE activity and siphoning rate from blue mussel, *M. edulis* as biomarkers.
3. Cholinesterases (ChEs) characterization of green mussel, *P. viridis*, from Pangkep district South Sulawesi Indonesia. This enzyme characterization was performed using two approach i.e. substrates and inhibitors differentiations.
Dimethoate is one of organophosphate (OP) pesticides that are in fact deliberately fabricated to inhibit ChE activity of target organisms. Albeit the pesticide frequently usage in upland the presence of the pesticides in aquatic habitat from river (Abdel-Halim et al. 2006) to coastal waters (Hernandes et al. 1993) has been detected. Some OP pesticides have also been recorded to induce immune system of mobile invertebrate such as lobster (De Guise et al. 2004). Hence, the use of selected biomarkers that elucidate neuro-immune response of non-point source eco-sentinel organisms such as blue mussel, *M. edulis* to detect dimethoate effects is of interest. The current study was set up to deal with laboratory test to recognize a potential extendable effect of dimethoate on marine organism by using the selected biomarkers.

One of the challenges to the use of biochemical or cellular biomarkers is the lack of ecological relevance due to the ability of organisms to recover from the neurological damages after being exposed to such contaminants (McHenery et al. 1997). Hence, it is demanding to study by integrating neurological response such as ChE activity and a higher level biological integrity that has a closed reasonable relation to the induced enzyme like feeding activity. The study also attempted to record the ability of the trichlorfon-exposed mussels to recover from the neurological and behavioral failures after incubation in artificial and natural seawater.

A characterization of ChEs from preferred sentinel organisms is a prerequisite of the use of ChEs from intended organisms as biomarker either in laboratory tests or field investigations (Bocquene et al. 1990; Strum et al. 1999; Rodríguez-Fuentes and Gold-Bouchot 2004). The characterization of these enzymes provides us information at least on types of the enzymes and their parameters by which misinterpretation on data that were derived from undefined ChEs as response to contaminants can be avoided.

The ChE and phagocytic activity are two biomarkers that have been employed frequently in stressor effects biomonitoring by using blue mussel, *M. edulis* in temperate regions. There are few investigations in tropical regions that used the two biomarkers by employing green mussel, *P. viridis*. 
Geographically *M. edulis* is distributed widely throughout boreal and temperate waters of both northern and southern hemispheres (Soot-Ryen 1955). In contrast, *P. viridis* is distributed widely in the Indo-Pacific region, from Japan to New Guinea and from Persian Gulf to South Pacific Islands (Siddall 1980). Although, the two mussels have different geographical distribution, their local distributions are similar. They generally live in marine intertidal, subtidal waters though occasionally inhabit deeper water particularly where there is notable water movement (Seed 1976; Rajagopal et al. 1998). They also share common characteristics by living and growing in cluster using well-developed byssal apparatus on a variety of substrata, such as rock, wood, concrete, metal, old submerged logs, and boats (Seed 1976; Rajagopal et al. 1998).

The two mussels can be differentiated distinctly by the external color of the shell. *M. edulis*’s shell is covered by the conspicuous, dark yellowish brown or black, proteinaceous periostracum, while the shell color of *P. viridis* is bright green to dark brownish-green near the outer edge and olive-green near the attachment point. The color of the shell is influenced by several genes, the age, and the habitat of the animal. Blue mussel that inhabits in the intertidal zone has a blue-black and heavy shell, while those that lives in the sublittoral zone possesses a brown with dark brown radial markings and thin shell (Gosling 2003). Old *P. viridis*’s shell tends to have more brown, while younger animal has vivid green or blue-green shell (Siddall 1980). Moreover, *M. edulis* is characterized by the presence of an anterior adductor muscle, while this organ is absent in *P. viridis* (Gosling 2003).

As a filter feeder, both *M. edulis* and *P. viridis* feeding on phytoplankton, small zooplankton and other suspended organic materials. As a temperate animal, blue mussel has an optimum temperature and salinity for filtration rate are 5-20 °C and 15-30 ppt, respectively (Bayne et al. 1976). The optimum filtration rate of green mussel occurs at temperature 30 °C and salinity 30-35 ppt (Rajagopal 1991). Accordingly, the growth of the two animals is influenced by temperatures. The optimum range of the growth of *M. edulis* is 3-20 °C (Almada-Villela et al. 1982), whereas *P. viridis* can grow appropriately on temperature 27-32 °C (Asikin 1982).
Both in *M. edulis* and *P. viridis* sexes are separate without external sign of dimorphism (Seed 1976; Rajagopal *et al.* 2006). However, hermaphrodites may occur in blue mussel population. Male and female gonads are distinguishable either in *M. edulis* or *P. viridis*. Ovaries are reddish in *M. edulis* and bright orange in *P. viridis* whereas testes are cream in *M. edulis* and milky white in *P. viridis*.

The hot spot monitoring in this present study attempted to apply the two selected biomarkers i.e. ChE and phagocytic activities which are originated from *M. edulis* in Indonesia waters which is populated by *P. viridis*. The use of *P. viridis* as eco-sentinel organism in stressor effects investigation campaigns in Indonesia is efficacious due to widely distribution of the animal from expected pristine to heavily polluted waters. The hot spot monitoring was conducted in three different sites. The expected pristine site is located at coastal area of Pangkajene Kepulauan (Pangkep) district, while the heavily polluted sites are located in Jakarta Bay, namely Kamal Muara and Cilincing. The three extreme environments are considered to be appropriate location models for applying the biomarkers to picturize adverse effects status of the deteriorated coastal environments.

1.4. **Objectives of the Studies**

The studies were aimed to elaborate the use of selected biomarkers, cholinesterase (ChE), phagocytic and siphoning activities of marine mussels to detect environmental stressors both in terms of *in vivo* and *in situ* applications. To achieve the objectives, the studies were composed as the following:

1. To detect the effects of dimethoate on ChE and phagocytic activity of *M. edulis*.

2. To evaluate the effects of trichlorfon on ChE activity and siphoning rate of *M. edulis*. The evaluation was focus on to know different sensitivity of ChE activity in different organs of *M. edulis* and transformation of inhibition of the ChE activity from innervated organs to inhibition of the siphoning rate.
3. To characterize ChEs enzyme of inervated organs of green mussel *P. viridis* as a rational basis for the use of the ChE activity as a biomarker.

4. To study the applications of selected biomarkers, phagocytic and ChE activities, from green mussel, *P. viridis* in hot spot biomonitoring in Indonesian coastal waters.

1.5. Purposes of the Studies

The studies on selected biomarkers contribute to establish rational basis of screening test and pollutant response detections in biological compartments of eco-sentinel organisms particularly marine mussel. In addition, since the state of the art of the use of biomarkers is to detect the pollutant effects in living organism in early destructive conditions the hot spot study underlie biomonitoring programs to detect early deterioration of pollutants effects on living organism. Therefore, the study gives environmental manager to characterize the hazards of environmental stressor at early state which trigger more detailed ecological risk assessments.

1.6. Hypothesis

The working hypothesis of the four studies are:

1. There are adverse effects of the OP pesticide, dimethoate, on nervous and immune system of blue mussels *M. edulis*.

2. OP pesticide, trichlorfon induces both ChE activity and siphoning rate of *M. edulis* in different sensitivity levels.

3. ChEs enzyme from different organs of *M. edulis* show different sensitivity to the OP Pesticide, trichlorfon.

4. There is relationship between the induced ChE activity from inervated organs of *M. edulis* and the siphoning rate.

5. Properties of ChEs enzyme of *P. viridis* differ from orthodox characteristic of vertebrate.

6. Selected biomarkers, phagocytic and ChE activities of *P. viridis* can be used to distinguish different effects of pollutants which exist in selected coastal area of Indonesia.
1.7. Novelties

The studies on the use selected biomarkers in terms of *in vivo* and *in situ* applications proposed new findings.

1. The OP pesticides are acknowledged as anticholinesterase of living organisms from vertebrate to invertebrate. In invertebrate particularly in marine mussel many studies were conducted to recognize the effect of pesticides on ChE activity of the mussels. However, the extension effect of the pesticides on higher biological integrity such as siphoning activity that have conceptually closed relate to nervous system has been not studied yet in terms of the correlation between the ChE activity inhibition of different organs of blue mussel and the siphoning rate. One part of the studies revealed that the transformation of inhibition of ChE activity induced by the pesticide in innervated organs to inhibition of the siphoning activity of blue mussel was observed.

2. The requirement for using ChE activity as a biomarker in new eco-sentinel organism is characterization of ChEs enzyme properties of the studied animal. Prior to application of the ChE activity from *P. viridis* in hot spot biomonitoring, the characterization of the ChEs of the indigenous sentinel organism, *P. viridis* was conducted. The study suggested that ChEs of *P. viridis* can be classified as a typical acetylcholinesterase (AChE) and an atypical butyrilcholinesterase (BuChe).

1.7. References


II. THE USE OF SELECTED BIOMARKERS, PHAGOCYTIC AND CHOLINESTERASE ACTIVITIES, TO DETECT THE EFFECTS OF DIMETHOATE ON MARINE MUSSEL (*Mytilus edulis*)

2.1. Abstract

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 phagocytosis activity were significant (*p* < 0.05) occurring at two concentrations of dimethoate (7.88 and 15.75 µg/l), but stimulation effects significantly (*p* < 0.05) emerged at the following concentrations (31.35 and 63.00 µg/l). The declining tendency of the ChE activity (23 % lower than the control) appeared when mussels were exposed to 7.88 and 15.75 µg/l dimethoate. Moreover, the significant inhibition of the ChE activity (*p* < 0.05) occurred at 31.35 µg/l dimethoate exposure. This study suggested that the phagocytosis and the ChE activity are useful biomarkers for assessing the affects of organophosphorous pesticide, dimethoate on neuro-immune system of blue mussels, *M. edulis*.

Key words: Dimethoate, Cholinesterase, Phagocytosis, Blue Mussels

2.2. 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 (Floesser-Mueller and Schwack 2001; Lartiges and Garrigues 1995). In spite of these compounds are much more unstable compared to organochlorine in the environment, their persistence toxicity on biota leads to damage ecosystem (Gaglani and Bocquene 2000). Accordingly, the biological response characterization of biota exposed by pesticide is an important step toward the evaluation of the risks. It is due to most of modern OP compounds that are deliberately synthesized to inhibit an important enzyme of nervous system, which is acetylcholinesterase (AChE) of target organisms (Galloway and Handy 2003). This enzyme plays a significant role on preventing an accumulation of a neurotransmitter compound i.e. 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 as an irreversible effect, because the time
needed to synthesis *de novo* this enzyme is longer than the time of dissociation of the OP-AChE complex (Gaglani and Bocquene 2000; Hyne and 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 and Key 2001).

OP compounds not only inhibit cholinesterase (ChE) activity, but also interfere the immune system of organisms (Banerjee *et al.* 1998; Galloway and 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 or cytotic T lymphocyte responses (Voccia *et al.* 1999). OP insecticides were used to control mosquitoes in coastal areas were detected in laboratory induced phagacytosis activity of lobster, 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*) which indicate that the pesticide brought about an effect similar to the production of anemia. Hatching rate of characid fish (*Prochilodus lineatus*) eggs and the hatched larve 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 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 were conducted to test the toxicity 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 and Guilhermino 2002) and common carp (*Cyprinus carpio*) (De Mel and 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. 2001b) 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 and Galloway. 2004; Brown et al. 2004) and to detect the potential immune suppression of some heavy metals and others pollutant in marine ecosystem using phagocytic activity (Pipe et al. 1999; Galloway and 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 to test the chronic effects of dimethoate on neuro-immune system of marine mussel, *M. edulis* using ChE and phagocytic activity assay.

### 2.3. Material and Methods

**Chemicals and Animal Preparation**

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

Marine mussels, *Mytilus 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 ± 1 °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 ± 1 °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 and Guilhermino 2002). The serial nominal concentrations covered also a realistic occurrence of the pesticide in 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 that consist of *Nannochloropsis acula*, *Isochrysis galbana*, and *Tetraselmis suecica*. The experiment was carried out in duplicate.

Cholinesterase Assay

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.* 2002). Mussels were dissected out and gill tissue (0.32 ± 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 °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 °C with 200 µl of 0.75 mM 5,5’-Dithio-bis-(2-Nitrobenzoic acid) prior to the reaction was started by 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 read 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 and 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 analyse 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 FITC (Fluoresceinisothiocyanate)-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 °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 read 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 phagocytic and the ChE activity. Dunn’s Multiple Comparison was used to recognize the differences among the treatments (Newman 1995).

2.4. Results

Phagocytic Activity

The current study showed that both hemocytes numbers and phagocytic activity of the blue mussels before treatment were not significantly different (Figure 2 and Figure 3) which provided a uniform state of the experiment. The exposure of dimethoate for 14 days to the animals depicted that the alteration of hemocytes density 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 4), but there was no significant difference of circulating hemocytes 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.
Figure 2. Circulating hemocyte density of *M. edulis* before the treatment. Data were expressed as median (25 % and 75 % quartile, 5 % and 95 % confidence interval).

Figure 3. Phagocytosis activity of *M. edulis* hemocytes before the treatment. Data were expressed as median (25 % and 75 % quartile, 5 % and 95 % confidence interval).
Figure 4. Circulating hemocytes 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 hemocytes from the treatments and from those observed in the control (p < 0.05).

Figure 5. Phagocytic activity of *M. edulis* hemocytes 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).
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 5). On the other side, the stimulation of the activity significantly occurred at 31.50 µg/l of dimethoate (p < 0.05) compared to previous levels and persisted significantly at the same level at 63.00 µg/l of dimethoate (Figure 5).

**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 6). Although, there were apparent reductions of the ChE activity about 23 % of the control from the mussels that were 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 evident (Figure 6).

![Figure 6. 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).](image)
2.5. 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 hemocytes density 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 density. 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 density compared to previous treatment (31.50 µg/l). The alteration of hemocytes density as response 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 hemocytes density of bivalve elevated as results of environmental stressors exposure (Coles et al. 1994a; Coles et al. 1994b; Pipe et al. 1999; Dizer et al. 2001a; St-Jean et al. 2002), whereas others have shown that the stressors declined the hemocytes density (Dizer et al. 2001b; Suresh and Mohandas 1990; Auffret et al. 2002). Undefined response of hemocytes density to environmental stressors may implied that the number of circulating hemocytes do not fundamentally reflect the total size of the hemocytes population in mussels 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 hemocytes numbers under environmental stressors, which depicted the elevation of mussel hemocytes density when exposed by 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 mussel hemocytes occurred at those concentrations. On the other hand, stimulated paghocytic 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, the first two low levels of dimethoate exposures had the circulating number 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 hemocytes numbers of two treatments were at the same level statistically. On the other hand, significant stimulation of hemocytes numbers at two highest contaminants (31.50 and 63.00 µg/l) has been not followed by distinct stimulation of the phagocytic activity compare to the control. Again, it might be due to the unphagocytic and/or death cells composed predominantly hemocytes population at two highest dimethoate concentrations, although the hemocytes 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 levels 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 were exposed to the pesticide for 96 h (Rickwood and Galloway 2004).

In fact, there are functional differences between mussel hemocytes types (Cheng 1984; Dyrynda *et al.* 1997; Pipe *et al.* 1997). The granulocytes are phagocytotic 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 was used in this recent study, did not involve a differentiation of hemocytos types so that the exact correlation between proportion of hemocytes 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 carbamate 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 these two types of pesticides in 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 effects arising from the presence of pesticides (Gaglani and 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 concentration just above the control. The decrease was 23 % compared to the control on mussels that were exposed to dimethoate at two concentrations, 7.88 and 15.75 µg/l respectively. However, these suppressions were not significant different with the control due to high variability response among individual mussels (Figure 6). In laboratory condition, it is broadly accepted that more than 20 % decrease in ChE activity indicates exposure to OP pesticides in different species (Bayers and Sikoski 1994; Ludke et al. 1975). Coppage (1972) also suggested that inhibition of the ChE activity from brain of fish in the range of 20 to 70 % indicated organophosphorous exposure. Moreover, Horsberg et al. (1989) reported that dead salmon concerning trichlorfon and dichlorvos exposure 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 gill.

The significant inhibition of the ChE activity clearly occurred when mussel exposed to 31.50 µg/L of dimethoate, decreased 47 % of the ChE activity compared to the control. The percentage of the inhibition tended to 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 the ChE activity exposed by 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 suggested that those were the sign of the recovery of the mussel immune system in light of phagocytic activity after exposed to the highest concentrations of dimethoate. Instead of following dose-dependent response curve, the pattern of dimethoate effect on the phagocytic activity which depicted the decreasing phagocytic activity at lowest concentrations and the stimulation at the highest concentrations agreed with U-Shaped hormesis dose-response pattern which were 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 by 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 other health parameters including phagocytic activity, it could be suggested that enhancing phagocytosis activity indicated continuing deleterious effects of the mussels health rather than improving the fitness of the mussels. Accordingly, the significant decrease of the phagocytic activity caused by dimethoate at the two lowest concentrations could be proposed as initial damage of dimethoate
disruption which was pursued by severe damages when mussels were exposed to the higher concentrations. The hypothesis of the initial damage was probably also strengthened by the fact that inhibition of the ChE activity of the mussel gills about 23% compared 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 phagocytosis and the ChE activity of the mussels probably occurred at concentration below 31.50 µg/l.

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

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2.6. Reference


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), γ-globuline, Zymosan (Z4250-1G) were obtained from Sigma, USA. Trichlorfon (PESTANAL®, 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®) in distilled water with 3 % salinity under temperature 5 ± 1 °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 IC₅₀ 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® 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 suspesion. 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.

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

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
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 nmoles 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 ± 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 ± 4.24, 58.86 ± 4.72 and 62.36 ± 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 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 $73.17 \pm 19.16$ 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 ($3.44 \pm 1.17$ nmol/min/mg protein) (Figure 9). At this concentration, the pesticide reduced 29 % of the ChE activity from the control level ($4.83 \pm 1.36$ 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 (striped 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).

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 μg/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 μg/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 μg/l and reduced slightly at concentration of 1000 μg/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 μg/l) (P < 0.05).
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 (stippled area with ± standard deviation) and 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
after exposing to Hg for 24 days took place after eight days transferring to clean water along with the ciliation 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 μg/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 μg/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 μg/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 control 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 $Y = -26.576 + 5.195 \times X_1 + 17.009 \times X_2$, 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 other 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


IV. CHARACTERIZATION OF CHOLINESTERASE ACTIVITY IN GREEN MUSSEL (*Perna viridis*) AS A POTENTIAL BIOMARKER IN MARINE BIOMONITORING

4.1. Abstract

Green mussels (*Perna viridis*) play an important economic and ecological role in the coastal areas of Indonesia. *P. viridis* has been used as an eco-sentinel organism for marine biomonitoring programme in Asia regions. To magnify its competency as a relevant organism in biomonitoring, we characterized the cholinesterases (ChEs) of *P. viridis* from a selected coastal area of Indonesia. Dissected organs which were gill, foot, mantle and posterior adductor muscle (PAM) were examined for substrate specificity and inhibitors sensitivity using selective and non-selective substrates and inhibitors. The highest level of the ChE activity was observed in gill and followed by foot, mantle and PAM. The substrate specificity approach using various Alkylthiocholines indicated that the ChEs of *P. viridis* constitute an acetylcholinesterase (AChE) and a butyrylcholinesterase (BuChE). Likewise, the inhibitors sensitivity approach using eserine, BW284C51, and iso-OMPA sustained the previous approach which recorded typical AChE and atypical BuChE. The results suggested that the *P. viridis* gill is the most suitable organ for employing the ChE activity as a biomarker in marine monitoring.

Key words: Green mussel, Acetylcholinesterase, Atypical Butyrylcholinesterase, Biomonitoring.

4.2. Introduction

Cholinesterases (ChEs) are enzymes, which play a major role for degrading one of important neurotransmitters, acetycholine (ACh) in the synaptic cleft. Degradation of ACh is vital not only to prevent undesired activation of neighboring neuron or muscle cells, but also to ensure proper timing of signaling to the post-synaptic cell (Chang and Strichartz 2005). The occurrence of two types of ChEs which are acetylcholinesterase (AChE) and butyrilcholinesterase (BuChE) and their variants have been characterized and recorded in the marine mussel body (Bocquene *et al.* 1990; Talesa *et al.* 2001; Brown *et al.* 2004).

There are two approaches that can be used to characterize ChEs types which are based on substrates and inhibitors selectivity (Sturm *et al.* 1999). Based on the substrates, AChE preferentially hydrolyzes acetylcholine rather than other choline esters and shows substrate inhibition at high concentration. On the other hand, BuChE preferentially hydrolyzes butyrylcholine, but it also hydrolyzes acetylcholine and other choline ester (Massouli *et al.* 1993; Chuiko
2000), which does not show substrate inhibition at high concentration (Legay 2000). Furthermore, the ChEs types can also be differentiated by using their specific inhibitors. Eserine is considered as a non-selective inhibitor for ChEs, while 1,5-bis (4-allyldimethylammoniumphenyl) pentan-3-one dibromide (BW284C51) and tetraisopropyl pyrophosphoramide (iso-OMPA) are considered as a selective inhibitor for AChE and BuChE, respectively (Brown et al. 2004).

In invertebrate particularly bivalves, the characterization of ChEs have been studied. The objectives of these studies are to get the basic understanding for using them as a biomarker of deleterious effects of organophosphorus and carbamate pesticides pollutants (Wachtendonk and Neef 1979; Mora et al. 1999; Moreira et al. 2001; Valbonesi et al. 2003; Brown et al. 2004). There are various biochemical properties and distributions of the enzymes in marine mussels, and commonly seem to be dominated by AChE and followed by BuChE (Bocquene et al. 1990; Brown et al. 2004). In the body tissues or innervated organs of mussels, the AChE form has been reported as a membrane-bound esterase (Talesa et al. 2001), whereas AChE as a globular esterase occurs freely in hemolymph has been observed in M. edulis and M. galloprovicinalis (Wachtendonk and Neef 1979; Talesa et al. 2001).

No matter of the ChEs form, they are the functional target of organophosphorus and carbamate pesticides that are used exceedingly to control the pest in agricultural and other anthropogenic activities (Chambers 1992). These two pesticides act as pseudo-substrates and inhibit ChE activity of target and non-target organisms by phosphorylating and carbamylating the active serine (Reiner and Radic 2000). Some heavy metals, polycyclic aromatic hydrocarbons (PAHs), surfactants and domoic acid, a neurotoxic of diatom, were reported to inhibit ChE activity of aquatic invertebrates as well (Guilhermino et al. 1997; Tabche et al. 1997; Hamzah-Chaffai et al. 1998; Akcha et al., 2000; Dizer et al. 2001; Moirera et al. 2004; Costa et al. 2007). Therefore, those evident gave raise to an idea for using the ChE activity as a diagnostic tools or termed as biomarker in biological effect monitoring programmes of aquatic systems.

Enormous attentions have been conferred to the ChE activity of mussels that inhabit the temperate regions as a biomarker in many biomonitoring
programmes of marine environment. ChEs of some tropical shellfish which were proposed as eco-sentinel organisms have been classified (Monserrat et al. 2002) and used as a biomarker in marine monitoring (Owen et al. 2002; Lau and Wong 2003). Nevertheless, in our knowledge characterization of ChEs from P. viridis that play a vital economic and ecologic role in the tropical region has not been established yet. Regarding the ChE activity, a direct application of techniques derived from temperate animals such as blue mussel (M. edulis) to tropical green mussel (P. viridis) in terms of biomarker in marine pollution monitoring should be performed carefully. It may bring up genetic and geographic difficulties. For instance, Valbonesi et al. (2003) reported different levels of the ChE activity of two bivalves, Ostrea edulis and M. galloprovincialis significantly, while no detectable ChE activity was observed in Tapes philippinarum. The sensitivity of the ChE activity from different species of bivalves to ChE-inhibitors is also different (Monserat et al. 2002). Likewise, some aquatic organisms occasionally have atypical ChEs (Rodryguez-Fuentes and Gold-Bouchot 2004). The present of atypical ChEs may mask the existence of pesticides effect on the ChE activity of sentinel organisms. In this context, the characterization of ChEs has to be established prior to a monitoring programe to avoid erroneous interpretations of data (Bocquene et al. 1990; Strum et al. 1999; Rodryguez-Fuentes and Gold-Bouchot 2004). Therefore, a research on green mussel (P. viridis) ChEs characterization is necessary and intriguing for underpinning the utilizing of the ChE activity in monitoring.

In some coastal areas of Indonesia such as Jakarta Bay, green mussels (P. viridis) play a significant role in small-scale farmer livelihood as marine culture organisms. Nowadays, these small-scale marine cultures are threatened by industrial and agricultural activities, which discharged xenobiotic waste such as pesticides into marine ecosystem (Munawir 2005). Meanwhile, the use of comfortable tools like biomarkers in marine biomonitoring for managing and protecting marine ecosystem has not been conducted yet. It is due to scarcity of information regarding a basic knowledge that substantiates the utilization of biomarkers considering tropical animals as an eco-sentinel organism. Therefore, the aim of this study is to characterize the ChEs of P. viridis from Indonesian
waters as a rational basis of the ChE activity employment as a biomarker of organophosphorus and carbamate pesticides and other ChE-inhibitors effects.

4.3. Materials and Methods

Chemicals

Acetylthiocholine iodide (ASCh), acetyl-β-methylthiocholine iodide (A-β-MSCh), butyrylthiocholine iodide (BuSCh), physostigmine (eserine), BW284C51 (1,5-bis (4-allyldimethyl-ammoniumphenyl) pentan-3-one dibromide), Iso-OMPA (tetraisopropyl pyrophosphoramide), 5,5'-Dithio-bis(-2-Nitrobenzoic acid) (DTNB) were purchased from Sigma. All others reagents used were analytical grade products.

Sample Collection and Preparation

The study was conducted on expected clean coastal area of Pangkajene Kepulauan (Pangkep) district in South Sulawesi (Figure 19). There are relatively minimal anthropogenic activities, which influence pollution in this place such as traditional fisheries that use static fishing equipments. Those equipments not only attract the target fish, but also attract undomesticated green mussels (*P. viridis*) which attach and dwell naturally on them.

Sample collection was conducted on 20 September 2005. Sixteen green mussels (5-6 cm) were handpicked from those fishing equipments and directly transferred to the laboratory of Marine Science and Fisheries Faculty, Hasanuddin University, Makassar Indonesia using cool box under humid condition. Gill, foot, mantle and posterior adductor muscle (PAM) were cut off, blotted dry and weighted before placing in 2 ml eppendorf tube containing potassium phosphate buffer (0.1 M/pH 8.0). The tissues were stored at –70 °C before transferring to the Ecotoxicology Department Laboratory, Technische Universitaet of Berlin, Germany using cool box that was filled with dry ice.

A Dounce homogenizer was used to homogenize 0.3 g of each tissue in 2 ml potassium phosphate buffer (0.1 M/pH 8.0). The homogenate was centrifuged for 10 min at 10 000 x g and the supernatant was harvested and stored at –80 °C prior to analysis of the ChE activity and the protein content. The supernatant was diluted in 1:2 of potassium phosphate buffer (0.1 M/pH 8.0) following the enzyme measurement.
Figure 19. Sampling station (ST) in Pangkajene Kepulauan (Pangkep).

**Cholinesterase Activity**

The enzyme activity was measured on a 96-well plate following the Ellman method (Ellman *et al.* 1961) and adapted for microplate. The enzyme measurement was carried out by various alkylthiol substrates in potassium phosphate buffer (0.1 M/pH 8.0) at 25 °C containing 0.75 mM 5,5'-Dithio-bis(-2-Nitrobenzoic acid) as a reagent. Subsequently, the enzymatic reaction rate was determined by photometry for microtiter plate (Spectra Thermo TECAN) in an interval of 30 s for 5 min at 405 nm. In each experiment, two blanks were included to quantify the reaction of thiols with DTNB and of spontaneous substrate hydrolysis sequentially. Three alkylthiocholine substrates, acetylthiocholine (ASCh), acetyl-β-methylthiocholine (A-β-MSch), butyrylthiocholine (BuSCh) which are selective for ChEs, acetylcholinesterase (AChE), butyrylcholinesterase (BuChE) were used to determine cholinesterase types. Substrate kinetic was observed in gill supernatant by utilizing substrate concentrations (ASCh) in the range of 0 – 12 mM. Inhibitor differentiation approach was also used by employing eserine, 1,5-bis (4-
allyldimethylammoniumphenyl) pentan-3-one dibromide (BW284C51) and tetraisopropyl pyrophosphoramide (iso-OMPA) which are selective for ChE, AChE and BuChE respectively. Incubation of enzymes preparation with 10 μM inhibitors for 30 minutes was performed prior to determination of residual activities as described before with 3 mM alkylthiocholine substrates. The various alkylthiocholine substrates and cholinesterase inhibitors that were used in this study according to Brown et al. (2004) were presented in Table 3.

Table 3. The various alkylthiocholine substrates and cholinesterase inhibitors.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholines Terase</td>
<td>Acetyl-β-methylthiocholine (A- β-MSCh)</td>
<td>Acetylthiocholine (ASCh)</td>
</tr>
<tr>
<td>AChE (EC 3.1.1.7)</td>
<td>ISO-OMPA</td>
<td>BW284C51 Serine</td>
</tr>
<tr>
<td>Butyrylcholines terase</td>
<td>Butyrylthiocholine (BuSCh)</td>
<td></td>
</tr>
<tr>
<td>BChE (EC 3.1.1.8)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Protein Measurement**

A protein content measurement was carried out by diluting 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 section 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, the samples were left in room temperature for 20 minutes to allow color development. The absorbance was read at 620 nm using photometry (Spectra Thermo TECAN).

**Data Analysis**

Determination of enzyme kinetic parameters of the ChE activity was performed in green mussel gill. The GraphPad Prism version 4.00 was used to determine enzyme kinetic parameters of ChEs that were $K_m$ and $V_{max}$ by plotting substrate concentrations to the enzyme activity in the term of a nonlinear regression analysis, where a hyperbola was fitted directly to substrate-velocity.
data. A Hanes-Woolf plot was created by plotting substrate concentrations data [S] toward [S/V] data. The line based on nonlinear regression analysis was overlaid in the plot to provide the best picture of estimating the enzyme kinetic parameters.

The statistical analyses were conducted using the GraphPad Prism version 4.00 edition software to determine the mean difference of the experiments (ANOVA). The tukey test was used to distinguish the differences between experiments. All determination were conducted in four replicates and expressed as mean and standard deviation.

4.4. Results

Enzyme Kinetic

The kinetic constants that are $K_m$ and $V_{max}$ were determined by plotting the enzyme activity of gill mussel supernatant against substrate concentrations (ASCh) (Figure 20). By employing substrate concentrations in the range of 0 – 12 mM, the $K_m$ and $V_{max}$ value were 0.1702 mM and 148.0 nmol/min/mg protein.

![Figure 20. Effect of acetylthocholine (ASCh) concentration as substrate on esterase activity in supernatant extract 10000 g (S10) from gill P. viridis. Insert is Hanes-Woofl-plot.](image_url)
Figure 21. Comparison of esterase activity of various tissue of green mussel with various substrates (3 mM). Pooled organs from sixteen green mussels were measured for the enzyme activity (nmol/min/mg of protein).

respectively. In light of tested concentrations, the ChE activity obeyed Michaelian-Menten behavior showing an indication of substrate inhibition. The transformation data from the Michaelis-Menten plot using the Hanes-Woolf method showed a linearity of the line (R = 0.999). The results also showed that the maximum activity of the enzyme was achieved when the substrate concentration was 3 mM (Figure 20). Accordingly, this concentration was used for further determination.

Substrate-Based characterization

The substrate-based characterization of ChEs enzymes have been studied by using three substrates analog, which are ASCh, A-β-MSch, and BuSCh. The results depicted that the highest ChE activity was recorded in the gill followed by foot, mantle, and PAM when using ASCh and A-β-MSch as substrates (Figure 21). In contrast, with BuSCh as a substrate, the gill had the lowest activity, while PAM presented the highest activity significantly succeeded by mantle and foot. Nevertheless, the highest ChE activity was generally detected when the enzymes were incubated with ASCh, while BuSCh substrate showed the lowest reaction with the enzymes. Comparison to BuSCh as substrate, A-β-MSch depicted a significant stronger reaction with the enzymes (Table 4). In all organs, incubation of the enzymes by A-β-MSch substrate exhibited enzymes activities relative to ASCh substrate ranging from 30.67 – 65.40 %. Accordingly, with BuSCh as a
substrate the enzyme activity relative to ASCh substrate showed the percentage of activity ranging from 0.18 – 26.79 %. The results indicated that the true AChE constitutes dominantly in four examined organs i.e., gill, foot, mantle, and PAM, while the BuChE coexists in those four organs. Eventually, the existence of BuChE activity in PAM was observed higher compare to other organs.

Table 4. Mean of cholinesterase activity of three substrates as measured in various organs of green mussel with comparison to ASCh.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>ChEs activity (nmol/min/mg P)</th>
<th>Enzyme activity relative to ASCh (%)</th>
<th>Significantly less than ASCh at p &lt; 0.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gill</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetylthiocholine (ASCh)</td>
<td>137.03 ± 8.6</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Acetyl-β-methylthiocholine (A-β-MSCh)</td>
<td>81.10 ± 8.30</td>
<td>59.18</td>
<td>Yes</td>
</tr>
<tr>
<td>Butyrylthiocholine (BuSCh)</td>
<td>0.25 ± 0.06</td>
<td>0.18</td>
<td>Yes</td>
</tr>
<tr>
<td>Foot</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetylthiocholine (ASCh)</td>
<td>47.82 ± 5.44</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Acetyl-β-methylthiocholine (A-β-MSCh)</td>
<td>25.75 ± 2.29</td>
<td>53.86</td>
<td>Yes</td>
</tr>
<tr>
<td>Butyrylthiocholine (BuSCh)</td>
<td>1.02 ± 1.14</td>
<td>2.13</td>
<td>Yes</td>
</tr>
<tr>
<td>Posterior adductor muscle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetylthiocholine (ASCh)</td>
<td>10.50 ± 0.31</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Acetyl-β-methylthiocholine (A-β-MSCh)</td>
<td>6.55 ± 0.16</td>
<td>62.34</td>
<td>Yes</td>
</tr>
<tr>
<td>Butyrylthiocholine (BuSCh)</td>
<td>2.81 ± 0.36</td>
<td>26.79</td>
<td>Yes</td>
</tr>
<tr>
<td>Mantle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetylthiocholine (ASCh)</td>
<td>22.46 ± 3.07</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Acetyl-β-methylthiocholine (A-β-MSCh)</td>
<td>14.69 ± 1.55</td>
<td>65.4</td>
<td>Yes</td>
</tr>
<tr>
<td>Butyrylthiocholine (BuSCh)</td>
<td>1.48 ± 0.44</td>
<td>6.61</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Inhibitor-Based Characterization

Three inhibitors which assumed to be an unselective (eserine) and selective (BW 284C51 and iso-OMPA) inhibitors of ChEs were used to confirm the substrate-based characterization of ChEs (Figure 22, Figure 23, & Figure 24). The figures demonstrated the usefulness of the used inhibitors in characterization of ChEs enzymes from different organs of *P. viridis.*
Figure 22. ChE activity of various organs of green mussel with acetylthiocholine (ASCh) as substrate and the inhibitors (10μM); Eserine, BW 284C51 and Iso-OMPA. The enzyme activity was measured by pooling organs from sixteen green mussels (nmol/min/mg of protein). Bars indicated standard deviation. * Significantly different from control ($P < 0.01$).

Figure 22 showed that the use of ASCh as substrate for hydrolyzing the enzyme which was inhibited by three different inhibitors. Eserine as general inhibitor for ChEs was able to inhibit the enzyme activity almost completely. A strong inhibition of the enzyme activity was still demonstrated by specific inhibitor for AChE i.e., BW284C51. On the other hand, iso-OMPA as specific inhibitor for BuChE delineated a weak inhibition, although it was significant statistically.

Interesting results were obtained when A-β-MSCh was used as substrate for hydrolyzing ChEs (Figure 23). As general inhibitor eserine inhibited the hydrolyzed enzyme almost entirely. Moreover, as specific inhibitor for AChE, BW284C51 inhibited the enzyme activity strongly. A partial inhibition of the enzyme activity was demonstrated by specific inhibitor of BuChE, iso-OMPA. It was intriguing since the ability of iso-OMPA inhibited the enzyme activity appeared a question on its specificity or a variant of BuChE that involved in hydrolyzing the enzyme when A-β-MSCh was used as substrate.
Figure 23. ChE activity of various organs of green mussel with acetyl beta metyl thiocholine (A-ß-MSCh) as substrate and the inhibitors (10μM); Eserine, BW 284C51 and Iso-OMPA. The enzyme activity was measured by pooling organs from sixteen green mussels (nmol/min/mg of protein). Bars indicated standard deviation (SD). *Significantly different from control (P <0.01).

Figure 24. ChE activity of various organs of green mussel with Butyrylthiocholine (BuSCh) as substrate and the inhibitors (10μM); Eserine, BW284C51 and Iso-OMPA. The enzyme activity was measured by pooling organs from sixteen green mussels (nmol/min/mg of protein). Bars indicated standard deviation (SD). * Significantly different from control (P <0.01).

Iso-Ompa as specific inhibitor of BuChE delineated a strong inhibition as strong as the inhibition by eserine when BuSCh was used as substrate for hydrolyzing the supernatant of the studied organs (Figure 24). In contrast, specific inhibitor for AChE, BW284C51 did not inhibit the hydrolyzed enzyme. It showed the specificity of BW284C51 as specific inhibitor of AChE.
Gill

Table 5 showed the effect of tested inhibitors (10 μM) on ChE activity on gill of *P. viridis* with different substrates. As an unselective inhibitor of ChEs, eserine inhibited hydrolysis of all tested substrates effectively. Whereas, BW 284C51, as a selective inhibitor of AChE affected not only hydrolysis of A-β-MSCh, but also suppressed hydrolysis of ASCh. However, the inhibition of A-β-MSCh hydrolysis by BW284C51 was significantly greater than that of ASCh hydrolysis by BW284C51. Iso-OMPA, which is considered as a selective inhibitor of BuChE affected significantly the ChE activity with BusSCh as a substrate, although it still showed a significant inhibition of ASCh and A-β-MSCh hydrolysis.

Table 5. The influence of anti-ChE substances on ChE activity green mussel gill with different substrates.

<table>
<thead>
<tr>
<th>Substrates-Inhibitors</th>
<th>ChE activity (nmol/min/mg protein)</th>
<th>Inhibition (%)</th>
<th>Significant different of inhibition degree of the inhibitors at p &lt; 0.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gill</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASCh</td>
<td>137.03 ± 8.6</td>
<td>0</td>
<td>Eserine &gt; BW 284C51 &gt; Iso-OMPA</td>
</tr>
<tr>
<td>ASCh-Eserine</td>
<td>3.57 ± 0.56</td>
<td>97.40</td>
<td></td>
</tr>
<tr>
<td>ASCh-BW 284C51</td>
<td>29.70 ± 4.82</td>
<td>78.33</td>
<td></td>
</tr>
<tr>
<td>ASCh-Iso-OMPA</td>
<td>104.56 ± 8.58</td>
<td>23.70</td>
<td></td>
</tr>
<tr>
<td>A-β-MSCh</td>
<td>81.10 ± 8.30</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>A-β-MSCh-Eserine</td>
<td>3.52 ± 0.36</td>
<td>95.65</td>
<td></td>
</tr>
<tr>
<td>A-β-MSCh-BW 284C51</td>
<td>10.41 ± 1.09</td>
<td>87.16</td>
<td>Eserine &gt; BW 284C51 &gt; Iso-OMPA</td>
</tr>
<tr>
<td>A-β-MSCh-Iso-OMPA</td>
<td>55.99 ± 4.60</td>
<td>30.96</td>
<td></td>
</tr>
<tr>
<td>BuSCh</td>
<td>0.25 ± 0.06</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>BuSCh-Eserine</td>
<td>0.02 ± 0.01</td>
<td>91.51</td>
<td></td>
</tr>
<tr>
<td>BuSCh-BW 284C51</td>
<td>0.21 ± 0.01</td>
<td>15.16ns</td>
<td>Iso-OMPA ≈ Eserine</td>
</tr>
<tr>
<td>BuSCh-Iso-OMPA</td>
<td>0.01 ± 0.01</td>
<td>95.44</td>
<td></td>
</tr>
</tbody>
</table>

*ns is not significant different*

Foot

The effect of examined inhibitors on foot of green mussels is demonstrated in Table 6. The hydrolysis of ASCh, A-β-MSCh, and BuSCh was significantly inhibited by eserine in foot. Moreover, BW284C51 demonstrated the lower level of inhibition on ASCh hydrolysis than that of eserine, but it showed the inhibition on A-β-MSCh hydrolysis as strong as the inhibition by eserine. As a selective
inhibitor for A-β-MSCh, BW284C51 did not show a significant inhibition on BuSCh hydrolysis. In contrast, Iso-OMPA inhibited all tested substrates by showing the lowest level of inhibition on ASCh and A-β-MSCh hydrolysis compared to other inhibitors, but depicted a significant high level of inhibition on BuSCh hydrolysis.

Table 6. The influence of anti-ChE substances on ChE activity of green mussel foot with different substrates.

<table>
<thead>
<tr>
<th>Substrates-Inhibitors</th>
<th>ChE activity (nmol/min/mg protein)</th>
<th>Inhibition (%)</th>
<th>Significant different of inhibition degree of the inhibitors at p &lt; 0.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foot</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASCh</td>
<td>47.82 ± 4.87</td>
<td>0</td>
<td>Eserine &gt; BW</td>
</tr>
<tr>
<td>ASCh-Eserine</td>
<td>2.46 ± 0.35</td>
<td>94.86</td>
<td>BW284C51 &gt; Iso-OMPA</td>
</tr>
<tr>
<td>ASCh-BW 284C51</td>
<td>9.46 ± 1.21</td>
<td>80.23</td>
<td></td>
</tr>
<tr>
<td>ASCh-Iso-OMPA</td>
<td>38.47 ± 3.05</td>
<td>19.56</td>
<td></td>
</tr>
<tr>
<td>A-β-MSCh</td>
<td>25.76 ± 2.29</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>A-β-MSCh-Eserine</td>
<td>2.20 ± 0.34</td>
<td>91.46</td>
<td>Eserine ≈ BW</td>
</tr>
<tr>
<td>A-β-MSCh-BW 284C51</td>
<td>4.13 ± 0.16</td>
<td>83.95</td>
<td>284C51 &gt; Iso-OMPA</td>
</tr>
<tr>
<td>A-β-MSCh-Iso-OMPA</td>
<td>19.11 ± 0.84</td>
<td>25.82</td>
<td></td>
</tr>
<tr>
<td>BuSCh</td>
<td>1.01 ± 0.14</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>BuSCh-Eserine</td>
<td>0.19 ± 0.09</td>
<td>80.44</td>
<td>Iso-OMPA ≈ Eserine</td>
</tr>
<tr>
<td>BuSCh-BW 284C51</td>
<td>0.81 ± 0.40</td>
<td>20.80 ns</td>
<td></td>
</tr>
<tr>
<td>BuSCh-Iso-OMPA</td>
<td>0.38 ± 0.07</td>
<td>63.11</td>
<td></td>
</tr>
</tbody>
</table>

ns is not significant different

PAM

PAM was one of the green mussel organs that showed the lowest ChE activity with ASCh and A-β-MSCh substrates, but showed otherwise with BuSCh substrate in this present study. The effects of assayed inhibitors are presented on Table 7. Eserine still demonstrates a strong effect on hydrolysis of examined substrates. This behavior is also acted by iso-OMPA, which is considered to be a specific inhibitor of BuChE with the lowest level of inhibition on ASCh and A-β-MSCh hydrolysis, but the highest level of inhibition on BuSCh hydrolysis is observed when the enzymes were incubated with the inhibitor. On the contrary, BW284C51 did show the inhibition of A-β-MSCh and ASCh hydrolysis, without delineating the inhibition on BuSCh hydrolysis.
Table 7. The influence of anti-ChE substances on ChE activity of green mussel posterior adductor muscle (PAM) with different substrates.

<table>
<thead>
<tr>
<th>Substrates-Inhibitors</th>
<th>ChE activity (nmol/min/mg protein)</th>
<th>Inhibition (%)</th>
<th>Significant different of inhibition degree of the inhibitors at p &lt; 0.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASCh</td>
<td>10.49 ± 0.37</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ASCh-Eserine</td>
<td>1.89 ± 0.23</td>
<td>81.97</td>
<td>Eserine &gt; BW</td>
</tr>
<tr>
<td>ASCh-BW 284C51</td>
<td>4.09 ± 0.37</td>
<td>60.97</td>
<td>284C51 &gt; Iso-OMPA</td>
</tr>
<tr>
<td>ASCh-Iso-OMPA</td>
<td>8.05 ± 0.45</td>
<td>23.37</td>
<td></td>
</tr>
<tr>
<td>A-β-MSCh</td>
<td>6.55 ± 0.16</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>A-β-MSCh-Eserine</td>
<td>1.78 ± 0.26</td>
<td>72.76</td>
<td></td>
</tr>
<tr>
<td>A-β-MSCh-BW 284C51</td>
<td>1.85 ± 0.41</td>
<td>71.64</td>
<td>Eserine ≈ BW</td>
</tr>
<tr>
<td>A-β-MSCh-Iso-OMPA</td>
<td>4.49 ± 0.28</td>
<td>31.44</td>
<td>284C51 &gt; Iso-OMPA</td>
</tr>
<tr>
<td>BuSCh</td>
<td>2.81 ± 0.36</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>BuSCh-Eserine</td>
<td>1.77 ± 0.21</td>
<td>37.08</td>
<td></td>
</tr>
<tr>
<td>BuSCh-BW 284C51</td>
<td>2.52 ± 0.21</td>
<td>10.29</td>
<td></td>
</tr>
<tr>
<td>BuSCh-Iso-OMPA</td>
<td>1.63 ± 0.33</td>
<td>42.03</td>
<td></td>
</tr>
</tbody>
</table>

ns is not significant different

**Mantle**

Although iso-OMPA did not show inhibition on ASCh hydrolysis in the mantle of *P. viridis*, it affected the hydrolysis of A-β-MSCh substrate in the lowest level of inhibition compared to that of eserine and BW 284C51 on the same substrate. Accordingly, a strong inhibition on BuSCh hydrolysis was depicted by iso-OMPA with similar level of eserine inhibition on the same substrate statistically. On the other hand, BW284C51 inhibited ChE activity with both A-β-MSCh and ASCh as substrates. In this organ, eserine inhibited hydrolysis of all assayed substrates with strong inhibition ranging from 80.44 – 94.86 % (Table 8).

**4.5. Discussion**

In general, ChEs of vertebrate can be classified into two types as acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8), also called pseudo-cholinesterase) based on substrate hydrolysis and inhibitor sensitivity. Notwithstanding, there are different properties of ChEs among species and its distribution within individual in invertebrate. For instance,
Table 8. The influence of anti-ChE substances on ChE activity of green mussel mantle with different substrates.

<table>
<thead>
<tr>
<th>Substrates-Inhibitors</th>
<th>ChE activity (nmol/min/mg protein)</th>
<th>Inhibition (%)</th>
<th>Significant different of inhibition degree of the inhibitors at p &lt; 0.01</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mantle</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASCh</td>
<td>22.46 ± 3.07</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ASCh-Eserine</td>
<td>1.18 ± 0.24</td>
<td>94.77</td>
<td>Eserine &gt; BW</td>
</tr>
<tr>
<td>ASCh-BW 284C51</td>
<td>9.95 ± 0.77</td>
<td>55.69</td>
<td>284C51</td>
</tr>
<tr>
<td>ASCh-Iso-OMPA</td>
<td>19.94 ± 0.72</td>
<td>11.23 ns</td>
<td></td>
</tr>
<tr>
<td>A-β-MSCh</td>
<td>14.69 ± 1.55</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>A-β-MSCh-Eserine</td>
<td>1.00 ± 0.19</td>
<td>93.18</td>
<td></td>
</tr>
<tr>
<td>A-β-MSCh-BW 284C51</td>
<td>4.31 ± 0.18</td>
<td>70.66</td>
<td></td>
</tr>
<tr>
<td>A-β-MSCh-Iso-OMPA</td>
<td>10.31 ± 0.44</td>
<td>29.79</td>
<td></td>
</tr>
<tr>
<td>BuSCh</td>
<td>1.48 ± 0.44</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>BuSCh-Eserine</td>
<td>0.40 ± 0.11</td>
<td>72.72</td>
<td></td>
</tr>
<tr>
<td>BuSCh-BW 284C51</td>
<td>1.01 ± 0.01</td>
<td>31.95 ns</td>
<td></td>
</tr>
<tr>
<td>BuSCh-Iso-OMPA</td>
<td>0.48 ± 0.18</td>
<td>67.68</td>
<td></td>
</tr>
</tbody>
</table>

ns is not significant different

in bivalve ChEs of the shellfish, *Murex brandaris* dominated by propionylcholinesterase (PrChE) since the enzymes hydrolyze PrSCh substrate rather than ASCh and BuSCh substrate (Talesa *et al.* 1990), while the common mussel *Mytilus sp* hemolymph contains exclusively AChE (Moreira *et al.* 2001). Different types of ChEs were also observed within organs of *Mytilus sp*, which AChE dominates in gill and BuChE constitutes mainly in digestive gland (Escartin and Porte 1997). On the other hand, in Antarctic scallop, *Adamussium colbecki*, two types of ChEs composite evenly between AChE and BuChE in three organs, gill, PAM, and digestive gland (Corsi *et al.* 2004). Those evidence suggested the properties of ChEs in mussels are species dependent and diverse between organs. As a consequence, for using ChE activity as biomarker in biomonitoring campaign it is essential to characterize the properties of ChEs and their distribution in organs of studied organisms.

This current study attempted to characterize ChEs types based on substrate hydrolysis and sensitivity to inhibitors in different organs of tropical green mussel (*P. viridis*) from expected clean coastal area of Indonesia. A measurable ChE
activity from *P. viridis* organs resulted from the present study is considered to be a rational basis of marine biomonitoring programme of neurotoxic substances.

**Substrate-Based Characterization**

Characterization of ChEs types of *P. viridis* using various alkylthiol substrates was preceded by measurement of the enzymes kinetic parameters. The hyperbolic regression analysis of the enzymes kinetic demonstrated that the ChEs activity of gill conformed Michaelian-Menten plot behavior by using ASCh as a substrate (Figure 20). An apparent $K_m$ value was 0.148 mM, which is in the same range of those reported ChEs in some aquatic animals (Table 9). Compare to the $K_m$ value of other bivalves, which have been reported in the previous studies, ChEs of *P. viridis* revealed the higher $K_m$ value. The $K_m$ value expressed the enzyme substrate affinity which a high $K_m$ value indicated a low binding power of the enzyme to the substrate, and *vice versa*. Hence, the results suggested that the enzyme substrate affinity of *P. viridis* gill is relatively lower than that observed in gill of other bivalves (Table 9). In addition, ChEs of *P. viridis* gill reached a maximum velocity ($V_{max}$) at level higher than that of other reported bivalves. This $V_{max}$ was observed at 3 mM substrate concentration and used for further experiments.

In fact, a $K_m$ and $V_{max}$ are properties of individual enzyme and not very useful enzyme parameters for comparing the enzyme. A $K_m$ reflects both binding of enzyme-substrate and the catalytic constant of the enzyme catalyzed reaction, while a $V_{max}$ is a value which depend on a catalytic constant. The more informative parameter for comparing enzyme is the ratio between $V_{max}$ and $K_m$ ($V_{max}/K_m$) which reflects the efficiency of the enzyme for catalyzing its reaction (catalytic efficiency). The present work revealed that $V_{max}/K_m$ value of *P. viridis* gill esterase is higher than those observed in previous studies (Table 9). This data suggested that the ChEs of *P. viridis* gill catalyzed the substrate more effective than those of other bivalves catalyzed and might reflect the higher ChEs activity compare to other bivalves. By comparing two bivalves Valbonesi *et al.* (2003) revealed that, the bivalve that has the higher $V_{max}/K_m$ value demonstrated the higher ChE activity. Nevertheless, the comparison of ChE activity data should be carried out carefully since it was often masked by differences between either
sample preparation methods or use of varying arbitrary units to describe the data (Brown et al., 2004). Therefore, inter-calibration among laboratories, which perform ChEs assay is needed.

Table 9. ChE kinetic parameters from aquatic animals.

<table>
<thead>
<tr>
<th>Animals</th>
<th>Sources</th>
<th>( V_{\text{max}} ) (nmol/min/mg protein)</th>
<th>( K_m ) (mM)</th>
<th>( V_{\text{max}}/K_m ) (min(^{-1}))</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqtinia equina</td>
<td>gill</td>
<td>216</td>
<td>0.098</td>
<td>2.20408 x 10(^{-3})</td>
<td>Talesa et. al. (1996)</td>
</tr>
<tr>
<td>Spongia officinalis</td>
<td>gill</td>
<td>142</td>
<td>8</td>
<td>1.775 x 10(^{-5})</td>
<td>Talesa et. al. (1996)</td>
</tr>
<tr>
<td>Crassostrea rhizophorae</td>
<td>gill</td>
<td>1.92</td>
<td>0.046</td>
<td>4.1739 x 10(^{-5})</td>
<td>Alves et al. (2002)</td>
</tr>
<tr>
<td>O. edulis</td>
<td>gill</td>
<td>4.82</td>
<td>0.093</td>
<td>5.1828 x 10(^{-5})</td>
<td>Valbonesi et al. (2003)</td>
</tr>
<tr>
<td>M. Galloprovicianlis</td>
<td>gill</td>
<td>18.36</td>
<td>0.076</td>
<td>2.4158 x 10(^{-4})</td>
<td>Valbonesi et al. (2003)</td>
</tr>
<tr>
<td>M. edulis</td>
<td>gill</td>
<td>9.198</td>
<td>0.013</td>
<td>7.0754 x 10(^{-4})</td>
<td>Mora et al. (1999a)</td>
</tr>
<tr>
<td>P. viridis</td>
<td>gill</td>
<td>148</td>
<td>0.1702</td>
<td>8.6957 x 10(^{-4})</td>
<td>Present study</td>
</tr>
<tr>
<td>Chasmagnathus granulate</td>
<td>Thoracic ganglia</td>
<td>1750</td>
<td>0.28</td>
<td>6.25 x 10(^{-3})</td>
<td>Monserrat et al. (1998)</td>
</tr>
</tbody>
</table>

Concerning biomonitoring of neurotoxicants in a laboratory and field scale, utilizing of enzyme kinetic parameters viz. \( K_m \), \( V_{\text{max}} \) and \( V_{\text{max}}/K_m \) on data interpretation of ChE activity as a biomarker would be a useful. In term of insect, Zhu and Gao (1999) found strong evidence that resistant strain of green bug (Schizaphis graminum) possessed high ChE activity associated with low affinity and high catalytic activity, and low inhibition to paraoxon as compared with ChE activity from the susceptible strain. The increasing activity of ChE induced by pesticides was also observed in the laboratory scale from larva of the grass shrimp, Palaemonetes pugio (Key and Fulton 1993) and freshwater mussel, Elliptio complanata (Moulton et al. 1996). Romani et al. (2005) reported that organophosphorus pesticide, chlorpyrifos, caused an elevation of ChEs activity of the bivalve, Scapharca inaequalvis at sublethal concentration instead of an
inhibition of the enzymes activity. The evidence will be confounding factors of using ChEs activity as a biomarker if the $K_m$, $V_{max}$ and $V_{max}/K_m$ were not taken into account since theoretically ChEs activity is inhibited by organophosphorous pesticides. In addition, Romani et al. (2005) observed the reasons of the elevation of ChE activity after the chlorpyrifos exposure which was linked to a significant rise in the synthesis of AChE-specific mRNAs followed by decreasing of the enzyme catalytic efficiency ($V_{max}/K_m$). This evidence indicated a resistance of the bivalve to the pesticide after exposed by chlorpyrifos (Romani et al. 2005), as the mechanism of resistance is mediated by amplification of esterase genes and often accompanied by a modification of kinetic parameters of acetylcholine hydrolysis (Fournier et al. 1992; Zhu and Gao 1999). Therefore, it is suggested that the enzyme kinetic parameters should be included in the ChE activity measurement for biomonitoring purposes to obtain an obvious picture of anti-ChE effects on the mussel ChEs activity.

The substrate-based study revealed that tested organs of *P. viridis* consist of two ChEs types, AChE and BuChE, which dominated significantly by AChE because A-β-MSCh substrate hydrolyzed the tested organs extract more effective significantly compared to BuSCh substrate. By considering hydrolysis of ASCh in the tissue extract as a total activity of ChEs enzymes, the activity of AChE only achieved 59 % of the total activity (Table 4). The rest is BuChE and may be propionylcholinesterase (PrChE). This result was in agreement with a previous study that reported an atypical of ChEs enzyme from ‘soluble’ fraction of *M. edulis* gill (Brown et al. 2004). The presence of AChE and BuChE in gill of brown mussel *P. perna* was also reported by Alves et al. (2002).

The distribution of two ChEs enzymes were observed variously in tested organs. The results showed that AChE activity was detected dominantly in all tested organs, which were investigated in the following order: gill > foot > mantle > PAM (Table 4). In contrast, the highest activity of BuChE was observed in PAM followed by mantle, foot and gill. The highest activity of AChE in gill that observed in this present work was in agreement with previous studies, which were conducted in gill of European counterpart of green mussel, *M. edulis* (Bocquene et al. 1990) and *M. gallorovincialis* (Escartin and Porte, 1997). The dominant of
the AChE activity from gill of *M. galloprovicianlis* was about 6-fold compared to the AChE activity from digestive gland has been also reported (Porte *et al.* 2001). However, Najimi *et al.* (1997) reported a high AChE activity observed in the digestive gland and muscle of African brown mussel, *P. perna*.

**Inhibitor-Based Characterization**

The inhibitor-based characterization has been conducted to confirm and complete the ChEs classification based-on substrates (Sturm *et al.* 1999). The presences of AChE and BuChE in all tissues of *P. viridis* have been demonstrated in this work and seem to be strengthened by the inhibitor approach. The strong inhibition of eserine on all substrates hydrolysis in all tissues indicated that all tested organs of *P. viridis* contained ChEs, which confirm a non-selectivity of eserine. Further classifying experiments by using two specific inhibitors, BW284C51 and iso-OMPA were considered as sensitive to AChE and BuChE respectively sustained what observed on the substrate-based study with little deviants. The extract tissues, which hydrolyzed A-β-MSCh substrate was not only sensitive to BW284C51, but also sensitive to iso-OMPA. Furthermore, the inhibition of iso-OMPA on A-β-MSch hydrolysis is significantly lower than that of BW284C5, which indicated a partial inhibition of iso-OMPA on that specific substrate. On the other hand, BuChE of all tissues was sensitive to iso-OMPA and did not demonstrate sensitivity to BW284C51. The results appeared a questioning on specificity of iso-OMPA, which considered as specific inhibitor of BuChE or pointed out an existing of an atypical BuChE in studied organs of *P. viridis*. Nevertheless, the question of iso-OMPA specificity is weakened by the fact that inhibition of iso-OMPA was partial. If iso-OMPA was non-specific inhibitor, it similarly as serine inhibited all substrates hydrolysis almost completely. Hence, the most acceptable explanation is the presence of an atypical BuChE in the studied organs of *P. viridis*. The capability of an atypical BuChE hydrolyzing both BuSCh and A-β-MSCh substrates, which were inhibited by iso-OMPA has been reported as well by Sturm *et al.* (1999) in marine teleost fish. That hypothesis was also supported by the fact that the atypical BuChE of *P. viridis* tissue was not sensitive to BW284C51, the specific inhibitor of AChE. Consequently, the insensitivity of the enzyme to anti-AChE could be postulated as
a characteristic of an atypical BuChE which has a lower affinity for many inhibitors and unnatural substrates since as an anti-AChE scavenger BuChE has broad specificity so that every anti-AChE is also anti-BuChE (Soreq and Glick 2000). Eventually, it is stated clearly that the inhibitor-based characterization is highlighted by substrate-based strategy for classifying ChEs, which identified at least two types of ChEs namely typical AChE and atypical BuChE were observed in studied organ of *P. viridis*.

In conclusion, regarding marine biomonitoring by using ASCh substrate, which is the unselective substrate of ChEs activity of *P. viridis* is preferable since it describes both true (AChE) and pseudo cholinesterase (BuChE). Furthermore, the occurrence of the atypical BuChE in *P. viridis* tissue may amplify the use of ChEs activity from this animal as a valuable tool for pesticides effect-based monitoring since atypical BuChE has a lower affinity for many inhibitors (Soreq and Glick 2000). Subsequently, the possibility of the BuChE confounds the data interpretation by scavenging the contaminants before reaching ChEs will be reduced. The omnipotence of discharged pesticides effects on studied organisms are not diminished by the scavenger activities so that the type II error can be avoided. Likewise, the present study revealed that gill has the highest ChE activity, which emphasized the employment of this organ as a suitable organ for risk-based monitoring tool. The advantage of this organ in term of biomarker study has also been strengthened by Lau *et al.* (2004), who found less sensitivity of protein of *P. viridis* gill to seasonal variation that produced higher protein compared to the whole body tissues.

### 4.6. References


V. HOT SPOT BIOMONITORING OF MARINE POLLUTION EFFECTS USING SELECTED BIOMARKERS, CHOLINESTERASE AND PHAGOCYTIC ACTIVITY, OF TROPICAL GREEN MUSSEL (*Perna viridis*)

5.1. Abstract

Selected biomarkers, Cholinesterase (ChE) and phagocytic activity have been investigated with the exposed green mussel *Perna viridis* in Indonesian coastal waters. An operative effect-based monitoring on two polluted sites and one reference area were investigated for aquaculture enterprises and human health aspects. Between two heavily polluted sites, green mussels from Cilincing indicated a lower level of the ChE activity than those from Kamal Muara. The phagocytic activity of green mussels from the polluted sites demonstrated a significant higher activity than that of green mussels from the pristine site, Pangkep. However, there were no significant differences of phagocytic activity between the polluted sites. This might indicate that the existing pollutants in Jakarta Bay were more neurotoxic rather than immunotoxic substances. The results showed clearly that both selected biomarkers are potential valuable tools for effect-based monitoring and pollution impacts in coastal zones of Indonesia. The hot spot biomonitoring contributes to a tailor-made toolbox focussing on risk-based coastal zone management in Indonesia.

Keywords: green mussel, biomarkers, coastal zone management, Indonesia.

5.2. Introduction

A biological approach has been used as a counterpart of a classic chemical approach for surveying marine pollution effects in many international programs. A chemical analysis solely is considered as an invaluable analysis for interpretation of the pollutant impact in marine ecosystem since it does not illustrate the harmful effects (Walker 1998; Damiens *et al*. 2004) and the fate of chemical compounds on living organism through biotransformation of xenobiotic substances within living organism body (Nicholson and Lam 2005). In many cases, the biotransformation may increase xenobiotic substances toxicity on organism via producing reactive metabolite compounds that are more toxic than original parent compounds (Belden and Lydy 2000). Moreover, the chemical approach is extremely expensive, applicable to only a small proportion of the toxic chemical in the environment, provides a little biologically meaningful information, and therefore overlooks the complexity of the system under surveillance (Butterworth 1995). For those reasons, the classic chemical analysis
should be accompanied by the biological approach which is so called biomarker that elucidates biological responses of environmental pollution.

Biomarkers have been considered as sensitive and suitable tools for detecting either exposure, or effects of, pollutants (Hansen 1995; Narbonne et al. 2001; Lagadic 2002) since they can provide more comprehensive and biologically more relevant information on the potential impact of pollutants on the health status of organism (Van der Oost et al. 1996). In respect to pollutants that has a lower stability in water such as organophosphate and carbamate pesticides, biomarkers are reliable tools for assessing the impacts of the pollutants on biota even if the existing of the pollutants in water cannot be detected (Sturm et al. 1999). It is because biomarkers can detect persistence responses and/or effects of the pollutants in such duration of biota lifetime (Depledge and Fossi 1994). Therefore, they have been used enormously in biosurveillance to assess the risk of marine ecosystem pollution (Cajaraville et al. 2000; Martin-Diaz et al. 2004).

Mytilid mussels have received tremendous concerns as a sentinel organism when applying biomarkers in many pollution monitoring programmes (Cajaraville et al. 2000; Livingstone et al. 2000; Dizer et al. 2001a). As sedentary and filter-feeder animals, marine mussels do not escape from contaminated water where they are living and can accumulate many contaminants to the level higher than existing in water (Widdows and Donkin 1992). Hence, the behaviors are providing realistic sentinel organisms that indicate the biologically available concentrations. The realistic bioavailability of contaminants in mussels is also demonstrated by the fact that they have inefficient detoxifying enzymes allowing only minimal metabolic transformation of contaminant within their tissue (Nicholson and Lam 2005). Consequently, mussels have been considered as notable eco-sentinel organisms for effect-based monitoring programme and have represented the sensitivity of detection harmful effect of pollutions.

The extensive use of mussels and biomarkers for that purpose were carried out in temperate region by using blue mussels, *Mytilus edulis*. There are few studies conducted concerning biomarkers in tropical region by using native species, green mussels *Perna viridis* (Nicholson and Lam 2005). It has been postulated that genetic and ecosystem differences of two marine mussels
generated complicated inherent difficulties, when an extrapolation of *M. edulis* data to *P. viridis* was conducted. Therefore, a hot spot investigation of biomarkers in tropical regions by employing *P. viridis* is demanded to enhance the understanding of biological response of indigenous species toward contaminants to enforce biosurveillance of marine pollution effects programs in tropical region.

This study applied selected biomarkers which are cholinesterase (ChE) and phagocytic activity to monitor effects of pollution in coastal areas of Indonesia. ChE activity has been widely used as a biomarker (biochemical response) for neurotoxic effects of organophosphorus and carbamate pesticides. There are some influences of ChE activity by several heavy metals, PAH and surfactants exposure (Guilhermino *et al.* 1998; Tabche *et al.* 1997; Akcha *et al.* 2000; Moirera *et al.* 2004). Moreover, the immune system is a vital part of the organism and associates intimately with the function of many organs and organ system (Fournier *et al.* 2000). In invertebrate, the phagocytic activity which is part of the immune system can be induced by wide range of xenobiotics. Hence, the phagocytic activity is considered as a less specific early indicator of immunotoxicity or as a biomarker (Oliver and Fisher 1999; Blaise *et al.* 2002). The two selected biomarkers were employed in the current study based on microtiterplate techniques in order to provide a rapid, cost-effective, justifiable (Blaise *et al.* 2002), and well-adapted application in developing countries.

### 5.3. Material and Methods

**Chemicals**

Acetylthiocholine iodide, 5,5’-Dithio-bis-(2-Nitrobenzoic acid) (DTNB), γ-globuline, Bovine Serum Albumin, Fluoresceinisothiocyanate were purchased from Sigma. Bradford reagent was purchased from Bio-Rad Laboratories GmBH, Germany. All others reagents used were analytical grade products.

**Study Area**

The study was conducted on three different areas of Indonesian coastal zone (Figure 25). Two sites of Jakarta bay, Kamal Muara and Cilincing (Figure 26) were chosen and considered as heavily anthropogenic polluted sites (station 2 and 3) since they received almost domestic and industrial wastes from Jakarta and
neighboring cities of Jakarta. Moreover, there were some studies based on chemical analysis have been conducted which determined Jakarta Bay was under threatened by anthropogenic pollutants (Williams et al. 2000; Sudaryanto et al. 2002; Munawir 2005). Whilst, many traditional fisheries activities such as green mussel aquacultures are situated along Jakarta Bay, which supplies sea food...
market demands of Jakarta and the surroundings. Hence, Jakarta Bay is considered also as highly valued fisheries resources of coastal area, which plays indispensables role for preserving marine food resources and economic basis of small scales fishermen populated along Jakarta Bay. On the other hand, a coastal area of Pangkajene Kepulauan (Pangkep) district in South Sulawesi was chosen as reference site (station 1; Figure 19) because there are relatively minimal anthropogenic activities that were performed in this place such as traditional fisheries, which use static fishing equipment.

**Sample Collection and Preparation**

The hot spot sampling was conducted from 7 – 20 September 2005. About 32 green mussels, *Perna viridis* (5 – 6 cm) were handpicked on traditional green mussel cultures along Jakarta Bay at Kamal Muara and Cilincing. Subsequently, around 32 green mussels (5-6 cm) at reference site were also handpicked from the population of wild green mussels attached naturally on traditional static fishing equipments.

The collected living green mussels were directly transferred to the laboratory using cool box under humid condition. Prior to dissecting out of the mussels, 1 ml of mussel hemolymph was withdrawn from mussel posterior adductor muscle (PAM) sinus using 1 ml syringe and 0.4 mm needle followed by phagocytosis assay as described below.

Gill, foot, mantle and PAM were cut off, blotted dry and weighted before placing them in 2 ml potassium phosphat buffer in eppendorf tube (0.1 M/pH 8.0). Prior to transferring the tissues to Ecotoxicology Department Laboratory, Technische Universitaet of Berlin, Germany using cool box that were filled by dry ice, the tissues were stored at – 70 °C.

**Cholinesterase Activity**

The enzyme activity was measured following the modified Ellman method (Ellman *et al*. 1961) for a 96-well plate. A Dounce homogenizer was used to homogenize 0.3 g of each tissue in 2 ml potassium phosphate buffer (0.1 M/pH 8.0). The homogenate was centrifuged for 10 min at 10 000 x g and the supernatant was harvested and stored at –80 °C prior to the 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 the 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’-Dithiobis(-2-Nitrobenzoic acid) prior to the reaction which started by addition of 50 µl of 3 mM Acethylthiocholine iodide. Accordingly, the plate was read by photometry for microtiter plate (Spectra Thermo TECAN) in an interval of 30 s for 5 min at 405 nm. Four independent measurements of the ChE activity were carried out for each individual of *P. viridis*, and the average activity were calculated.

**Protein Measurement**

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 a separate well section of the microplate. A blank was made by placing 10 µl of distilled water into the blank section of the microplate. After the addition of 5% Bradford-reagent solutions (200 µl) into the microplate, the samples were left in room temperature for 20 minutes to allow color development. The absorbance was read at 620 nm using photometry (Spectra Thermo TECAN).

The ChE activity is expressed as nmoles of product developed per minute per mg of protein (nmol/min/mg protein). The ChE activity was measured on each tissue to recognize which tissue has the largest ChE activity.

**Phagocytic Activity**

Phagocytic activity of hemocytes was determined by a microplate-based fluorescence measurement method (Hansen 1992; Anderson and Mora 1995). This method is based on the number of fluorescence labeled yeast cells that were phagocytosed by mussel hemocytes. The yeast cells were treated and labeled by Fluoresceinisothiocyanate (FITC) (Anderson and Mora 1995) and kept in aliquots at -80 °C. After withdrawing hemolymph from the PAM sinus of the mussels using 1 ml syringe and 0.4 mm needle, 100 µl of hemolymph was dropped into
96-microplate wells. Five replicates were used to analyze the phagocytic activity and 3 replicates were used for the protein analysis. The density of hemocytes from each mussel was calculated by using a 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 wells, 25 µl of the FITC-labeled yeast was added into each phagocytic activity section of the microplate wells. A standard was made by adding 100 µl of phosphate buffer saline (PBS) and 25 µl of the FITC-labeled yeast into the 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°C at dark condition. At the end of the incubation, 50 µl of 1% glutaradehyd was added into each phagocytosis section of microplate wells, while 50 µl of methanol was dropped into the protein section of microplate wells. Before transferring to the laboratory in Germany, the plates were covered by a film and wrapped in aluminum and stored at 5°C in darkness. Accordingly, the fixatives were removed carefully and replaced by 125 µl of PBS when samples have arrived in the laboratory. For quenching the fluorescence background of unphagocytosed cells, 25 µl of 0.6 mg/mL trypan blue dissolved in PBS was added to each well of the microplate. The plate was incubated for 20 minutes prior to removing of all supernatants. The fluorescence of the ingested FITC-labeled yeast cells were read at excitation of 485 nm and emission of 535 nm using a microtiter plate fluorometer (Dynatech, Fluorolite 1000).

**Protein Measurement**

A protein content measurement was carried out using hemocytes only. Prior to the measurement, the buffer was removed carefully and 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 the lysed hemocytes and the serial dilutions of protein standard (Bovine Serum Albumin) were added to 96-microplate wells. Accordingly, 200 µl of 5% Bradford-reagent solution were added into the plate and incubated for 20 minutes to allow color development. The absorbance of protein was measured at 620 nm using photometer (Spectra Thermo TECAN). Accordingly, the phagocytic activity was expressed as
Relative Fluorescence Units (RFU) and finally calculated as a Phagocytic Index:
RFU/mg hemocyte protein.

Statistical Analysis

The statistical analyses were performed using non-parametric test, Kruskall-Wallis to distinguish the difference of ChE and phagocytic activity among the sites. If there were differences among the sites, the test was continued by Dunn’s multiple comparison test to determine the different between two sites.

5.4. Results

Cholinesterase Activity

It has been reported that the ChE activity level differed among organs in marine mussels (Bocquene et al. 1990; Brown et al. 2004). The current study was started by recognizing which organ of green mussel, P. viridis that posses the highest ChE activity. It has been performed using P. viridis tissues from expected clean area. The results presented in Figure 27 demonstrated the median of the

![Figure 27. Cholinesterase activity of different organs of green mussel, Perna viridis from Pangkep Indonesia. Data were expressed as median (25 % and 75 % quartile, 5 % and 95 % confidence interval).](image)

ChE activity in the gill which had the significant highest ChE activity namely 83.56 nmol/min/mg protein followed by the foot (46.16 nmol/min/mg protein), the mantle (27.35 nmol/min/mg protein) and the PAM (4.94 nmol/min/mg protein).
Accordingly, the gill was used as a tissue target for measuring the ChE activity since the highest ChE activity of such organ should be the most suitable for measurement of the ChE activity inhibition (Brown et al. 2004).

Statistical analysis showed the difference ChE activity in the gills of the samples (p < 0.05) (Figure 28). The animals collected from the reference site had the significant highest ChE activity (83.56 nmol/min/mg protein) followed by the green mussel collected from heavily polluted areas, Kamal Muara (49.92 nmol/min/mg protein) and Cilincing (27.20 nmol/min/mg protein). Between two heavily polluted sites, the animals inhabited in Kamal Muara showed significant less inhibition of the ChE activity than those from Cilincing (p < 0.05).

Figure 28. ChE activity of green mussel gill, *Perna viridis* from the pollution gradient of Indonesian waters. Kamal Muara and Cilincing are expected as polluted areas, while Pangkep is expected as a clean area. Data were expressed as median (25 % and 75 % quartile, 5 % and 95 % confidence interval). * indicate the different enzyme activity among the sampling sites (p < 0.05).

**Phagocytic Activity**

In the present study, the phagocytic activity expressed as phagocytic index, and hemocytes numbers and total cell protein content were measured simultaneously. The results were represented in Figure 29 and Figure 30. Statistical analysis of the median of circulating hemocytes numbers exhibited no difference numbers of hemocytes ranging from 2,070,000 – 2,370,000 cells/ml.
Figure 29. Circular hemocytes of green mussel *P. viridis* collected from the selected areas of Indonesia waters; Pangkep, Kamal Muara, and Cilincing. Data were expressed as median (25 % and 75 % quartile, 5 % and 95 % confidence interval).

Figure 30. Phagocytotic Index of green mussel *P. viridis* collected in the selected area of Indonesia waters; Pangkep, Kamal Muara, and Cilincing. Data were expressed as median (25 % and 75 % quartile, 5 % and 95 % confidence interval). * indicate the different phagocytic activity of mussel hemolymph collected from polluted sites to the reference site (Pangkep) (p < 0.05). Y-axis is logarithmic scale.
In contrast, the median of the phagocytic index demonstrated significant different phagocytic activities of *P. viridis* collected from gradient pollutions of Indonesian coastal waters (p < 0.05). The animals collected from two heavily polluted sites in Jakarta Bay showed significant higher phagocytic index than those collected from reference sites. Nevertheless, there was no significant different phagocytic index within the polluted site (p = 0.118). The highest phagocytic index was demonstrated in hemocytes of *P. viridis* from Cilincing (23410.10 RFU/mg protein) which followed by Kamal Muara (7566.84 RFU/mg protein) and reference site, Pangkep (1714.19 RFU/mg protein).

5.5. Discussion

**Cholinesterase Activity**

Cholinesterases (ChEs) are enzymes that hydrolyze neural transmitter acetylcholine (ACh) to choline and acetic acid for terminating neural transmission impulse in the synaptic gap of cholinergic synapses and neuromuscular junctions. ACh play an important role both as excitatory and inhibitory transmitters of the gill muscle of bivalve (Gainey *et al.* 2003). In blue mussel, *Mytilus edulis*, ciliary movement of the gill is controlled by acetylcholine, dopamine and 5-hydrotryptamine (Aiello 1990). Organophosphorous and carbamate pesticide inhibit ChE activity which may lead to severe physiological impairment of marine animals (Dauberschmidt *et al.* 1997) such as reduction in feeding efficiency of marine mussels (Donkin *et al.* 1997).

Since ChEs was purified by Wachtendonk and Neef (1979) in marine mussels hemolymph, a measurement of ChE activity in marine mussels has been used as a biomarker in laboratory test (Galloway *et al.* 2002; Rickwood and Galloway 2004) and several international monitoring programs in the field (Dizer *et al.* 2001a,b; Romeo *et al.* 2003).

Characterization of ChEs in bivalve has been conducted in some bivalves e.g. in *M. galloprovincialis* the ChE specific activity was predominantly localized in the gills compare to others organs (Mora *et al.* 1999; Porte *et al.* 2001). Moreover, the ChE activity from *M. galloprovincialis* gill was observed more sensitive to organophosphorous pesticides than that from the digestive gland (Escartin and Porte 1997). In *M. edulis*, Bocquene *et al.* (1990) found that the
highest ChE activity occurred in the gill compare to others organs such as the hepatopancreas, the mantle and the adducent muscle. By characterizing and comparing the ChEs in different organ of *M. edulis*, Brown *et al.* (2004) found that ‘mitochondrial’ fraction of foot had the highest ChE specific activity with very low recovery of activity. Accordingly, the gill ‘microsomal’ activity had the second highest ChE specific activity with useful level of recovery and therefore was the most suitable fraction for biomarker application. The highest ChE activity in the gill compared to others organs such as the adducent muscle and the digestive gland were observed in the Antarctic scallop *Adamussium colbecki* (Corsi *et al.* 2004). Compared to the foot, the gill of the bivalve, *Scapharca inaequivalvis*, demonstrated the higher specific ChE activity level as well (Romani *et al.*, 2005). Eventually, Bonacci *et al.* (2008) observed that the highest ChE activity also occurred in the gill of scallop (*Pecten jacobaeus*) compared to others organs which were the adducuent muscle and the digestive gland.

The current study compared the ChE activity of green mussel, *P. viridis* in different organs such as the gill, the foot, the mantle and the PAM. The results demonstrated that the gill of *P. viridis* had significant higher of the ChE activity compared to others organs such as the foot, the mantle and the PAM. Porte and Albaiges (2002) demonstrated that the ChE activity from the gill of blue mussels (*M. galloprovincialis*) was more sensitive than that of digestive gland and it revealed a certain correlation with the concentration of fenitrothion in whole mussels. It has been reported that the gill of *P. viridis* that were collected from Hong Kong waters had the higher ChE activity than that of the whole tissue and this ChE activity was not size-dependent (Lau and Wong 2003). This is conceivable because mussels use their gills not only as a respiratory apparatus but also as filter feeder organ thereby ambient water filtered and managed for gaseous exchanges and sifting food (Bayne *et al.* 1976). Since the gill are the front line of contact with contaminants and the first line of defense (Lau and Wong 2003), detoxification compounds such as ChEs are necessary to be produced to protect other organs. Consequently, the production of ChEs not only provides as the control of neurotransmission, but also serves as contaminants detoxification particularly for organophosphorus and carbamate pesticides (Soreq and Seidman
In addition, it has been reported that the protein level of *P. viridis* gill was not seasonal dependent which lead to reduce the intrinsic variability of the biochemical responses in different growth phase throughout the year (Lau *et al.* 2004). Those evidence set up the gill as a *par excellence* tissue for biomarkers application to minimize effects caused by the natural reproductive cycles and the dilution effect due to large variation in the total tissue protein (Lau *et al.* 2004). The selection of the gill as tissue target for conducting biomarkers were also shown by the nature of the gill, which comes into contacts with relatively large volumes of seawater compared to the rest of the animal so that conferring them with the potential for being a suitable target tissue for xenobiotic substance exposure.

In consequence, the present study employed the gill of *P. viridis* to investigate pollutants effect to ChE activity in gradient pollution of Indonesian coastal area. The results indicated that the ChE activity of the gill of *P. viridis* from pristine site was significantly higher than that from the gill of *P. viridis* which inhabit polluted sites. The inhibition of the ChE activity from the gill of *P. viridis* collected from Kamal Muara was about 49.2 %. Statistically, the greatest inhibition of the ChE activity was indicated in mussels from Cilincing, which was about 72.41 %. By exposing brown mussels (*Perna perna*) to furadan (carbamate pesticide), Alves *et al.* (2002) observed the ChE activity of the gill was suppressed by 35 %. Hence, it is suggested that the ChE activity is a sensitive tool since it can in fact distinguish gradient levels of two heavily polluted areas.

Ludke *et al.* (1975) classified the percentage of ChE activity inhibition based on comparison of the individual value with the activity of the normal population for providing the interpretation of the environmental risk. The following are the risk criteria of inhibition percentage of ChE activity that were proposed by Ludke *et al.* (1975):

- 0 – 20 % = Zone of normal variation
- 20 – 50 % = presence of exposure or zone of reversible effects
- 50 – 100 % = life-threatening situation or zone of irreversible effects.

In respect to estuarine fishes, Coppage (1972) suggested that inhibition level of the ChE activity in the range of 20 – 70 % could be classified as an
indication of organophosphorous exposure. Subsequent studies observed that the inhibition of the ChE activity in the fish brain, which reached 70 – 90 % indicated mortality (Coppage *et al.* 1975; Coppage and Matthews 1975). Sandahl *et al.* (2005) observed that the inhibition of the ChE activity in brain and muscle from juvenile coho salmon (*Oncorhynchus kisutch*) was correlated well with the behavior disruption i.e., feeding and swimming ability when the fish were exposed by chlorphyrifos. At the lowest concentration (0.6 μg/l), chlorphyrifos caused 12 % inhibition of the muscle ChE activity reducing 27 % of the swimming rate, while no mortality was observed when fish exposed by the high concentration (2.5 μg/l) inhibiting 67 % of muscle’s ChE activity. By conducting microcosm study using mixtures of selected organophosphorous pesticides, Sibley *et al.* (2000) observed that 10 % mortality was correlated with approximately 50 % inhibition of AChE activity, while 50 % mortality was correlated with approximately 90 % inhibition of AChE activity of fathead minnows. Fleming *et al.* (1995) found the die-off freshwater mussels (*Elliptio steinstansana*) from sites that were influenced by agricultural activities with the inhibition of ChE activity from 65 – 73 % compared to the reference site. Based on the criteria and the results of those studies, it is suggested that discharged pollutants into coastal area of Jakarta Bay indicated neurotoxic compound causing from reversible to irreversible effects of the neurological activity of the green mussel population. The mussels that populated in Kamal Muara indicated reversible effects, while those from Cilincing showed irreversible conditions.

The link between the inhibition of ChE activity of sentinel organism and the discharged neurotoxic compounds from agricultural, urban and industrial activity to aquatic environment has been suggested by many studies (Fulton and Key 2001). However, the relationship between ChE activity and higher level biomarker such as feeding rate in green mussel has not been studied yet. Therefore, a chronic in vivo study on the response of ChE activity in green mussel and other behavioral biomarkers such as feeding rate to the serial concentrations of pollutants, which picturize suspected pollution area concentrations, is indispensable to translate the inhibition of ChE activity induced by pollutants into ecological perspective. The translatable of ecological consequence of the
suppressed ChE activity is a vital consideration in ecological risk assessment in the coastal zone.

**Phagocytosis Activity**

Green mussel hemolymph contains both hemocyte and humoral defense factors which are responsible for the defense system. Hemocytes circulating in hemolymph are the principal cellular effectors of invertebrate immunity (Mitta et al. 1999) which have a capability to perform phagocytosis of foreign materials (Cheng 1984; Carbalal et al. 1997) and cytotoxicity via the production of radicals (Winston et al. 1996).

Phagocytosis of mussel hemocytes can be affected by various chemical stressors in the aquatic environment (Anderson and Mora 1995). Biphasic patterns of mussel phagocytic responses induced by xenobiotic have been demonstrated in many laboratory studies (Cole et al. 1994; Pipe et al. 1999; Parry and Pipe 2004). Theoretically, the phagocytic activity will be stimulated when mussels exposed to low level of contaminants, while it will be suppressed when mussel exposed to high level of contaminants. Consequently, measurement of the phagocytic activity, which is as part of immune system of mussel has been used as a biomarker of xenobiotic substances effect (Anderson and Mora 1995; Oliver and Fisher 1999; Blaise et al. 2002; Gagne et al. 2002).

In spite of mussel hemocytes playing an important role in the phagocytic activity, it is difficult to depict the correlation pattern between circulated hemocytes number and the phagocytic activity of mussel. The current study showed that there was no different numbers of circulating hemocytes of green mussel, which were collected from both polluted and clean sites. However, a significant difference of the phagocytic activity between the collected green mussels from polluted sites and those from clean site were evident. The data showed that discharged pollutants in Jakarta Bay have stressed cultivated green mussels, which stimulated significantly their phagocytic activity compared to the phagocytic activity of the green mussels collected from the clean site. The modulation of mussel phagocytic activity was in accordance with Luengen et al. (2004) who observed the elevation of phagocytic activity of mussels that collected from polluted sites. The elevation of phagocytic activity induced by the pollutants
may be a part of mussel’s strategy to sequester the toxic materials from vulnerable organs (Oliver et al. 2001). Nevertheless, Dizer et al. (2001b) found that high number of circulating hemocytes of mussels collected from control site followed by relatively low phagocytic activity, while relatively low number of hemocytes from polluted sites had a high phagocytic activity, they could not depict clearly the relationship between hemocytes number and the phagocytic activity of mussels.

The complicated relationship between hemocytes number and the phagocytic activity of mussels may result from dynamic association/dissociation between hemocytes and bivalve tissues that enable to change the total size of the hemocytes population within bivalve body over short time (Ford et al. 1993). The population could not be simply depicted by circulating number of hemocytes, which were drained from the PAM sinus as the mussel has the open circulatory blood system, which circulate the blood to whole organs. In addition, commonly the population of mussel hemocytes are composed by phagocytotic and unphagocytotic hemocytes which can be altered by xenobiotic substances (Pipe et al. 1999). Unfortunately, most of the techniques to measure the phagocytic activity including the technique used in the present study were based on the mixture of hemocytes sub-population so that an estimation of capability level of each sub-population of hemocytes was not possible.

Although, the present study enabled to distinguish the phagocytic activity of green mussels dwelled in polluted and clean sites, the difference of the phagocytic activity within the polluted site could not be differentiated significantly. Having taking into account the data from the ChE activity, which enable to distinguish the magnitude effects of released pollutants within the polluted sites, it is tempting to suggest that released pollutants in Jakarta Bay seem to be ChEs inhibitors, which raised greater impact on the ChE activity rather than the paghcoeytosis activity. For that purpose, the chemical analysis of water/sediment samples and relevant pollutants within mussel’s tissue should be taken into account. Regardless of the chemical analysis approach, the ChE activity indicated a more responsive tool compare to the phagocytic activity so that it could distinguish between two heavily polluted sites. However, it is hard to
justify that the ChE activity is more sensitive compared to phagocytic activity as was observed by Perez and co-workers (2004) in ChE activity of invertebrate, Scrobicularia plana (clam) and Nereis diversicolor (marine worm). The authors delineated higher sensitivity of ChE activity compared to others biomarkers that were used in biomonitoring of Spain waters. Therefore, the useful results that recorded by the current study are the information on neurotoxicity and immunotoxicity compounds which were present in Jakarta Bay and the magnitude impact of neurotoxicity contaminants to induce an effect is greater than the immunotoxicity contaminants.

Conclusively, the results suggested that the use of the selected biomarkers is a reliable and preferential strategy in the ecological risk assessment of released xenobiotic compounds in coastal waters due to their ability to elucidate bio-effects of neuro-immuno systems disruptors.

5.6. Reference


VI. GENERAL DISCUSSION

6.1. Biomarker as a Counterpart of a Classic Chemical Analysis Approach

The explosive growth of human populations over the past millennium enhances the use of chemical compounds such as pesticides for increasing the quality and quantity of food production, and other applications of anthropogenic activities. Organophosphorous (OP) pesticides are worldwide used pesticide class in agriculture to combat and control the pest and also to eliminate vector animals which cause epidemiological diseases (Chambers 1992). Although the main use of the pesticides is by agricultural sectors in upland, the occurrence of the pesticides in aquatic ecosystem cannot be ignored. The waste of the applications of the pesticides in upland can reach aquatic ecosystems through run off from the field, spray drift, and leaching from contaminated ground water. Moreover, the pesticides are in fact used extensively in coastal area such as in the shrimp cultures (Gräslund and Bengtsson 2001). Hence, the development of aquaculture entrepreneurship along coastal zone, which contribute enormous quantities of the pesticides released into aquatic ecosystem, cannot be omitted. The existing of the pesticides in water ecosystem is also contributed by accidental spills during transportation which are not frequently encountered but they dispense significant quantities of the pesticides in the ecosystem (Racke 1992). The occurrence of the pesticides in coastal area stimulates the requirement to adequately understand the deleterious effects of discharged pesticides to the inhabitant animals.

The advantage of the OP pesticide is a non-persistency in environment so that the use of this pesticide can reduce the residual effects both on terrestrial and aquatic habitat. However, the use of the pesticide not only raises benefits for humankind but also produces immense discharged pesticides waste into environments. During the pesticides application, not more than 5 % of the pesticides used can come up to targeted organisms because of losses from volatilization or washing out (Porte and Albaiges 2001). As a consequence, the excessive wastes can potentially be discharged and induce deleterious effects on non-target organisms, which ultimately devastate ecological homeostasis of point-source and non-point-source habitat. Although, OP pesticides are in general degraded rapidly in environments, they are not specific pesticides, which were
designed deliberately to kill living organisms effectively through disturbing their nervous system. The inhibition effect of OP pesticides on the nervous system was considered an irreversible effect, because the time needed to synthesis de novo of neurotransmitter enzymes i.e., acetylcholinesterase (AChE) being longer than the time of dissociation of the OP-AChE complex (Gaglani and Bocquene 2000; Hyne and Maher 2003). Therefore, in living organism point of view the effects of the pesticides persist longer than the existing of the pesticides in environment.

Classical monitoring of OP pesticides based on chemical observations and analysis is thought to be insufficient mainly due to the physicochemical properties of the pesticides, particularly in water. Rapid degradation of the pesticides in the environment is the main factor that masks deleterious effects of the released pesticides, which were recorded in monitoring campaign, to living organisms (Fulton and Key 2001). Moreover, once the pesticides enter the bodies of organisms, the harmful effects of the pesticides are mostly enhanced by biotransformation, which increases toxicity of the parent compounds. Consequently, in terms of biomonitoring the chemical analysis of the pesticides which performed solely in the environment and/or the bodies of organisms describes only a level of the pollutants without solving critical questions on cause of environmentally induced deleterious effects in living organisms (Decaprio 1997; Cajaravile et al. 2000; Nicholson and Lam 2005). In respect to bioavailability of the pesticides in whole animals or tissue, the chemical analyses cannot provide an accurate measurement due to the complexities of multi-cellular organisms (Schlenk 1999) thereby cannot reflect truly the overall toxicity of complex mixtures compounds (Lam and Wu 2003). Besides, financial factor can be a potential problem to perform chemical based monitoring of the pesticides especially in developing countries such as Indonesia. Hence, to solve those problems, measurable biological responses of living organisms to contaminants reflected from biochemical to behavioral levels are proposed to be an important counterpart of classic chemical analysis in biomonitoring programs to elucidate the effects of the contaminants on biota and the ecosystem. It is acknowledged that pollutants interrupted organism integrity at the biochemical level leading to effects at the individual level such as reduction of life-history characteristics
including growth and Darwinian fitness can be manifested ultimately at the ecosystem levels (Wepener et al. 2005). Nowadays, the responses are termed as biological markers or biomarkers, which can be used to devise rapid, effective screening assay, can be a complement to other testing techniques by significantly reducing the number of samples that may require a more elaborate, definitive or specific evaluation (Lam and Wu 2003; Hansen 2008). Eventually, biomarkers can be considered as useful tools for understanding the complex interactions that govern organism responses to environmental stressors and their sublethal effects on organism health (Werner et al. 2003).

Theoretically as a complementary tool of chemical based-monitoring approach, the relationship between biomarkers and detected contaminants in either media (water or sediment) or body of sentinel organisms should be evident. The evident are useful to envisage how the contaminants cause effects on the animals in which the response are identified and recorded as biomarkers in natural conditions. In consequence, the cause-effect link between contaminants content measured in media or animal body burden and biomarkers is not postulated, but it is proved a posteriori instead. Pipe et al (1995) found the correlation between immunocompetence assay i.e. hemocytes numbers, phagocytosis activity and release of reactive oxygen metabolites and the levels of contaminants (DDT, PCB, lindane and some heavy metals) measured in digestive glands of mussels. Another study conducted by Oliver et al (2001) also depicted positive relationships between level of contaminants including various PAH, PCB, heavy metals in the tissue of the studied organism and defense-related characteristics of oyster (Crassostrea virginica). However, to acquire the evidence of the causal link between biomarkers and the levels of OP pesticides in a field study is not easy. It is because the physicochemical properties of the OP pesticides in media or in body of organisms are unstable. Besides, the bioaccumulations and depuration patterns of the pesticides in organisms depend upon many factors including route of uptake, time of exposure, occurrence of others xenobiotic compounds in the environment that can act synergistically or antagonistically to rise effects in organisms and seasonal variations. From the organism perspective and the time of exposure, the rising of the pollutants effects in general are
depended on living organism ability to react to the pollutant exposures which result in three conditions such as induction, adaptation and recovery. Wu et al. (2005) proposed six types of temporal changes of biological responses when living organisms exposed by pollutants including slow induction-adaptation, fast induction-adaptation, slow induction-fast recovery, slow induction-slow recovery, fast induction-fast recovery, and fast induction-slow recovery. Fast induction-slow recovery type of butyrylcholinesterase (BuChE) activity from freshwater fish was probably observed by Strum et al. (2000) from the river at Braunschweig, Lower Saxony, Germany. The authors found that inhibition of BuChE activity from the eco-sentinel fish (Gasterosteus aculeatus) was detected before June during the peak contaminations of parathions through runoff events, but inhibition of the enzyme activity still persisted when the occurrence of the pesticide was not detected in August. The present study demonstrated also the persistent inhibition of ChE activity from trichlorfon-exposed mussels (Mytilus edulis) after incubation in uncontaminated media for seven days (Chapter II). The factors noticed above will be hindered for obtaining the obvious patterns of causal link between the levels of the pesticides and used biomarkers.

The difficulties for revealing the clear-cut causal link between biomarkers and the levels of the OP pesticides in a field study do not invalidate the use of the selected biomarkers in the field scales. Many biomarkers including ChE and phagocytosis activity and siphoning rate have been proved in the laboratory studies to be response of pesticides exposures (e.g. Chapter I and II). Hansen (2003) demonstrated that in vivo test of the effluent exposure caused effects on inhibition of ChE activity from trout muscle tissue. Hence, what should be done is the inter-laboratory comparison works both in vitro and in vivo that will play vital roles to underpin the acceptances of biomarkers in science, technology and governmental legislatures (Hansen 2003) since they can provide information in characterization of causative agents. In some cases, the field studies may not demonstrate the observable link between the levels of OP pesticides and biomarkers, but they provide the link between biomarkers and the gradient usages of the OP pesticide as observed by McHenery et al. (1997) in ChE activity of M. edulis from dichlorvos contaminated areas of salmon culture. The inhibition of
the ChE activity of *M. edulis* correlated positively with the application levels of dichlorvos in those areas (McHenery *et al.* 1997). Therefore, the knowledge of those obstacles in terms of physicochemical properties of xenobiotic compounds, biological responses of organisms, and the historical use of the contaminants at studied sites is efficacious in interpretation and evaluation data from field studies must be understood before employing biomarkers in biomonitoring.

The primary use of biomarkers in biomonitoring campaigns are to characterize potential hazards of environmental stressors, to identify current status of the ecosystems and to trace or to predict relevant environmental changes induced by the environmental stressors (Picado *et al.* 2007). Den Besten and Munawar (2005) extracted from such reviews on biomarkers concepts (Depledge and Fossi 1994; Den Besten 1998) four different concepts to apply biomarkers in ecotoxicological contexts:

1. Biomarkers in combination with bioassay as parameters in water or sediment-quality monitoring (trend analysis).
2. Biomarkers that lead the investigations from screening to detailed assessment (tiered approaches or weight-of-evidence approaches).
3. Biomarkers linked with chemical analysis (hyphenated approaches or toxicity identification evaluation).
4. Biomarkers as diagnostic tools.

The concept that underlying the use of biomarkers, which was chosen in the current dissertation particularly in the field study, is in accordance to second concept, which is the application of biomarkers in tiered approaches. Tiered approaches provide a step-by-step application of different biomarkers that can be very effective for estimating water quality and environmental health in field areas suitable for regulatory and standard monitoring (Den Besten and Munawar 2005).

The hot spot strategy of biomonitoring attempted to depict the use of the selected biomarkers i.e. phagocytosis and ChE activity from green mussels (*Perna viridis*) as an early warning system. The study was underpinned by characterization of the ChEs enzymes based upon substrate and inhibitor approach revealed that the enzymes consist of two types of ChEs which are typical acetylcholinesterase and atypical butyrylcholinesterase. The results indicated that the activity of the
enzymes was not distributed evenly in different organs of the green mussels. Gill revealed the highest ChE activity compared to others organs including foot, mantle and the posterior adductor muscle (PAM). Hence, gill was used as the target organ in the hot spot biomonitoring. Furthermore, the results recorded that the ChE activity can discriminate different levels of neurotoxicants that occurred in selected sites of Jakarta Bay, while phagocytosis activity indicated otherwise. It should be kept in mind that the results did not indicate sensitivity of one of the selected biomarkers. However, it might point out that the mixtures xenobiotic compounds existed in Jakarta Bay tend to be more neurotoxic rather than immunotoxic.

In the tiered approach context, the results are valuable to trigger and underpin further researches to identify specific contaminants that were profoundly present in Jakarta Bay. If the specify of ChE activity is considered to be a biomarker of OP and Carbamate (C) pesticides, then subsequent researches are probably to refine the investigation on the range of OP and C pesticides that were used in the surrounding area of Jakarta Bay based on the information on daily or historical applications. Nonetheless, many studies showed that ChE activity is no longer specific for OP and C pesticides, but this biomarker can be influenced by others xenobiotic compounds including heavy metals, surfactant, PAH etc. It depicts not only the potential of the use of ChE activity as a biomarker is extended, but also reveal that ChEs enzymes could be a potential target of other xenobiotic compounds such as heavy metals, PAH, OP and C pesticides. Furthermore, concerning Jakarta Bay where contaminants from 13 rivers are converged, further research should be orientated to diagnose potential adverse effects of the complex-mixtures of xenobiotic compounds to living organisms rather than to refine the causative agents that discharged to the Bay. It is because biomarkers illustrate not only response to the biologically active fraction of one or more toxicants, but also combine interactive effects of the complex-mixtures of toxicants that were experienced by organisms (Bartell 2006).

Hypothetical time response of organism to contaminants that have been proposed by Wu et al (2005) was an example of effort to schematize potential adverse effects of the diverse contaminants to biological responses in such range
of times, providing magnitude and duration plot of the adverse effects in organisms. In this regard, a screening test of Jakarta Bay’s compartments e.g. sediments and waters by using suite hierarchical biomarkers from molecular to behavioral levels should be a further research to obtain prognosis pictures of diverse array of discharged xenobiotic compounds. The research will provide a better estimation of the hazard magnitude of the sites of concern as well. On the other hand, despite the fact that chemical analysis approach that conducted in routine environmental monitoring campaign is not a cost-effective procedure it will improve the hazard estimation of the studied sites by integrating the chemical stressors levels and the biomarkers data.

The serial information resulted from the routine monitoring on both chemical and biomarker approaches and the screening test are therefore valuable for recognizing the initial levels of damage and duration of deleterious effects induced by discharged contaminants, providing a good environmental protection approach and preparing remediation strategies along with the application of strategic plans to reduce anthropogenic waste that enter to aquatic environment like in Jakarta Bay. By integrating data from laboratory test and biomonitoring in terms of chemical analysis and biomarkers, Huscheck and Hansen (2005) generated a classification of the aquatic ecosystem i.e. Berlin river system based on ecotoxicological viewpoint. Of course to achieve a better classification of ecotoxicological status of such regions, conducting more comprehensive studies that integrate many aspects with reference to ecological point of view are needed, although delineating the prognosis of xenobiotic compounds and their bioavailability inventory remain efficacious to increase public and government awareness to minimize the wastes in such degrees that do not have possibility to perturb the ecosystem.

6.2. Mussel as Eco-sentinel Organism

The selection of an appropriate species in ecotoxicological assessment of potential hazardous of chemical compounds that will be used or used in anthropogenic activities is distinctly profound factor in determining any robust results in laboratory and filed studies. In terms of bioaccumulator monitoring,
Connell et al. (1999) proposed requirements that should be fulfilled by the eco-sentinel organisms are as follows.

- The organisms should accumulate the pollutant without being killed by the environmental level present.
- The organisms should be sedentary in order to be representative of the area in which they are growing.
- The organisms should be readily found throughout the area being monitored.
- The organisms should be relatively long lived.
- The organisms should be of reasonable size to ensure that enough material is available for analysis.
- The organism should be easy to handle, both in the field and in laboratory situations.
- The organisms should be able to tolerate brackish water, where pollutants are frequently found.
- There should be a simple correlation between the concentration of pollutants in the environment, and the levels found in the tissue of the living organisms.

All the requirements above are favor to marine mussels as an eco-sentinel organism that oriented to use in biomonitoring of effects of OP pesticides particularly using biomarkers except the last requirement. As stated previously that the physicochemical property of the pesticides which is particularly unstable characteristic of the pesticides may prevent to obtain well-correlations between the concentration of the pollutant in the environment and body tissue burden. Particularly, the hard obstacles will appeared when the relationship between contaminant residues in environment and/or the tissues of organisms and biomarker responses will be plotted since the intrinsic and extrinsic factors such as seasonal variations will influence both biological cycles and processes, and physico-chemical properties of residues.

Farrington et al. (1983) elucidate the reasonable characteristic of using marine bivalves including *Mytilus edulis*, *Perna viridis*, and others bivalve like Oyster (*Crassostrea* and *Ostrea*) species in biomonitoring as follow.
• Bivalves are worldwide distributed animals which permits comparison data without generating profound differences due to the different life histories and relationships with their habitat.
• They are sedentary and are therefore to be more effective animals than mobile species as accumulators of chemical pollution status for a given area.
• As filter feeder, bivalves siphon large volume of water and accumulate many chemicals by factors of $10^2$-$10^5$ compared to seawater in their habitat. Hence, this is a simplified measurement of trace xenobiotic compounds in their body compared to seawater.
• As long as the chemicals of concern are measured in the bivalves, biological availability of the desired chemical constituents can be obtained which is not resulted from measurement of the chemical substances in the environment including water and sediment.
• Assessment of the contaminant level in the body of bivalves is considered a more accurate reflection of the contaminant level in the environment since bivalves show low level of enzyme activity for metabolizing organic contaminants compared to fish and crustacean.
• Bivalves present in relatively stable populations that provide repeated sampling for short and long term study on changes in concentrations of pollutant chemicals without threaten their live.
• They are relatively resistance to the enviromantal stressors so that they still exist in areas where other organism cannot preserve their live.
• They are well-transplanted animals which can be transferred from pristine to polluted areas, vice versa, either maintained using mooring or cage in intertidal or sub-tidal habitat, providing expansion of investigated area of interest.
• They are important economic species consumed globally and assessment of their body-burdens of contamination is a relevant factor for public health consideration.
Concerning marine mussels, blue mussels (M. edulis) that populated mostly in temperate regions have been acquired more attention in research activities for understanding and developing suitable tools in biomonitoring campaign compared to their relative green mussels, P. viridis that inhabit subtropical and tropical area. The huge numbers of studies on M. edulis in ecotoxicological context provide considerable data that elucidate the sensitivity of diverse series of biomarkers to contaminants effects, permitting comparison data among different sites of concern. However, extrapolation data resulted from animals that live in temperate regions to animals which dwell in tropical regions are difficult and then opinionated. Hence, breakthrough efforts should be done especially in Indonesia to endorse and intensify scientific activities. Studies on biomarkers and indigenous mussel i.e. green mussel P. viridis for providing efficacious tools in effects biomonitoring in Asia region particularly in Indonesia are recomended. The use of green mussels as a sentinel organism have been performed in Jakarta Bay actually for detecting concentrations of heavy metals and their effects using biomarker lysosomal stability (neutral red retention) (Rees et al. 1999). The current study (Chapter III and IV) along with the previous study conducted by Rees et al. (1999) attempted to confirm a potential use of indigenous species in biomonitoring campaigns.

In Asia regions indeed there are some studies which successfully demonstrated the use of P. viridis as an eco-sentinel organism in biomonitoring programs. Green mussels, P. viridis, named previously as Mytilus viridis have been successfully employed as the eco-sentinel organism to detect heavy metal, PCB and persistent pesticides in river mouths of Thailand (Menasveta and Cheevaparanapiwat 1979), coastal waters of Singapore (Sivalingam 1982) and South India (Ramesh 1990). In Jakarta Bay, Hutagalung (1989) observed high concentrations of Hg and Cd in soft tissue of P. viridis. The results depicted that the small green mussels accumulate the heavy metals higher than the bigger animals which indicate that the size of the sentinel organism should be taken into account. By using transplantation strategy, Kannan et al. (1989) found bioaccumulation of highly toxic contaminant PCB in the tissue of P. viridis that were transplanted in two sites of concern in coastal waters of Hong Kong.
Furthermore, the green mussels *P. viridis* that were collected from coastal waters off peninsular Malaysia demonstrated different absorption of heavy metals including Cu, Pb, and Zn in different organs in which crystalline style accumulated the highest levels of Cu compared to the remaining organs such as food, mantle, gonad, and gill (Yap et al. 2006). Positive correlations between concentrations of some heavy metals i.e. Cd and Pb residues in tissue of *P. viridis* and Cd and Pb in sediment have been investigated from west coast of Peninsular Malaysia (Yap et al. 2002). The results clearly indicate that whole body of *P. viridis* is a good material and indicator for investigation of heavy metals in the aquatic environment.

Recently, *P. viridis* is not only used as eco-sentinel organism for detecting contaminants body burden, but also employed for investigating the biological responses that so called biomarkers. Different array of biomarkers have been studied in *P. viridis* ranging from molecular (Ching et al. 2001; Siu et al. 2004; Krishnakumar et al. 2006) to behavioral (Nicholson1999;Vijayavel et al. 2007). In laboratory scale, it has been observed that exposing *P. viridis* to Benzo[a]pyrene showed DNA damage in terms of DNA adduct and DNA strand breaks following exposure to serial dilutions of the contaminants and in range of determined times (Ching et al. 2001). Other organ which is hemocytes cell from *P. viridis* showed also DNA damage after exposed by the same contaminants (Siu et al. 2004). In the field scale, such battery of biomarkers which are sister chromatid exchange (SCE), chromosomal aberration, micronucleus (MN), hemic neoplasia (HN), and comet assay have been used in biomonotoring south west coast of India which indicated low levels of the battery of the biomarkers (Krishnakumar et al. 2006). The study implied that the studied site received minimal genotoxic and carcinogenic wastes which resulted in the low effects on the used biomarkers (Krishnakumar et al. 2006). The progress studies of antioxidant enzyme from green mussels that inhabit Hong Kong waters as a biomarker to detect trace organic pollutants concentrations has been demonstrated both in laboratory and field scales (Cheung et al. 2001, 2002; De Luca-Abbott et al. 2005). Field validation by transplanting the green mussels from expected
clean area to polluted area indicated that the light relationship between antioxidant responses and organic pollutants body burden occurred (Luca-Abbott et al. 2005). Furthermore, well-correlation between Benzo[a]pyrene and total PAH concentrations and oxyradical scavenger glutathione (GSH), chlorinated hydrocarbons and Glutathione S transferase (GST)/GSH have been reported (Cheung et al. 2001, 2002), but more study should be performed before other antioxidative enzymes can be used as biomarkers since they demonstrated a lack correlation with the tissue concentration of organic pollutants (Cheung et al. 2002). Cardiac activity of green mussel has been used to detect the effects of pollutants in the field by transplanting the animals to polluted and clean water which showed no contaminants effects on the cardiac activity (Nicholson 1999). In contrast, others behavioral activity i.e. siphoing rate of green mussel has been employed in laboratory test which pointed out inhibition of the behavioral biomarker following silver and chromium exposures (Vijayavel et al. 2007). Last but not least, non-destructive biomarkers namely lysosomal membrane stability and phagocytosis activity which measured hemocytes of green mussel have been studied in laboratory scale following cooper exposure (Nicholson 2003). The study elicited that administered copper concentrations destabilized lysosomal compartment of hemocytes and impaired phagocytosis activity.

All the studies on biomarkers were conducted on the green mussels that inhabit subtropical regions where variability of annual seasonal conditions are more pronounced than in tropical regions. It is acknowledged that seasonal variations bring about variations of biochemical responses of the studied species through endogenous biological cycles and process and induced by other exogenous factors such as ambient physico-chemical environments. As results, biomarkers responses of eco-sentinel organisms that populate subtropical regions are attributable to partly seasonal variations in addition to anthropogenic stresses. For examples, seasonal variations of the oxidative stress indices including lipid peroxidation (LPX), hydrogen peroxide (H$_2$O$_2$), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione transferase (GST), glutathione reductase (GR), reduced glutathione (GSH) and ascorbic acid (ASA) from P. viridis have been detected in green mussels from coastal waters of Goa,
India (Verlecar 2008). Previous study from Hong Kong waters accounted for also seasonal variability in battery of antioxidative biomarkers i.e. antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), the lipid peroxidation product malondialdehyde (MDA), a phase II detoxification enzyme glutathione-S-transferase (GST) and cholinesterase (ChE) in *P. viridis* (Lau *et al.* 2004).

Interestingly, the study revealed that protein content of gill tissues were less sensitive to seasonal variations compared to protein of whole body which indicated an advantageous of gill tissues as an organ target in using the biomarkers. Those examples suggested that seasonal variations are important factors and thus should be taken into account when comparing data from different seasons are conducted. Long term studies on tracing influences of seasonal variations on biomarkers particularly in green mussels in coastal waters of Indonesia appeared to be necessary for a more accurate application of biomonitoring strategies. It seems that the hot spot strategy which was performed at certain season is one of cost-effective preferable approaches for conducting effect biomonitoring using biomarkers that introduce general pictures of studied sites (Chapter IV).

### 6.3. Organophosphate Pesticides Pollution and Biomarkers

The general formula of OP pesticides is presented in Figure 31 shows the structural basis of the OP pesticides that contain a central pentavalent phosphorus atom (Baird and Cann 2005). The R of most of the OP pesticides are represented by methyl or ethyl groups, while R’ is methyl, ethyl, phenyl, amino, substituted amino or alkylthio (Fukuto 1990). The leaving groups which are sometime symbolized by OX or X define the majority of the structural differences between individual OP pesticides (Cocker *et al.* 2002). The oxygen atom in the OX group can be substituted by S with some compounds such as the living group of dimethoate. Likewise, the OP pesticides that contain the thio moiety (P=S) configuration like dimethoate (Figure 32) require metabolic activation to the corresponding oxon (P=O) before they can inhibit enzyme activity (Galloway and Handy 2003). It is due to the lower electronegativity of sulfur in P=S moiety compared to oxygen in P=O moiety results in a more electropositive phosphorus atom in P=O moiety, which facilitates attack on phosphorus by nucleophilic
agents such as the serine hydroxyl of AChE (Fukuto 1999). On the other hand, trichlorofon (Figure 33) is the OP pesticide that has an oxon moiety (P=O) which can react to and inhibit acetylcholinesterase (AChE) directly.

\[
\begin{align*}
S & \text{ or } O \\
RO & \\
R'O & \text{P--OX}
\end{align*}
\]

Figure 31. General structure of OP pesticide (Connell 2005).

\[
\begin{align*}
\text{CH}_3\text{O} & \text{P--O--S--CH}_2\text{C--N--CH}_3\text{O} \\
\text{CH}_3\text{O} & \text{P--CH--C--Cl} \\
& \text{OH} \quad \text{Cl}
\end{align*}
\]

Figure 32. Dimethoate.

Figure 33. Trichlorofon.

OP pesticides undergo chemical alteration when they enter into water system or penetrate to body of living organisms. For instance, trichlorofon is reconfigured in water to be dichlorvos which toxicity is more potent than the parent compound. Besides, once OP pesticides penetrate the body of living organisms, they are subjected to be metabolized by some enzymes such as cytochrome P450-dependent monooxigenases system that lead to mostly enhance the toxicity by forming oxon configurations (P=O) which so-called desulfuration (Fukuto 1990; Galloway and Handy 2003). Desulfurations of the OP pesticides inside the body of living organisms subsequently bind and inhibit one of the prominent neurotransmitter enzymes acetylcholinesterase (AChE). Inhibition of the enzyme by OP pesticides takes place due to the active site of the enzyme which is serine-hydroxyl group at catalytic center of the enzyme is phosphorylated (Chambers 1992). Accordingly, the prominent function of the enzyme to breakdown neurotransmitter compound i.e. ACh become choline and acetic acid is unavailable which subsequently cause the accumulation of ACh in
synaptic cleft. The reactivation of phosphorylated AChE takes place very slow which causes prolonged inhibition. The situation is more complicated when one alkyl group of the phosphoryl moiety is split non-enzymatically which brings on the formation of negative charge and ultimately sustains it (Peña-Llopis 2005). This reaction is called aging or dealkylation which then makes the reactivation of the enzyme is completely unavailable. The deleterious effects of OP pesticides inhibition become more fatal when the accumulation of ACh occurs at very high concentrations in the synaptic cleft which cause what so-called desensitization that blockage some cholinergic transmissions (Chambers 1992). Synaptic blockage causes to tetanus, paralysis of the diaphragm muscles and respiratory failure which ultimately followed quickly by the death of organisms (Walkers et al. 2001).

Mechanisms of OP pesticides actions to AChE activity through activation of biotransformation enzymes provide rationale basis for the use of the alteration of the enzymes as biomarkers in laboratory test (Sturm and Hansen 1999; Frasco and Guilhermino 2002) and field biomonitoring programs (Triebskorn et al. 2001; Binelli et al. 2005; Schiedek et al. 2006). However, the current dissertation focus only on the enzyme that shows a principal mode of action of the OP pesticides on nervous system which is AChE activity from two mussels species i.e. green mussel (Perna viridis) and blue mussel (Mytilus edulis) as a biomarker. It is due to the fact that the OP pesticides are fabricated determinedly to be effective inhibitors of AChE by binding the nucleophilic active site serine of the enzyme (Galloway and Handy 2003). Moreover, the disruption of AChE activity is considered as much reasonable explanation of the nervous system failure that will be manifested on higher levels of biological organization such as feeding activity and changes in inter-individual relationship (Lagadig 2001). Interestingly, the current research observed that reduction of siphoning rate which is the behavioral biomarker exhibits correlations with inhibition of ChE activity from mantle, gill and posterior adductor muscle of M. edulis after exposed by trichlorfon (Chapter II). Observable correlation between ChE activity of hemolymph and physiological biomarker i.e. cardiac activity from crab (Carcinus maenas) has been measured by Lundebye et al. (1997). Therefore, measured
activity of this enzyme provides a quantification of disability induced by the pesticides that might be reflected in population or in higher level.

There is emerging evidence that OP pesticides not only inhibit AChE activity but also disrupt immune system of aquatic organisms (De Guise et al. 2004; Gagnaire et al. 2006; Chang et al. 2006; Ivan et al. 2007; Canty et al. 2007). Accordingly, it has been postulated that pesticides disrupt immune-competence of organisms through direct and indirect mechanisms by phosphorylating and inhibiting critical protein involved in immune function (Pruett 1992; Galloway and Handy 2003). Directly the pesticides impair the immunity via inhibition of serine hydralase class of enzymes which play key roles in immune function, hampering membrane associated esterase of lymphocytes and monocytes which alter structural and functional immunocytes population, generating oxidative damage to immune organs, modulation of signal transduction pathway controlling cell proliferation and differentiation (Rickwood and Galloway 2004; Galloway and Handy 2003). Indirect mechanisms of the pesticides to induce immune-competence of living organism are by alteration of cholinergic tone to lymphoid organs, influencing on GABA (gamma amine butyric acid)-mediated transmission, and alteration of metabolism (Galloway and Handy 2003). In invertebrates, phagocytosis is a principal mechanisms of cellular defense which was performed by circulating hemocytes (Galloway and Depledge 2001). The disruption effects of OP pesticides to phagocytic activity will be reflected in the impairment of the immune function of living organism. Therefore, phagocytosis which operates through well-characterized phases, including recognition of non-self material, chemotaxis, adhesion to cell membrane receptors, ingestion, and destruction of the foreign agent (Russo and Lagadic 2004) has been used as biomarker to detect the impact OP pesticides and other xenobiotic compounds (Galloway and Depledge 2001; Blaise et al. 2002).

The applicability of the phagocytosis activity from blue mussels M. edulis as biomarker has been studied in the current studies by exposing the blue mussels to OP pesticide, dimethoate. The study revealed intriguing results by emerging non-dose dependent response pattern of the phagocytosis activity that so called U-Shape hormetic-like response (Figure 5). In contrast, inverted U-Shape hormetic-
like responses of phagocytic activity have been observed in *M. edulis* when the animals were exposed to OP pesticide, chlorfenvinphos (Rickwood and Galloway 2004). Hence, to take into account the hormetic-like response as one of phenomena that likely appeared in vivo study concerning phagocytosis activity of blue mussels is necessary.

### 6.4. Cholinesterase Activity: An Enzymatic Biomarker

The terminology of Cholinesterases (ChEs) is known as an expression of two types of acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BuChE; EC 3.1.1.8 is known as pseudocholinesterase as well). This dissertation uses the generic term of cholinesterase (ChE) activity as integrative meaning of the two types of the enzymes activities namely AChE and BuChE activity. Historically, Dale (1914) was the first scientist who postulated the existence of ChEs by observation on the ability of the horse serum to hydrolysis acetylcholine (ACh). Further research conducted by some researchers like Mendel and Rudney suggested that the serum contains two types of enzymes which have competency to hydrolysis ACh (Legay 2000). Nowadays, the enzymes that are attributed to the class of serine hydrolases can be classified based on substrate and inhibitors. AChE is capable to hydrolyze ACh more rapidly compared to other choline esters, inactive on butyrylcholine (BuCh), and shows substrate inhibition (Sutherland *et al*. 1997). To characterize AChE in tissues of *M. edulis* Brown *et al*. (2004) used synthetic analog substrate acetyl-β-metilthiocholine (AβMSch) as specific substrate for AChE. Conversely, BuChE can hydrolyze both two substrates ACh and BuCh (Massoulie *et al*. 1993), and exhibit activation in excessive substrates (Sutherland *et al*. 1997). In spite of the catalytic properties of BuChE vary from species to species, BuChE is more active on the synthetic substrates -- propionylcholine or butyrylcholine -- than on acetylcholine (Massoulie *et al*. 1993). Hence, the characteristic of BuChE can be distinguished by using synthetic analog substrate butyrylthiocholine (BuSCh) (Brown *et al*. 2004). The inhibitors that can be used to determine the two enzymes are BW284C51 or ethopropazine which are specific for AChE, while iso-OMPA and bambuterol are specific for BuChE (Legay 2000).
On vertebrate and invertebrate cholinergic synapses AChE play a well-defined role in hydrolyzing neurotransmitter compound i.e. ACh. The capability of AChE to hydrolyze ACh is about four thousand molecules hydrolyzed per active site and per second (Legay 2000) which turnover time of 150 μ second, lead it one of the most efficient hydrolytic enzymes known (Chang and Strichartz 2005). In addition to cholinergic function, AChE from marine mollusk, Aplysia, has a role in promoting neurite growth of adult neurons (Srivatsan and Peretz 1997). Although the main cholinergic function of AChE is very clear, the role of BuChE in cholinergic transmission remains questionable. However, it is to have an capability to hydrolyze some anti-cholinesterase drugs and ACh, and it is therefore suggested that BuChE is considered as an endogenous scavenging enzyme by detoxifying and sequestering the anti-cholinesterase compounds before reaching AChE at physiologically vital target sites (Massoulie et al. 1993; Çokuğraç 2003) or as a backup to AChE in supporting and governing cholinergic transmissions by hydrolyzing ACh (Li 2000).

Dichotomy of AChE and BuChE is specific and clear for vertebrates, particularly mammals which perhaps derived from duplication of one ChEs gene in an ancestor of the gnathostome (Massoulie et al. 1993). Nonetheless, this orthodox classification is difficult and inadequate to be applied to other animals since an unusual ChE activity that exhibit atypical, intermediate properties has been recorded along with AChE in for examples freshwater and marine teleost fishes (Leibel 1988; Sturm et al. 1999; Rodríguez-Fuentes et al. 2004; Jung et al. 2007). Likewise a single AChE has been observed in copepod, Tigriopus brevicornis (Forget et al 1999), grass shrimp, Palaemonetes pugio (Key and Fulton 1993) and hemolymph of M. galloprovincialis (Moreira et al. 2001). Brown et al (2004) reported that the extract of gill ‘microsomal’ of blue mussel M. edulis consist of a single atypical AChE which is less sensitive to specific AChE inhibitor i.e. Acetyl-β-methylthiocholine. The current study by using substrates specificity and inhibitors sensitivity approach revealed a deviant of mammalian-based classification of ChEs in green mussels (Perna viridis) as well. The result showed that ChEs of tropical green mussels consist of at least two types of ChEs which are a typical AChE and an atypical BuChE (Chapter III).
‘True’ AChE and the atypical BuChE were also observed in earth worm *Alolobophora chlorotica* (Rault *et al.* 2007).

ChE enzymes exist in varying amount in various organs of animals. In general AChE is observed profoundly in muscles and in the nervous system along with a lower level of BuChE, particularly at early developmental stage (Massoulie *et al.* 1993). Rault *et al.* (2003) observed ChEs in six species of earth worm whit higher enzyme activity occurring in nervous tissues compared to crop or gizzard.

Brain and muscles of three marine fishes *Limanda limanda, Platichthys flesus* and *Serranus cabrilla* showed different ChEs (Sturm *et al.* 1999). The authors concluded that the brain of these fishes consist of ChEs with all respect to the orthodox classification of AChE, while the muscles contain a typical AChE and an atypical BuChE. The different distributions of ChEs were also observed in different organs of scallops, *Pecten jacobaeus* (Bonacci *et al.* 2008). Gill and digestive gland of the scallops contained a classical-classification of AChE and an atypical of BuChE that showed a partial sensitivity to Iso-OMPA (specific inhibitor of BuChE), whereas the adductor muscle consisted of AChE which has most characteristics of true AChE such as a high sensitivity to BW28C51 and were resistance to Iso-Ompa (Bonacci *et al.* 2008). Green mussels *P. viridis* that collected from coastal waters of Pangkajene Kepulauan district revealed a different distribution of ChEs enzyme in different organs in which the highest ChE activity occurred in gill, followed by foot, mantle, and PAM (Chapter III).

The variations types and distributions of ChEs within organisms as observed in the studies (Chapter III) are quite clear evidence that envisage an indispensability of ChEs characterization on sentinel organisms of concern prior to the use of the enzymes as a biomarker. Upon the characterization, the preference of ChEs types which will be used in biomarker studies can be determined reasonably to avoid erroneous interpretations due to involvement of unexpected esterase that were measured in ChE activity generating high or low sensitivity to anti-cholinesterase. Besides, the characterization on different organs of a potential eco-sentinel organism like tropical green mussel provides information about the organs that are available and suitable in terms of neurotoxic
biomarker applications. The recent study showed that among the green mussel’s organs such as foot, mantle and digestive gland, gill demonstrated the highest ChE activity which contained a typical AChE and an atypical BuChE. The advantages of the occurrence of two types of ChEs in green mussel’s organs in terms of biomonitoring of marine pollutions have been discussed in Chapter III.

In the recent hot spot study (Chapter IV) that was conducted to prove different level of pollutions between heavily polluted sites in Jakarta Bay and Pankajene Kepulauan (Pangkep) revealed that ChE activity of gill of green mussels successfully differentiated pollution effects on cholinergic system of green mussels in sites of concern. The ChE activity not only was capable to distinguish the level of pollution effects between Jakarta Bay and expected clean site in Pangkep, but also it elucidated the different level of pollution impacts between two heavily polluted sites in Jakarta Bay, i.e. Kamal Muara and Cilincing. Although the results illustrated inspiring and promising evidence for the use of biomarkers particularly ChE activity in biomonitoring of pollutions campaigns, further studies to answer questionable factors such as seasonal variations, endogenous factors that influence the performance of biomarkers in field conditions, and ecological relevance of the biomarkers response are required before they are applied in routine biomonitoring of marine pollutions program.

Insufficient ecological perspective concerning lower levels of biological organizations is one of the major problems in using of biomarkers in ecological risk assessment (ERA) (Hansen 2008). Theoretically the lower the level of biological organizations such as ChE activity posses a high sensitivity to environmental stressors, but they are not easy to be extrapolated or interpreted to higher levels such as population structures or ecological integrity. However, the information of the early effects of such contaminants in the organismic levels is to underpin efforts for getting rid of the contaminants problems in the very early stage so that the problem can be recognized in advance and solved in proper time. Hence, in response to this matter setting up quantification relationships between biological responses across the level of biological integrity is necessary (Silbey et al. 2000).
Chapter II demonstrated the results of the trichlorfon effects across biological integrity namely ChE activity and siphoning rate of blue mussel, *M. edulis*. The correlation between the ChE activity of certain organs such as mantle, gill and posterior adductor muscle (PAM) and the siphoning rate were observed after exposed to serials concentrations of the pesticide for 96 h. Among the organs the ChE activity of mantle elucidated a predominant role in the siphoning activity. Accordingly, these results indicated that the effects of the pesticide on the biochemical or cellular level could be transformed to the higher level of biological integrity viz. behavior. Plotting the percentage of the siphoning and the ChE activity of mantle as depicted in Figure 34, the ChE activity inhibition (24 %) can be associated with siphoning activity disruption (50%). This relationship envisages the behavioral consequence of ChE activity inhibitions of blue mussels induced by the OP pesticide, trichlorfon, which prove the inhibition of ChE activity is not depict the exposure of the animals by the pesticide, but in fact it rather demonstrates the effects of the pesticide to biological organizations from a nervous system to a behavioral level.

Figure 34. Reduction of the siphoning activity in relation to the ChE activity inhibition from mantle of blue mussels, *M. edulis* after exposed to trichlorofon concentration for 96 h.

Recovery study (Chapter II) demonstrated that inhibition of the ChE activity turned back to level of the control after transferring the exposed mussels
to the clean media for seven days, except the ChE activity from foot and mantle at the highest concentration (1000 μg/l). It implies that the point where 24 % of the ChE inhibition which relate to 50 % of siphoning rate reduction is in the level of which the ChE activity inhibition recovers and thereby can be classified as a reversible condition when the pesticide exposure does not take place continuously. In contrast, the highest concentration of trichlorfon which causes an irreversible effect on the ChE activity of mantle by reduction of the activity approximately 34 % associates with recovery of the siphoning rate. It is indicated that the recovery of siphoning activity is not only supported by mantle, but also aided by gill. As results the recovery of the ChE activity of gill accommodates the mussels to perform the siphoning activity in the normal way as indicated by turning back the siphoning rate at the level of the control although other organ (mantle) that also play vital roles in the siphoning activity does not recover. It is also suggested that a paradoxical situation between biochemical and behavioral biomarkers occurred in which there were an indication of behavioral responses recovery, at same the time at cellular level, the biochemical response delineated an enhancing level of abnormality (Depledge 1999). Those results showed the advantageous of integrating biomarkers across biological organizations in relevant and multiple organs of eco-sentinel organism which provide more cautious explanation that keep away from miss judgments based on a single organ observation.

The hypothetical classification is in agreement with other experiments that have been reviewed in Chapter I and IV. The paucity of the current research is not including the exposure concentrations which cause mortality. Hence, the inhibition of ChE activity cannot be integrated and extended to the ultimate reversible endpoint i.e. the death of the organism that will be efficacious in determining the degree of nervous system impairments that cause mortality. Silbey and co-workers (2000) have correlated a biochemical biomarker viz. the ChE activity and mortality of fathead minnows. The authors observed that 10 % of the ChE activity inhibition was associated to 50 % of mortality, while 50 % of the ChE activity reduction was correlated to 90 % of mortality. What follows is further long-term researches are needed on marine mussels by integrating more than two biomarkers across biological organizations which allow categorizing the
level of nervous impairment along with some distinct interpretable endpoints which possess a closer link to ecological relevance such as reproduction, growth, and mortality. Peakall (1992) has provided an example of the classification that divides the biological responses of organisms to contaminants into normal, reversible and irreversible conditions (Figure 35). This kind of classification is useful for providing information on interpreting data obtained from particularly in situ studies, if the reference site is not adequate for providing comparable data due to endogenous or exogenous factors such as seasonal variations.

Figure 35. Hypothetical dose response of organophosphate pesticides (modified from Peakall 1992).

6.5. Phagocytic Activity as Biomarker

Along the controversial classification of bivalve hemocytes, Blaise and co-worker (2002) have simplified the classification of the hemocytes into progenitor (stem cells), phagocytic, hemostatic, and nutritive cells. Based primarily on the review of morphological bivalve hemocytes studies, Hine (1999) has concluded that the hemocytes of bivalves can be classified into two main groups which are granular and agranular hemocytes. The granular hemocytes (granulocytes) contain numerous vesicles and specific granules (Renwrantz 1990), while the agranular contain few or less granules which are called also as hyalinocytes since under light microscope, they comprise hyaline cytoplasm of silky appearance (Hine 1999). In addition, he also recorded that the agranular haemocytes can be
differentiated into blast-like cells, basophilic macrophage-like cells, and hyalinocytes (Hine 1999). In *M. edulis*, the granular hemocytes can be divided into small granulocytes that are basophilic and large granulocytes which are eosinophilic (Pipe 1990; Pipe *et al.* 1997). The size of the two blood cell types varies in *M. edulis*, the diameter of hyalinocytes is ranging from 4 – 10 μm (majority 8 μm), and the granulocytes diameter is between 4 and 24 μm (majority 10-13 μm)(Renwrantz 1990). Concerning phagocytic activity, the granulocytes comprise much more abundant phagocytic cells compare to hyalynocytes (Blaise *et al.* 2002). Carballal *et al.* (1997b) observed that the granulocytes of *M. galloprovincialis* showed an important phagocytic activity, while the hyalynocytes did not. In *M. edulis*, Wotton *et al.* (2003) observed that granulocytes composed predominantly in the blood cells, consisting of 90.58 % from whole cells which have a greater phagocytic activity compared to others bivalves i.e. the edible cockle, *Cerastoderma edule*, and the razor-shell, *Ensis siliqua*.

In marine mussels phagocytosis is the first line of internal cellular defense mechanisms that is mediated by cellular fraction of the hemolymph, hemocytes, for eliminating invading microorganism and foreign materials (Carballal *et al.* 1997b; Oliver and Fisher 1999; Wotton *et al.*2003). The fundamental process of phagocytic activity consists of four phases which are a chemotactic response, recognition, internalization and internal digestions (Renwrantz 1990), which involved humoral components of marine mussels immunity such as lysozyme activity, lectins, and the phenyloxidase system (Carballal *et al.* 1997a; Blaise *et al.* 2002). The hemocytes may be attracted by released microinvader’s substances and subsequently the microinvaders are opsonized to be bound to the peripheral ectoplasm of the phagocytic cells (Renwrantz and Stahmer 1983; Renwrantz 1990). After adhesion to the surface of the phagocytic cell, those foreign materials are internalized smoothly towards the centre of the cell where they are engulfed and undergo intracellular degradation and ingestion by action of digestive enzymes or the production of highly reactive oxygen metabolites (Renwrantz 1990; Coles *et al* 1994).

Immune function of marine invertebrate can be disrupted by many types of anthropogenic stressors including heavy metals, PAH, pesticides, leading to
impairment of bacterial clearance which enhance sensitivity and vulnerability of
the animals to be infected by microorganisms-associated diseases (Coles et al
1994; Parry and Pipe 2004; De Guise et al. 2004; Gagnaire et al. 2007). For
example, after the bacterial challenge oyster mortality was higher in pesticide-
treated oysters compared to untreated oysters (Gagnaire et al. 2007). Furthermore,
as a predominant internal defense organ, hemocytes of bivalves play vital roles in
combating pathogens, wound healing, shell repair, and transporting xenobiotic
compounds from entry organs to target organs where they are accumulated or
sequestered (Oliver and Fisher 1999). Those activities relied on phagocytic
ability of the hemocytes which has been known to be affected by environmental
stressors in the aquatic ecosystem (Anderson and Mora 1995; Blaise et al. 2002;
Thiagarajan et al. 2006). Hence, the phagocytic activity has been used as a
biomarker to detect wide range effects of anthropogenic contaminants including,
heavy metals (Sauve et al. 2002), Organophosphorous pesticides (Rickwood and
Galloway 2004: Gagnaire et al. 2006; Chapter I and II), polycyclic aroromatic
hydrocarbon (PAH) (Sami et al. 1992; Grundy et al. 1996, Gagnaire et al. 2006),
polychlorinated byphenil (PCB) (Gagnaire et al. 2006) and tributyltin (TBT)
(Cima et al. 1998; St-Jean et al. 2002).

This biomarker is also considered as a non-destructive biomarker due to
the fact that the use of this biomarker is relied on hemolymph and the blood cells
so that scarifying the sentinel organism is not required. Therefore, repetitive
sampling for recording time series response of the phagocytic activity in the same
animal is possible.

Since alterations of phagocytic ability of mussels induced by contaminant
exposures lead to increase susceptibility of the organism to pathogenic diseases
which ultimately causes the death of organisms, the use of the phagocytosis as a
biomarker can be an early warning system for jeopardy of declining mussel
population induced by diseases. Accordingly, this biomarker will be a useful tool
in biomonitoring particularly in the place where mussels are cultivated like in
Jakarta Bay, providing information on the magnitude of the risk which is
threatening the sustainability of mussel enterprises. The hot spot strategy that
applied in the recent study indicated that the phagocytosis of green mussels (P.
viridis) which were cultured in Jakarta Bay both in Kamal Muara and Cilincing underwent stimulation when compared to that of green mussels from pristine area in Pangkajene Kepulauan district. Thus, the results pointed out a warning of the danger that threatened the cultivated green mussels in Jakarta Bay which probably also threatened the livelihood of small scale farmers inhabiting alongside Jakarta Bay coast. By reason of the study was addressed to recognize the applicability of the biomarkers in the field study by comparing biological responses of the mussels collected from two different levels of polluted areas, it did not embrace all parameters that were demanded in the ecological risk assessment. Hence, there is space for further studies on the link between alterations of the phagocytosis and number of opportunistic pathogens and level of relevant chemical compounds in the green mussel body. The result of the studies may support environmental managers or government in establishing scientific based management to protect and enhance ecological and economical resiliencies of the aquatic ecosystem and human being.

Ecologically the susceptibility of the mussels to invasive pathogens which at least lead to inhibition of mussel growth or mass mortality threatened the aquatic ecosystem due to the fact that mussels play significant roles in maintaining the aquatic ecosystem for example as a biofilter for anthropogenic contaminants. It is because of the ability of mussels to filter huge amount of water where they are living. As a result, elevation of turbidity and level of contaminants which are linked to industrial and demographic growth will be worsened by decreasing or loss of marine mussel population. Furthermore, marine mussel population both in wild or cultivated population provide a habitat for epifauna that attach on the surface of clumping mussel shell such as polychaete worm, bernacles and others bivalves by enlarging the physical component, producing structurally complex entities that are capable attracting and harboring a diverse assemblages of associated fauna and flora (Seed and Suchanek 1992). This micro habitat is ecologically important and economically relevance by maintaining food web association.

Due to their feeding behavior as filter-feeder, marine bivalves accumulate enormous number of invasive pathogens from waters where they are living which
may raise health problems to the bivalves and human beings that consume them. The marine bivalves possess humoral and cellular internal antimicrobial mechanisms to overcome that challenge, such as phagocytic ability with excellent capacity for killing invasive pathogens. However, some microbial pathogens can evade from hemocytes killing by applying some fundamental strategies namely producing toxic substances that impair the hemocytes, constructing a capsule which is an “armor” to protect from opsonized surface attachment by hemocytes and producing antioxidant substances to prevent oxidative burst activity of hemocytes (Canesi et al. 2002). The capabilities of microbial pathogens to combat with internal defense systems of the hemocytes enable them to exist in the bivalve’s body. For example, three years study that has been conducted by Guyader and co-workers (2000) have recorded that hepatitis A virus (HAV), Norwalk-like virus (NLV), enterovirus (EV), rotavirus (RV), and astrovirus (AV) occurred in oyster (Crassostrea gigas) and mussels (Mytilus galloprovincialis). Furthermore, the authors observed that the bivalves (Mytilus galloprovincialis) which were sampled from area that received routinely human sewage were more highly contaminated. This evidence illustrate that pathogens induce diseases in human beings is capable to contaminate marine bivalves without any lethal consequences on the bivalves. Subsequently, the pathogens will be transferred back to people who consume the bivalves. In other words, the bivalves that populate the polluted area like Jakarta Bay provide not only food and income for those who live along coast of Jakarta Bay but also act as passive carriers of human pathogens (Canesi et al. 2002). The danger of microbial pathogens will emerge profoundly along with elevation of uncontrollable released contaminants in polluted area like Jakarta Bay.

The data from the recent study in Jakarta Bay on phagocytic activity of green mussels are of interest since the study observed that the contaminants from Jakarta Bay stimulating the phagocytic activity of the green mussels. Although the pattern of phagocytosis responses of the bivalves to contaminants are still obscure whether increasing or decreasing (Luengen et al. 2004), the common evidence reveal that phagocytosis stimulation will occur when the bivalves are exposed to low concentration of contaminants, and increasing the level of
contaminants dosed-dependently will be followed by decreasing phagocytic activity. Rickwood and Galloway (2004) observed this pattern when they exposed blue mussels to serial dilutions of organophosphate pesticide, chlorfenvinphos, for 24, 48, 96 h. If Jakarta Bay is considered as heavily polluted area, the xenobiotic compounds that were released in Jakarta Bay will reduce the phagocytic activity of the mussels, but the results of the current study showed otherwise.

In regard to marine pathogenic micro-organism, bivalves act as a reservoir of the animals (Sindermann 2006). Enteroviruses were observed in high number in both sediments and green mussel (P. canaliculatus) near the New Plymouth sewage outfall with maximum virus levels 32,000 pfu 100g⁻¹ of wet mussel tissue and 56 pfu 100g⁻¹ of wet sediments (Lewis et al. 1986). Furthermore, Setyobudiandi et al (1999) have reported the occurrence of pathogenic bacteria in green mussels that were cultivated in Jakarta Bay. The bacteria pathogens are Shigella sp, Escherichia coli, Salmonella sp. and Vibrio sp. Recently, the correlation between the occurrence of pathogens and contaminants in bivalves has been reported by Kim et al. (2008). The authors recorded that the correlation between the occurrence of pathogens and heavy metals and pesticides were more frequent compared to that of PAH. The existence of pathogens entered along with released sewages in Jakarta Bay can be one possible explanation to interpret the hot spot biomonitoring data. The stimulation of the phagocytic activity of the green mussels is probably the outcome from responses of the green mussels to compensate the invasive pathogens. Moreover, this compensation is perhaps beyond the response of the green mussels to cope with the xenobiotic compounds to achieve a homeostatic condition that results in increasing the phagocytic activity. Probably, the stimulation of phagocytosis is a strategy that is used by mussels to get rid of contaminants (Oliver et al. 2001) and fend off other foreign materials such as pathogens.

In vitro study conducted by Gagnaire et al. (2006) reported that an elevation of phagocytic activity of oyster occurred after exposed to mixture of pesticides for 4 h. In recent study organophosphate pesticides, dimethoate, produced an U-shape hermotic-like response of phagocytic activity of blue
mussels after 14 days exposure (Chapter I). Besides, Luengen et al. (2004) observed that stimulation of phagocytosis of marine mussels occurred at high polluted areas compared to low polluted area. To unravel the relationship between mussels phagocytosis and body burdens of contaminants and pathogens further studies on the effect of invasive pathogens along with mixture of xenobiotic compounds of the Jakarta Bay compartments in controlled and field scales are needed. The studies may also help in establishing ecological relevance of the phagocytic activity that will be efficacious in ecological risk assessment at least schematizing a pattern of the phagocytic response to environmental stressors that will be as a standard. However, the data from recent study on phagocytic activity of the green mussels in Jakarta Bay might be utilized as an early warning system of the possible occurrence of the xenobiotic compounds and invasive pathogens effects that may raise health problems in people particularly who consume the green mussels.

6.6. Siphoning Activity: Behavioural Biomarker

The siphoning activity is frequently called also as filtration or pumping activity. A term of clearance activity is usually attributed to this activity as well. Gosling (2003) has defined filtration or pumping rate as the volume of water flowing through gill in a unit of time, while the clearance rate is that volume of water completely cleared of particles per unit of time. When all particles presented to gills are cleared from suspension, the clearance rate is the same as the filtration rate (Gosling 2003).

As mentioned previously that the suspension feeder, mussels, play significant role in aquatic ecosystem by siphoning suspension material including chemical compounds, organic materials, and pathogenic organisms from the ambient water due to their high ability to filtrate the water. As results the levels of suspended materials, including phytoplankton, organic and non-organic materials can be controlled by this activity leading to increase deposition of organic materials in sediments and enhance the quality of filtrated materials by mineralization (Ostroumov and Widdows 2006). This bio-deposition activity supports significantly to enhance water clarity and enrich the ecosystem by cycling nutrients between water column and benthic habitat (Ostroumov 2005).
For example in seagrass bed, it was reported that the suspension of feeding bivalves can elevate seagrass productivity via increasing nutrient pools and reducing epiphytic loads on the leaves (Peterson and Heck 2001). Therefore, it can be stated that the siphoning activity of mussels play ecological roles not only on reducing xenobiotic compounds and suspended particulate materials but also enhancing water productivity by providing nutrient for the growth of primary producers in the aquatic ecosystem.

Siphoning activity of mussels is a biological activity which involves at least three organs including mantle, gill and posterior adductor muscle (PAM). Since those three organs are innervated organs, the failure of the nervous systems of those organs such as due to being exposed to anti-cholinesterase compounds will raise an impact on the siphoning activity. Accordingly, it indicates the siphoning activity is a good biomarker that can be related to and traced from low level biological organizations such as the nervous systems responses. In individual levels, the siphoning activity plays an important role in energy acquisition for vital function such as growth and reproduction (Widdows et al. 1987). Besides, from individual to ecological context, alteration of mussel behavior from normal conditions can lead to eliminate growth, reproduction, and survival of individual, resulting in decreasing mussel population that ultimately reduce ecological functions of the mussels as a transformer of organic and non-organic materials in the aquatic ecosystem. Consequently, the application of the siphoning activity as a biomarker of contaminants effects will provide a suitable tool for ecological risk assessments because of its closed relation to population or community and other ecological levels. Between cellular levels such as cholinesterase (ChE) activity and ecological contexts, siphoning activity may act as a connector since the destructive effects of contaminants in nervous system might be manifested to inhibit siphoning activity which may raise deleterious effects on community or ecosystem.

Siphoning activity of mussel is also considered as a non-destructive biomarker. The use of this biomarker is not necessary to kill the studied organism thereby damaging samples populations can be avoided. It is particularly important when the eco-sentinel organisms are animals under restricted by law in
conserving their population. Likewise, the use of non-destructive biomarkers provide consecutive sampling on the same individual which enable the time course of an effect to be studied accurately (Peakall and Walker 1994).

Both siphoning rate of freshwater or marine bivalves have been used as behavior biomarkers to detect effects of contaminants in laboratory and field scales from heavy metals (Mouabad et al. 2001; Anandraj et al. 2002; Vijayavel et al. 2007), PAH (Widdows et al. 1987; Toro et al. 2003), surfactants (Ostroumov and Widdows 2006: Ostroumov 2006) to organochlorine, organophosphate and carbamate pesticides (Bourdelin 1996; McHenery 1997; Donkin and Widdows 1997; Toro et al. 2003; Chapter II; Cooper and Bidwell 2006; Canty et al. 2007). The field study that was conducted by Toro and co-workers (2003) demonstrated that the siphoning rate of Coromytilus chorus was affected by occurrence of residues of organochlorines (OCs) and polynuclear aromatic hydrocarbon (PAHs) in their tissue. Furthermore, the authors suggested that the failure of the siphoning rate was reflected in physiological responses related to energy balance which had a significant correlation to the organic pollutants in the tissues (Toro et al. 2003). In contrast, McHenery et al (1997) and Canty et al (2007) were unable to show the effects of OP pesticides on the siphoning rate of mussels due to technical problems in the experiment, whereas Donkin and Widdows (1997) and Cooper and Bidwell (2006) did observe the effects of the pesticides on that biomarker. However, the correlation between inhibition of ChE activity and the siphoning rate were not observed (Donkin and Widdows 1997; Cooper and Bidwell 2006). The data illustrate that the correlation between ChE activity of the targeted organ and siphoning rate under exposure of OP pesticides is not simple. The use of a single organ as a surrogate of whole organisms in relation to animal behavior under pressure of OP pesticides contaminant is not adequate.

Chapter II has described that around 40% of siphoning process was performed by non-organismic factors such food particles and water movements. Whilst, the ChE activity of the tested organs i.e. gill and mantle demonstrated that the two organs played roles about 60% in siphoning activity. Moreover, the results of the study revealed that mantle possessed more dominant roles compared to gill in siphoning activity when the activity mechanism is simplified under
assumption that ChE activity played significant roles in nervous system of organs of interest. Perhaps, the results of the study highlighted the uncertainty of the exact role of ChE in controlling gill activities (Aiello 1990), even though the role of this neurotransmitter compound cannot be ruled out completely in gill activities.

Another possible explanation is on the method that was used to measure the siphoning activity. The used technique is centered on measuring the numbers of particles in media that can be siphoned by mussel. This method in fact does not take considerations on the significant role of gill to filtrate, ingest the particles as food and/or flocculate and then excrete the particles as pseudo-feces. It takes into account mainly the flowing particles along with water into the shell cavity. As a consequence, it is reasonable when the result revealed the dominant role of mantle in the siphoning activity. In the siphoning activity mantle is the main organ to maintain the flow of water into the shell cavity by protruding or retracting mantle (Jørgensen et al. 1988). Donkin et al. (1997) observed that *M. edulis* showed a relaxation state with lack of adductor muscle tones when exposed to dichlorvos. Under contaminant effects the valve gape is not necessarily related to an elevation of siphoning activity (Redpath and Davenport 1988) since the nervous system of siphoning organs such as gill and mantle are disrupted. Hence, it is worthwhile to suggest that when ChE activity from *M. edulis* is used as a biomarker in relation to higher biological response such as siphoning activity for detecting the effect of OP pesticides, the first priority of mussel’s organs which should be involved in the measurement is mantle, followed by gill and PAM. Integration of relevant organs such as those three organs in relating the siphoning activity of mussels will provide more detail explanation on the effects of the contaminants of concern to relevant organs which were involved in the siphoning performance. The recent study revealed that the sensitivity of the mussel’s organs to trichlorfon in term of ChE activity was different. Therefore, the use of battery of the relevant organs can also refine data interpretation by minimizing under and/or over estimating on the effect of the contaminant to animals when a single organ was used as representative of whole organism.
Overall, the relationship between reduction of the siphoning activity which is behavior that plays key roles both in terms of individual or ecosystem and inhibition of ChE activity from organs of concern of *M. edulis* induced by the OP pesticide suggesting that it is an ecological relevant parameter. As a consequence it is promising to establish ecosystem health standards by involving those two selected biomarkers along with others relevant biomarkers from molecular to individual levels.

6.7. Conclusion

In laboratory test, ChE and phagocytic activity from *M. edulis* were able to detect the effects of OP pesticide, dimethoate. The serial dilutions of dimethoate promoted effects on the ChE activity dose-dependently, while hormetic-like responses of the phagocytic activity were observed.

The different sensitivity of different organs to trichlorfon in terms of cholinergic enzyme, ChEs, has been detected in the studied *M. edulis*. Gill was the most sensitive organs compare to other studied organs including foot, mantle, posterior adductor muscle (PAM), hemolymph and digestive gland. The inhibition of the ChE activity of the organs turned back to the level of control after the animals transferred to the clean media except foot and mantle.

Reduction of siphoning activity of studied mussels was also observed after the animals were exposed to serial dilutions of trichlorfon. It was intriguing that the sensitivity of the siphoning activity to the OP pesticide was in range of sensitivity of the ChE activity to the OP pesticide. The recovery of the siphoning activity was observed after the animals were put in clean media as well.

The exposure of trichlorfon experiment revealed correlation responses between the siphoning activity and the ChE activity of the relevant organs including mantle, gill and posterior adductor muscle. The correlations are efficacious in promoting overview that enzymatic biomarkers such as ChE activity can be translated to higher biological integrity and population levels via a good connector namely the siphoning activity. From the siphoning activity the way for interpreting ecological significance of the ChE activity inhibition can be deducted.
Characterization of ChEs enzyme from tropical green mussels (P. virisis) based on substrates specificity and inhibitors sensitivity strategies displayed that there were two types of ChEs enzyme namely typical Acetylcholinesterase (AChE) and atypical Butyrylcholinesterase (BuChE). These enzymes were present in varying mount in studied organs of the mussels in which the typical AChE was present dominantly in gill, while the atypical BuChE existed profoundly in the posterior adductor muscle (PAM).

Hot spot monitoring strategy which were conducted in two heavily polluted areas of Jakarta Bay and pristine site in Pankajene Kepulauan demonstrated that two selected biomarkers i.e. ChE and phagocytic activity from P. viridis were suitable biomarkers that were able to distinguish the effect of environmental stressors between the heavily polluted sites and the reference site in dry season.

6.8. Recommendations

1. The studies revealed the usefulness of the selected biomarkers either in laboratory or filed scale to detect the effects of environmental stressors. Hence, it is recommended to employ the selected biomarkers in screening test in laboratory scale and biomonitroing program.

2. Since the selected biomarkers that were used in the studies in term of hot spot monitoring strategy, it is recommended to perform long term studies on tracing influences of seasonal variations on biomarkers particularly in green mussels form Indonesian waters. The study will extend the application of biomarkers in different seasons.

6.9. References


Menasveta P, and V. Cheevaparanapiwat. 1979. On the accumulation of heavy metals, DOT, and PCBs in the green mussel (Mytilus viridis Lin.). mullets (Mugil dussumerii Val.). and bottom sediments rolled from the four river mouths Thailand. Bangkok: Chulalongkorn University, Final Report for National Environmental Board of Thailand by Institute of Environmental Research.


Appendix

Appendix 1. Protocol of measurement of cholinesterase activity.

**Principle**

Acetylcholinesterase catalyze the hydrolysis of acetylthiocholine. Upon hydrolysis these substrates analog produce acetatic acid and thiocholine. Thiocholine in the presence of the highly reactive 5,5'-dithio-bis(-2-nitrobenzoic acid) generates the yellow anion of 5-thio-2-nitrobenzoate. The yellow color can be quantitatively monitored by spectrophotometric absorption at 405 nm.

**Solutions for measurements in mussel tissues**

**Potassium phosphate buffer (0.1 M) / pH 8.0**

0.422 g/l KH₂PO₄, 16.878 g/l K₂HPO₄

**DTNB-stock solution (7.5 mM)**

297.2 mg 5,5'-Dithio-bis(-2-Nitrobenzoic acid) (Sigma D-8130) and 112.5 mg NaHCO₃ in 100 ml potassium phosphate buffer (0.1 M/pH 8.0). This stock solution can keep at -20°C. For 5 microplates at the assay you need 10 ml.

**DTNB-working solution (0.75 mM)**

The solution has to be prepared at the day of the assay. Add 90 ml potassium phosphate buffer (0.1 M/pH 8.0) to 10 ml DTNB-stock solution and incubate the solution at 25°C.

**ASCh-stock solution (30 mM)**

216.9 mg Acetylthiocholine iodide (Sigma A-5751) in 25 ml potassium phosphate buffer (0.1 M/pH 8.0). This stock solution can keep at –20°C for 1 month. For 5 microplates at the assay you need 2.5 ml.

**ASCh-working solution (3 mM)**

2.5 ml ASCh-stock solution will be added to 25 ml with potassium phosphate buffer (0.1 M/pH 8.0) at the day of the assay. If a cholinesterase activity is not measurable you have to prepare a fresh ASCh-working solution. 21.69 mg ASCh will be solved in 25 ml potassium phosphate buffer (0.1 M/pH 8.0). For 5 microplates you need 25 ml ASCh working solution.
Solutions for measurements of protein

Bradford-reagent

20 ml Protein-Assay (BIO-RAD 500-0006) will be added to 100 ml with distilled water. It is enough for 5 microplates.

Protein-stock solution

1 mg γ-globuline (Sigma G-5009) will be solved in 1ml distilled water. The solution can be kept at -20°C.

Protein-standard solution

The protein-stock solution is to dilute with distilled water at the following concentrations: 500 µg/ml, 250 µg/ml, 125 µg/ml.

Materials

- Microplate photometry
- Filter 405 nm and 620 nm multichannel pipette
- Microvolume pipettes, 1000, 200, 100, 10 µl glassware

Preparation of tissue extract

Mussel’s tissue from Mytilus edilus (200-300 g) is homogenized with 2 ml of potassium phosphate buffer (0.1 M/pH 8.0), centrifuged for 10 min at 10000 g, and a 1:2 dilution of the supernatant used in the enzyme assay. The supernatant can be kept at –20°C for later measurements. Mytilus hemolymph can be used without dilution.

Measurement of the enzyme activity

At the beginning switch on the microplate reader and the computer. Start TECAN software and select the kinetics program. Load the ACCH.template. Put in 50 µl of each diluted sample into each well of microplate. Each sample is assayed in four replicates. In each blank well of microplate put in 50 µl of potassium phosphat buffer. Add 200 µl DTNB-working solution (0,75 mM) to each well. Incubate the microplate at 25°C for 5 min. Start reaction by adding 50 µl ASCh-working solution to each well, set microplate into microplate photometry and start the measurement. The measurements will be carried out in an interval of 30 s for 5 min at 405 nm.
## Measurements of protein

The tissue extract of mussels will be diluted 1:10 with distilled water. Put in 10 µl of the diluted extracts and the protein standard solutions into microplate. Each Sample and protein standard solution is assayed in four replicates. Blank wells receive 10 µl of distilled water. Add 200 µl Bradfort-reagent to each well. Incubate the microplates for 5 to 30 min. The measurements will be carried out at 620 nm. If the values above the protein calibration curve, repeat the measurements with a higher dilution.
Calculation of cholinesterase activity

For each sample average absorption values of the reaction (OD_{reac}) and average blank values (OD_{Blank}) will be plotted against a time axis. The proportion of the curve in which the absorption in reaction replicates increases linearly is noted (t_1 to t_2). For convenience, assay conditions should be adjusted in such a way that the reaction is linear over the whole time of observation. From the linear proportion of the curve, the rate of increase in absorption (\Delta OD / min) in reaction replicates is calculated:

\[
\Delta OD/min = \frac{[OD_{reac(t2)} - OD_{reac(t1)}] - [OD_{Blank(t2)} - OD_{Blank(t1)}]}{(t_2 - t_1)}
\]

Calculation of rough activity values:

In the calculation an absorption coefficient of 13.6 mM^{-1}*cm^{-1} and a path length of 0.9 cm is used.

Rough activity [nM*min^{-1}*ml^{-1}] = \left[\frac{\Delta OD/min}{(13.6*0.9)}\right]*1000

Calculation of specific activity:

spec. act. [nM*min^{-1}*mg^{-1}] = roughactivity/[(volume/300)*proteinconcentration]

“Volume” is the volume of tissue extract used per microplate well in [\mu l] and “proteinconcentration” is the protein concentration of the tissue fraction in mg/ml.

**Materials**

- 1 ml tuberculin-syringes (0.1 ml step marks)
- Needles (Diameter 0.4 mm).
- Hemocytometer (according to Neubauer new).
- Pipettes 10 µl, 100 µl.
- 8-Channel- variable Pipette for 100 µl and 200 µl
- Pipette tips (2-200 µl).
- 96-Well-Microtestplate / flat bottom (Greiner 655101)
- Cover film for Microtestplate.
- Beaker glass and bottle.
- 15 and 50 ml disposable plastic reaction tubes (Greiner, Nunclon, Falcon etc).
- ELISA reader, Filter 620 nm (like TECAN STTC or Spektra) with Data transfer to Computer.
- Microtestplate Fluoreszenzreader; Ex 485 nm, Em 535 nm (Dynatec Fluorolite) with with Data transfer to Computer.

**Chemicals**

- Sodium chloride solution 0.9%. 9 g NaCl / l A. bidest.
- Sodium carbonate buffer. 10.6 g Na₂CO₃ and 20 g NaCl per 1 l Aqua bidest. Adjusting of pH 9.6 with 6 N HCl.
- Fluoresceinisothiocyanate (FITC)-solution. Fluoresceinisothiocyanate (Sigma F-3651) has to be kept in dark. 10 mg are solved in 100 ml Sodium carbonat buffer.
- *Mytilus* Hemolymph buffer (MHB) -Buffer for *Mytilus* Hemocytes:
  - TRIS/HCl (40 mmol/l): 6.30 g/l.
  - NaCl (400 mmol/l) (equivalent amount for 890 mosm): 23.38 g/l.
  - CaCl₂ x 2H₂O (2 mmol/l): 0.29 g/l.
  - pH 7.4: the osmotic pressure 890 mosm.
- Trypanblue (0.6 mg/ml). 60 mg Trypanblue (Merck 1.11732 for Microscopy) is solved in 100 ml MHB.
• Fixation of cells (only for field monitoring). 1 % Glutaraldehyde for fixing haemocytes and phagocyted yeast cells.

• Protein-standard (1mg/ml). 10 mg Bovine Serum Albumin (BSA) from (Sigma A-2153) are diluted in 10 ml Aqua bidest. The Protein-Standard can be stored frozen in batches of 500 µl at -20ºC. For each day of testing the protein-standard has to be prepared freshly from a new batch in Aqua bidest.

- 1000 µl/ml
- 500 µl/ml
- 250 µl/ml
- 125 µl/ml
- 62.5 µl/ml
- 31.25 µl/ml
- 15,625 µg/ml

• Lysis reagent : 0.1N NaOH

• Bradford-Reagent. Protein is measured with Coomassie Blue brilliant Blue G450 according to Bradford with the BioRad (500-0006) assay (20 % in Aqua bidest).

**Laboratory exposition/Keeping Mussels**

Mussels from un- or low-contaminated reference sites are necessary. Prior the testing, an one week adaption period is required (minimum). Mussels are kept in artificial Seawater (Tropic Marine® in Aqua dest.) with a salinity according the salinity at the origin (Salinity in the German bight can seasonally differ between 2.6 % and 3.3 % ). Offshore condition is 3.3%. Cold season simulation is 8-12 ºC. Warm season simulation is 15-20ºC. Aeration of the tanks is done by commercial pumps

Maximum density is 30 Mussels / 10 l. Mussels are fed with life phytoplankton (Aquatin® for marin filter feeders). 75-150 µl Plankton per Mussel are calculated (3 ml per 25 l).
Due to transportation stress effects, an exchange of 75% of the water each 2-3 days could be necessary. After adaptation took place a 25% water replacement/week is enough. A daily control of the tanks is required.

FITC-conjugated yeast (used for field monitoring as well as for lab work)

Preparing Yeast

10 g of dry yeast (Dr.OETKER) are mixed in 150 ml 0.9% NaCl and heat inactivated by cooking for 30 min. The inactivated Yeast becomes washed 6 times by 10 min centrifugation 1000 U/min followed by decantation of the supernatant and bloating of the pellet in 100 ml 0.9% NaCl-Solution.

Purity of the suspension needs to be checked by microscopy before the last centrifugation. After the last centrifugation the pellet is bloated in Sodium bicarbonate buffer with a concentration of ca. 2 x 10⁹ cells per ml. The suspension can be stored at -20°C.

FITC conjugation of Yeast cells (Stock solution)

120 ml of the thawed yeast becomes washed with 2 times with 0.9% NaCl –solution as described before. After the last centrifugation, the pellet becomes bloated in the freshly prepared FITC-Solution (FITC, Sigma F7250) and warmed up at 37°C for 30 min. Five times of washing (as described above) are necessary to remove unconjugated FITC. FITC conjugated yeast has to be kept in the dark and can also be stored frozen in -20°C.

Yeast concentrations used in the Test

As observed regularly, hemocyte concentrations can range between 10,000 and 1,000,000 cells/100 μl. Therefore five different yeast concentrations are needed to make sure that there is always a matching Yeast concentration in the optimum range (between 1:10 and 1:25) available. All concentrations are based on a predilution with a concentration of 1 x 10⁹ cells/ml made from Stock.
<table>
<thead>
<tr>
<th>Yeast cell suspension</th>
<th>Hemocytes in 100 µl Hemolymph</th>
<th>Hemocytes to yeast cells</th>
<th>Yeast cells in 25 µl x 10^6</th>
<th>Yeast cells in 1000 µl x 10^6</th>
<th>Volume of prediluted yeast [ml]</th>
<th>MHP [ml]</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>10 000 – 25 000</td>
<td>1:10 – 1:25</td>
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<td>10</td>
<td>0,2</td>
<td>19,8</td>
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<tr>
<td>2</td>
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<td>1:10 – 1:25</td>
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<td>0,5</td>
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</table>

**Procedure:**

Obtain hemolymph (approximately 1 ml) from the posterior adductor muscle into a syringe. Drop off haemolymph on the Neubauer count chamber. Hemocytes have a tend to clot fastly. Aliquot 100 µl of each sample into 6 microplate wells. Four replicates for phagocytosis and two replicates for measuring the protein concentration. Two replicas are left for measuring the yeast standard. Incubate hemolymph for 30 min for adhesion of haemocytes. Aliquot 25 µl of yeast working solution into 4 microplate wells for phagocytosis activity as control: aliquot of 100 µl MHB into 8 microplate wells (one column) and 25 µl yeast dilution into 4 wells. Incubate the plate for 90 min at 21°C in darkness.

**Fieldwork**

At the end of the 90-minute period, 50 µl of Glutaraldehyde became added to each well with yeast. In addition 50 µl of Methanol became added to the wells with hemocytes only. The plates have to be covered with film and wrapped in Aluminum. Store the plates at 10°C in darkness up to 4 weeks. Prior to fluorescence measurement the fixative solution has to be removed carefully and is replaced by 125 µl of MHB.

**Quenching (both field monitoring and Lab work)**

At the end of the incubation time or after replacement of the fixative solution (remove methanol too) 25 µl of trypan blue were added to all wells with
yeast to quench the background fluorescence of unphagocytic cells. Quenching is best after 20 minutes in a dark condition.

Prior to measurement all supernatants are carefully removed. Measuring fluorescence of yeast cells uses a reader for 96-wells microtest plate (Excitation of 485 nm and an Emission of 535 nm). Keep lamp voltage or gain constant for all plates to ensure comparable data sets.

**Measuring Protein**

Measuring protein is carried out in wells with hemocytes only. A negative control and the serial dilution of the protein standard are made on the same plate. Prior to measuring the hemocytes are lysated with 50 µl of 0.1n NaOH. From each well (protein standard and lysed sample a 10 µl aliquot is transferred to a second plate. Blank is made with 10 µl of Aqua bidest. Staining starts with the addition of 200 µl of Bradford reagent (working solution). Staining is best after around 10 minutes at room temperature. Extinction is measured at 620 nm.

**Calculation of Phagocytotic activity**

**Fluorescence**

Due to the use of five different yeast concentrations the relative Fluorescence of phagocyted yeast needs to be corrected by subtraction of the background fluorescence of an adequate control of quenched yeast (without hemocytes). The result is expressed as Relative Fluorescence Units (RFU)/ml.

\[
RFU = \frac{\text{Fluorescence of Sample} - \text{Fluorescence of Control}}{100 \mu l \text{ volume of sample}} 
\times 1000 \mu l
\]

Amount of hemocytes expressed as proteine concentration

The protein concentration [mg/ml] is calculated from the standard curve.

The phagocytic activity is expressed as **Phagocytic-Index**

\[
\text{Phagocytic-Index} = \frac{\text{Relative Fluorescence Units (RFU)}}{\text{Protein [mg / ml]}}
\]
### Microtestplate (For field monitoring)

<table>
<thead>
<tr>
<th>BSA</th>
<th>LW-Tr</th>
<th>P-1</th>
<th>P-2</th>
<th>P-3</th>
<th>P-4</th>
<th>P-5</th>
<th>P-6</th>
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<td>MW-2</td>
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<td>NK-5</td>
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</table>

**BSA:** Proteine standard

**LW-Tr:** Blank (125 µl MHP + 25 µl trypanblue-solution)

**MW:** Meßwert (100 µl Hemolymph + 25 µl yeast-suspension + 25 µl trypanblue-solution/Fixed with 50 µl Glutardialdehyde)

**P:** proteine measurement (100 µl hemolymph/fixation 50 µl Methanol)

1–8: Animal number.

**NK:** Negativ control (100 µl MHP + 25 µl yeast-suspension + 25 µl trypanblue-solution)
Appendix 3. Correlation between the trichlorfon induced ChE activity of mussel’s organs and the siphoning rate.

**Descriptive Statistics**

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>N</th>
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<tbody>
<tr>
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<tr>
<td>Mantle</td>
<td>3.1858</td>
<td>.62347</td>
<td>36</td>
</tr>
<tr>
<td>PAM (Posterior Adductor Muscle)</td>
<td>2.1568</td>
<td>.40975</td>
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<tr>
<td>SR (Siphoning Rate)</td>
<td>42.6476</td>
<td>18.51585</td>
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**Correlations**

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<td>.570(**)</td>
<td>.458(**)</td>
<td>.656(**)</td>
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<tr>
<td>Sig. (1-tailed)</td>
<td>.</td>
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<td>.002</td>
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<td>Sum of Squares and Cross-products</td>
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<td>36</td>
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<td>36</td>
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<tr>
<td>Mantle</td>
<td>.570(**)</td>
<td>1</td>
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<td>.761(**)</td>
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<td>Pearson Correlation</td>
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<td>.000</td>
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<tr>
<td>PAM</td>
<td>.458(**)</td>
<td>.415(**)</td>
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<td>.510(**)</td>
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**Correlation is significant at the 0.01 level (1-tailed).**
Appendix 4. Backward multiple regression procedure on the trichlorfon induced ChE activity of mussel’s organs and the siphoning rate.

Descriptive Statistics

<table>
<thead>
<tr>
<th>Organs</th>
<th>Mean</th>
<th>Std. Deviation</th>
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<tbody>
<tr>
<td>Sr (Siphoning Rate)</td>
<td>42.6476</td>
<td>18.51585</td>
<td>36</td>
</tr>
<tr>
<td>Gill</td>
<td>2.8938</td>
<td>1.17549</td>
<td>36</td>
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<tr>
<td>Mantle</td>
<td>3.1858</td>
<td>.62347</td>
<td>36</td>
</tr>
<tr>
<td>Pam (Posterior Adductor Muscle)</td>
<td>2.1568</td>
<td>.40975</td>
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Model Summary

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<tr>
<th>Model</th>
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<th>Adjusted R Square</th>
<th>Std. Error of the Estimate</th>
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<td>.672</td>
<td>.641</td>
<td>11.09752</td>
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<tr>
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a Predictors: (Constant), PAM, Mantle, Gill
b Predictors: (Constant), Mantle, Gill
c Dependent Variable: SR

ANOVA

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<tr>
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<th>Mean Square</th>
<th>F</th>
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a Predictors: (Constant), Pam, Mantle, Gill
b Predictors: (Constant), Mantle, Gill
c Dependent Variable: SR

d Coefficients

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<tr>
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a Dependent Variable: SR
### Excluded Variables

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a Predictors in the Model: (Constant), Mantle, Gill  
b Dependent Variable: SR

### Residuals Statistics

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a Dependent Variable: SR