CHAPTER V

DISCUSSION

5.1 Quantification of commercial chlorpyrifos

Commercial chlorpyrifos commonly found at local pesticide retailer was used in this study in order to obtain similar ingredients applied in the field. After the commercial insecticide was analyzed, the result revealed that the actual concentration of chlorpyrifos in the insecticide was about 32% less than it was indicated in the product's label. This could possibly due to the degradation of chlorpyrifos or the problem with the product's quality control. Therefore, the concentration of chlorpyrifos indicates in this study was the actual concentration detected by GC analysis in comparison with technical grade chlorpyrifos.

Mazanti et al., (2003) investigated the presence of chlorpyrifos at 1.0 and 0.1 mg/l in the aquaria and at 0.1 mg/l in the outdoor macrocosms. The results indicated that chlorpyrifos disappearance was rapid in all of the systems and followed a two-phase sequence. Initial half-lives varied from 0.16 day to 0.38 day and showed similar rates in the aquaria and the outdoor systems. The second phase of the chlorpyrifis loss pattern was slower (18–20 days) in all the treatments. Within the first 10 days of the experiment, chlorpyrifos was reduced by between 80% and 84% in the high and low insecticide treatments. In addition, the aquaria receiving no tadpoles showed losses of chlorpyrifos slightly lower than the tanks with tadpoles. It was suggested by the authors that the greater initial loss of chlorpyrifos (about 44%) has been caused by the aeration system. However, the rapid losses of chlorpyrifos in all of the outdoor pond treatments were comparable to those observed in the laboratory aquaria.

Chlorpyrifos has relatively low hydrolysis (16–30 days) and low photolysis (11 days) half-life values, indicating the high escaping tendencies in the aquatic system. It is also important to notice that the hydrolysis rate of loss is also pH-dependent but was not a single parameter to extrapolate hydrolysis rate constant (Liu et al., 2001). However, from the study of Macalady and Wolf (1985), the authors reported the hydrolysis rate of chlorpyrifos increased with the increasing of basic condition (alkalinity), where half-life of chlorpyrifos was 53 d at pH 5.90, 141.6 d at

pH 6.11, and 10 d at pH 9.77 in distilled water at 25 °C. Literature values for loss by hydrolysis of chlorpyrifos applied as a spray mix to ponds (Giddings *et al.* 1997) are also similar to the studied of Mazanti et al., (2003) (6d).

The loss of chlorpyrifos in this study was rapid which was in agreement with those studies. However, the two phase reduction of chlorpyrifos was not obviously observed probably due to the very low concentrations of chlorpyrifos used in this study.

5.3 Acute toxicity test of chlorpyrifos

The acute toxicity studies of chlorpyrifos to non-target aquatic organisms have been performed mostly on adult fish. The results of LC_{50} were varied among test fish species. For examples, the 96 h LC_{50} for fathead minnows, *Pimephules promelus*, sheephead minnows, *Cyprinodon variegates*, and Indian carp *Catla catla*, *Labeo rohita*, and *Cirrhinus mrigala* were 122.2, 136, 350, 470, and 650 µg/l, respectively while that of some atherinid fishes such as *Menidia menidia*, *M. peninsulae*, *M. beryllina*, and *Leuresthes tenuis* were 1.7, 1.3, 4.2, and 1.3 µg/l, respectively, which were much more sensitive to chlorpyrifos than most fish (Clark et al. 1985; Jarvinen et al. 1988; Tilak et al. 2004).

The estimated acute value shows that the 96 h LC_{50} of chlorpyrifos for *P. monodon* obtained from this study is 20.74 µg/l which is over 6 times lower than the fathead and sheephead minnow LC_{50} and almost 30 times lower than *Cirrhinus mrigala* LC_{50} but it is about 10 times higher than that of the atherinid fishes. Therefore, the sensitivity of *P. monodon* to chlorpyrifos is ranged between the most and the least sensitive tested fish.

Only a few investigations have been carried out with aquatic crustaceans and most of them were conducted on larval stages. The 96 h LC_{50} of chlorpyrifos for larvae of mysid shrimp (*Neomysis integer*) and grass shrimp (*Palaemonetes pugio*) were 0.13 and 0.15 µg/l, respectively (Roast et al. 1999; Key and Fulton 2006). This indicates that *P. monodon* is more tolerant to chlorpyrifos than these two crustaceans. However, at the higher concentration, *P. monodon* was more sensitive to the pesticide than some tested crustaceans. The 24h- LC_{50} value of chlorpyrifos for *P. monodon* (52.43µg/l) obtained from this study was lower than that found in *Artemia salina*

(3.19 mg/l) (Varo et al. 2002) and *Litopenaeus stylirostris* larvae (2.26 mg/l) (Reyes et al. 2002). At the juvenile stage, the 96 h LC_{50} indicate that *P. monodon* was more tolerance to the pesticide than other penaeid shrimp e.g. *P. aztecus*, and *P. duorarum* and mysid shrimp *Mysidopsis bahia* (Table 5.1).

It should be noted that the sizes and the developmental stages of the tested animals as well as the experimental conditions are different. Therefore, the comparison of the results in this study and that earlier reported in open literatures has to be considered. The difference in sensitivity to chlorpyrifos among these aquatic species suggests the consideration in the application or contamination of chlorpyrifos in aquatic. To prevent environmental and health risk from chlorpyrifos contamination, acute toxicity can be used as information for the zoning criteria of pesticide used in aquaculture area or aquatic environment. In Thailand, there is still no water quality standard on the allowable contaminated concentration of organophosphorus pesticide and chlorpyrifos. The information of acute toxicity of *P. monodon*, an economic shrimp cultured in agricultural area where the pesticides have been heavily used, should be useful.

Table 5.1 LC_{50} of chlorpyrifos on aquatic crustacean

Species	**96 h LC_{50} (µg/l)**
P. monodon (juvenile)	20.74
Mysid Shrimp *Mysidopsis bahia* (adult)	0.040
Mysid Shrimp *Mysidopsis bahia* (juvenile)	0.045
Brown Shrimp *Penaeus aztecus* (juvenile)	0.20
Grass Shrimp *Palaemonetes pugio* (juvenile)	1.5
Pink Shrimp *Penaeus duorarum* (juvenile)	2.4
Blue Crab *Callinectes sapidus* (juvenile)	5.2
Waterflea *Daphnia magna*	0.10

Daphnia magna	1.7
Scud *Gammarus lacustris (mature)*	0.11
Stonefly *Classenia sabulosa (2nd year)*	8.2
Stonefly *Pteronacnarcys Californica (2nd year)*	50
Eastern Oyster *Crassostrea virginica* (shell-deposition)	84
Eastern Oyster *Crassostrea virginica* (embryo-larvae)	2,000

Source: US EPA 2002b

5.4 Assay for AChE activity

In the shrimp exposed to lethal concentration of chlorpyrifos (68.1 and 681 µg/l), it was clearly indicated that AChE activity from the gill of the shrimp decreased in corresponding to the increased concentrations of chlorpyrifos. The AChE activities observed in shrimp exposed to 68.1 and 681 µg/l of chlorpyrifos were significantly lower than that of control shrimp after 30 min of exposure. This result is similar to many studies reported earlier in various organisms including grass shrimp, *P. pugio* (Key and Fulton 2006) and worm, *Eisenia foetida*, (Rao et al. 2003). In grass shrimp larvae at 18-day-old, 50% inhibition of AChE activity was detected after 24-h exposure of chlorpyrifos at the concentration of 0.27 (0.21-0.35) µg/l. The mortality of the shrimp was also correlated with the inhibition of AChE activity (Key and Fulton, 2006). The inhibition of AChE activity of lethal concentration-exposed worm, *Eisenia foetida*, was found to increase from 62% to 91% after exposure to chlorpyrifos at the concentration of 0.063 µg/cm^2 for 12 to 24 h (Rao et al., 2003).

For sub-lethal exposure, the AChE activity of *P. monodon* decreased approximately 50% (from 4.15 to 2.12 nmol/min/mg protein) after exposing to chlorpyrifos at sub-lethal concentrations (0.681 µg/l) within 72 h when compared to control shrimp. This effective concentration was many times lower that the lethal levels while most studies on other aquatic invertebrates indicated that the effect of chlorpyrifos can be observed when AChE inhibition is at near-lethal levels (Fulton

and Key 2001). A variety of studies with estuarine fish have suggested that brain AChE inhibition levels of > 70% are associated with mortality in most species. Selected species, however, appear capable of tolerating much higher levels (> 90%) of brain inhibition. Sublethal effects on stamina have been reported for some estuarine fish in association with brain AChE inhibition levels as low as 50%. Most studies suggest, however, that these effects are observed only when brain AChE inhibition is at near-lethal levels. However, in asian clam, *Corbicula fluminea*, the cholinesterase (ChE) activity was reduced 58% and 42% of control levels during a 24-h exposure to sub-lethal concentration at 0.5 and 1 mg/l chlorpyrifos, respectively (Cooper and Bidwell, 2006).

The use of the inhibition of AChE activity as biomarkers for the determination of xenobiotic contamination has been increasingly adopted as part of the environmental monitoring program. Field study conducted at Vilaine River estuary, France, has used the colorimetric method (Ellman et al., 1961) to measure acetylcholinesterase activity in copepods (*Tigriopus brevicornis*) as biomarker for detecting the contamination of many neurotoxins. This included triazines, amids, oxadiazoles, piperidines, morpholines, phenylamids, oxazolidine derivative, and methylparathion. The inhibition levels of acetylcholinesterase activity from copepods in agricultural contaminated area were 70-80% higher than that of the reference site (Forget et al., 2003). The sensitive reduction of AChE activity in *P. monodon* at the sub-lethal concentration (0.681 µg/l) found in this study indicates the potential use as biomarker of chlorpyrifos exposure.

Several studies have shown that AChE inhibition in the animals still occurred days after the exposure had ended (Reddy and Rao 1988; McHenery *et al.* 1991; Abdullah *et al.* 1994). In channel catfish, *Ictalurus punctatus* and blue crab, *Callinectes Sapidus*, the effects persisted for several weeks after a single acute exposure to an organophosphorus compound, *S,S,S-tri-n-butyl* phosphorotrithioate (Habig *et al.* 1986). In *P. monodon*, the inhibition of AChE did not appear to persist after 96 h of exposure. This is relatively similar to the recovery time of *Daphnia magna* exposed to chlorpyrifos at severe level (24h- LC_{50}, 1.4 nM) which were between 24 and 96 h to achieve 50% and near complete recovery levels, respectively

(Barata et al., 2004). More experimental work is still needed to confirm the recovery period of acetylcholinesterase in this shrimp.

5.5 Single cell gel electrophoresis analysis (Comet assay)

The constant decrease of haemocyte viability in all treatments is correspondent to the increase concentration of chlorpyrifos, indicating the cytotoxic effect of chlorpyrifos on the haemocytes of *P. monodon*. Presently, there are very few literature reports on the assessment of *in vitro* cytotoxic effects of chlorpyrifos on any non-target aquatic invertebrates. A few numbers of studies have been reported on higher organisms. Harford et al. (2005) investigated the *in vitro* exposure of chlorpyrifos to the head kidney cells of 4 native Australian freshwater fish. The result of Murray cod (*Maccullochella peelii*) which was the most sensitive species among these fish indicated that viability of head kidney cells and the number of lymphocytes was reduced 50% and 15%, respectively after exposing to 10 mg/l of chlorpyrifos. Study *in vitro* of Guizzetti et al. (2005) on the effect of chlorpyrifos in human astrocytoma cell line 1321N1 cells revealed that about 11% of dead cells were observed after 24 h exposure with 17.53 mg/l (50 μM) of chlorpyrifos. Giordano et al. (2007) studied on the toxic effect of chlorpyrifos on neuronal cells of rat. The result indicated that the viability of the cells reduced almost 50% after exposing to the test chemical at approximately 35.057μg/l (0.1 μM) for 24 h. In the present study, the viability of *P. monodon* haemocyte reduced to 52.5 % after in vitro exposing to 0.170 μg/l of chlorpyrifos for 24 h. The viability result of the haemocytes was not so different from the result from other species but the effective concentration was much lower. By comparing with the result of *in vitro* cytotoxic effect of chlorpyrifos to other organisms, *P. monodon* haemocyte was many folds more sensitive than most cells from the species mentioned earlier. This is presumably because *P. monodon* is in the same phylum as insects which are the main target of chlorpyrifos. Therefore, it is more susceptible to the pesticide than higher animals.

Although the viability of the cells decreased in all treatments in responding to the increasing levels of chlorpyrifos, the declining of dead cells after 6 h of exposure can be noticed. This could be due to the repair mechanisms of the detoxifying enzymes which could bind or metabolize chlorpyrifos, thereby reducing the toxic

effect to the cells. The result of recovery time observed in this study is similar to a number of cyto- and genotoxicity investigations. Repair mechanism was observed on mice leucocytes after 48 h of chlorpyrifos exposure (Rahman et al., 2002). Similar results were also reported on various cell types from several organisms exposed to different pesticides. This includes the study on the different stages of the embryos of grass shrimp (*Palaemonetes pugio*) exposed to various concentrations of benzo[a]pyrene (BP), Cr (VI) and hydrogen peroxide (Giordano et al., 2007), repair of the leucocyte-damaged DNA in Swiss albino mice administered with chlorpyrifos at 0-8.96 mg/kg was also found after 48 to 96 h (Rahman et al., 2002), human lymphocytes (Błasiak et al., 1999), and monocrotophos in fish (Saleha et al., 2001) using comet assay.

In comparison to other organisms, most studies on the genotoxicity of chlorpyrifos have been tested *in vivo* in several organisms, including *Drosophila melunogaster* (Woodruff et al., 1983), mice (Rahman et al., 2002), and shrimp larvae (Reyes, et al., 2002). *In vitro* studies on DNA damage caused by pesticide on aquatic crustaceans are relatively scarce due to the difficulty on preparing appropriate media for maintaining cells in the test conditions. Chloryrifos was reported to be genotoxic substance in mice leucocytes (Rahman et al., 2002). The damage of DNA due to the pesticide could cause alterations in haemocyte DNA by phosphorylation (Wild, 1975). A similar study by Hartmann et al. (1995) also demonstrated significant DNA damage in human blood cells exposed *in vitro* to chloryrifos. A number of similar reports on *in vitro* genotoxicity of the pesticide using comet assay have been performed on cultured human lymphocytes (Betti et al., 1994; Anderson et al., 1998; Kassie et al., 2000; Jamil, 2001; Naravaneni and Jamil, 2005; Prabhavathy et al., 2006).

When comet assay is used for evaluating the genotoxicity, DNA fragmentation, occurred by non-genotoxic effect should be considered. In the event that a positive comet assay response is obtained, it is possible that the increase in migration is not associated with genotoxicity. As the result of viability in this study, approximately 75% or more of haemocytes were still viable within 6 h at the highest level of chlorpyrifos exposure (0.170 µg/l). By conducting the assay with the exposure time not longer than 6 h where the viability of haemocytes is still high, false positive during the comet assay in this study was limit. In general, treatment of cell

cultures with concentrations resulting in <70% relative viability were considered to be too cytotoxic and were not evaluated. This experimental condition was recommended by the International Workshop on Genotoxicity Test procedures to limit concentration of test compounds to ≥ 70% viability (Tice et al., 2000).

The potential problem of possible false positive cells due to excessive cytotoxicity arises from extensive DNA fragmentation upon cell death. In the Comet assay, the microscopic image resulting from necrotic or apoptotic cells appeared as comets with small or nonexistent head and large, diffuse tails (Olive et al., 1993; Fairbairn et al., 1996). These cells are commonly called ghost cells. It was shown for *in vitro* tests that such cells can be present upon treatment with cytotoxic, non-genotoxic agents (Hartmann and Speit, 1997; Henderson et al., 1998). However, different cells, or cell line, may behave differently. Data from *In vitro* studies suggest that false positive results due to cytotoxicity vary depending on cell type. This can be seen on the Comet assay of rat hepatocytes (Frei, 2001), V79 Chinese hamster cells (Hartmann et al., 2001), mouse colon cells (Roser et al., 2001), and L5178Y mouse lymphoma cells (Kiskinis et al., 2002) which excessive cytotoxicity did not cause positive results. In contrast, false positive results were reported in TK-6 cells (Frei, 2001), rat lymphocytes (Quintana et al., 2000) and Jurkat cells (Choucroun et al., 2001). In this study, the number of ghost cells found in the comet assay was not in correlation with the increasing levels of chlorpyrifos. The result support the notion that extensive DNA fragmentation induced by cytotoxicity does not lead to elevated DNA migration (false positive cells) when ghost cells are not considered in genotoxicity evaluation.

Scoring of comets can be conducted in several ways including the percentage of DNA in the comet tail, the length of the tail, and DNA tail moment (product of the fraction of DNA in the tail and tail length). Tail moment is considered to be one of the best indices of induced DNA damage among the various parameters (De Boeck et al., 2000). For the evaluation of DNA damage in this study, 2 parameters were monitored; tail length and tail moment. Significant results with regard to comet tail length and tail moment were observed with chlorpyrifos exposure over a range of concentrations from 1 to 6 h. Similar results were obtained from both parameters. Increases were found when measuring Comet tail length, but the greatest changes were in tail

moment which showed that the extent of DNA damage was proportional to the concentration of chlorpyrifos.

5.6 Isolation, characterization, and expression analysis of well-characterized and novel genes induced by chlorpyrifos in *P. monodon*

Molecular targets focused in this study have classified into 2 groups: 1) genes of *P. monodon* homologous to well-characterized genes reported in a number of organisms to be sensitive to chlorpyrifos exposure, and 2) novel and known genes differentially expressed in *P. monodon* after chlorpyrifos exposure.

Group I

Genes in the first group consists of cytochrome P450, beta glucuronidase, glutathione-s-transferase, acetylcholinesterase, vitellogenin, heat shock protein 70, and heat shock protein 90. Three important tissues, haemocyte, gill, and hepatopancreas, were chosen for determining the tissue distribution of these genes because these tissues are the main organs that initiate the defense mechanisms with the xenobiotics. Haemocyte of the shrimp is involved with many defense systems such as innate immunity and self non-self recognition mechanisms (Buethong, 2004). Gill is the first line of defense which always contacts to surrounding environment. It also involved in large numbers of exchanges and osmoregulations. Hepatopancreas functions in the same manner as liver and pancreas in higher animals. It is responsible for many enzyme productions and mechanisms. Detoxifications are carried out in or by enzymes secreting from hepatopancreas (Manisseril and Menon, 1995).

Esterase

Esterase activity has been successfully used for monitoring exposure to organophosphate in many organisms (Scaps et al., 1997; Escartin and Porte, 1996; Guilhermino et al., 1996). Esterase is a hydrolase enzyme that splits esters into an acid and an alcohol in a chemical reaction. When these two components are mixed with water, it is called hydrolysis. A wide range of different esterases exist that differ in substrate specificity, protein structure, and biological function.

Three types of esterase including acetylcholinesterase(AChE), butyrylcholinesterase (BChE), and carboxylesterases (CbE) were reported to be affected by pesticide. The main function of AChE is to hydrolyse acetylcholine at synapses in nerve transmission while BChE, of also called pseudoAChE, releases fatty acids from triacylglycerols, phospho- and sphingolipids. CbE hydrolyze short-chain fatty acids from xenobiotics and participate in their detoxication (Maxwell, 1992; Hyne and Maher, 2003; Vioque-Fern´andez et al., 2007). Cholinesterases (ChEs) are a ubiquitous class of serine hydrolases which physiologically remove acetylcholine from the synaptic cleft.

ChEs are widely distributed among vertebrate and invertebrate animals (Bocquene et al., 1997). In vertebrate two isoforms occur, acetylcholinesterase (AChE) which preferentially hydrolyses acetyl esters such as acetylcholine, and butyrylcholinesterase (BChE) which preferentially acts on butyrylcholine. ChEs are often highly polymorphic enzymes in invertebrates; only one gene encodes ChEs in insects, while three genes have been reported in nematodes (Massoulie et al., 1993). In mammals, however, acetylcholinesterase (AChE) was reported to present various molecular forms. This gene provided a large variety of transcripts depending on promoter usage (Meshorer et al. 2002) in combination with alternative splicing in the 3' region (Li et al. 1993).

Alternative acceptor sites of splicing in the 3' region leads to three different variants, including $AChE_R$ (readthrough), $AChE_H$ (hydrophobic), and $AChE_T$ (tailed), which possess the same catalytic domain but distinct C-terminal domains The $AChE_R$ variant results from the absence of splicing after the last catalytic exon and produces a non-amphiphilic monomeric (Rna1) enzyme, which normally represents a minor AChE species. The $AChE_H$ variant produces glycophosphatidylinositolanchored dimers, mostly found in blood cells. The $AChE_T$ variant terminates with a 40-residues peptide, which is highly conserved among vertebrates. $AChE_T$ subunits produce amphiphilic monomers (T1), homomeric dimers (T2) and tetramers (T4), as well as hetero-oligomers with collagen ColQ and with the transmembrane PRiMA (Proline Rich Membrane Anchor) protein. These diverse molecular forms have specific localizations and probably specific functions. In the muscle, the size of the T4-

PRiMA pool depends on the activity of the muscle and may exert a specific adaptive function, distinct from that of collagen-tailed forms (Jasmin and Gisiger 1990; Jasmin et al. 1991).

Carboxyesterase is another class of serine-dependent esterases which is related to AChE. It hydrolyzes a wide range of xenobiotic substrates. Carboxyesterase activity is present in a variety of tissues in vertebrate and invertebrate species (Liu et al., 1987). A number of studies have demonstrated that carboxyesterases also protect against the toxicity of organophosphorus pesticides (Maxwell, 1992).

P. monodon esterase enzyme found in this study shares distinctive motifs with a number of esterase enzymes including carboxylesterase, acetylcholinesterase, butyrylcholinesterase, and juvenile hormone esterase. Its full length putative sequence is identified with the close similarity to putative esterase of Tribolium castaneum (35 %), JH esterase of *Psacothea hilaris* (34%), and AChE of *Strongylocentrotus purpuratus* (35%) (see BLAST result in appendix C).

Based on the putative molecular structure, *P. monodon* esterase obtained from this study is more similar to CbE, and JH esterase than AChE. In crustaceans CbE has been reported to hydrolyze methyl farnesoate, a molt regulating hormone (Homola and Chang, 1997). Similar role might be applied to esterase found in *P. monodon*. More information such as genomic and proteomic as well as specific function of this enzyme is needed for the classification.

In this study, the result of tissue distribution revealed that the expression of esterase in *P. monodon* was found in haemocyte and gill but not in hepatopancreas. Wheelock (2005) studied the carboxylesterase and AChE activity in juvenile Chinook salmon (Oncorhynchus tshawytscha) following exposure to chlorpyrifos. High doses of chlorpyrifos (100 microg/l) was acutely toxic, causing 100% mortality within 96 h. Exposure to chlorpyrifos at a high dose (7.3 microg/l), but not a low dose (1.2 microg/l), significantly inhibited AChE activity in both brain and muscle tissue (85% and 92% inhibition, respectively). In contrast, liver carboxylesterase activity was significantly inhibited at both the low and high chlorpyrifos dose exposure (56% and 79% inhibition, respectively). In bivalve mussel species, CbEs displayed a greater

sensitivity than ChEs to inhibition by organophosphorus pesticides exposure in bivalve mussel species (Ozretic and Krajnovic-Ozretic, 1992; Escartin and Porte, 1997; Basack et al., 1998). Dose and time dependent exposure of chlorpyrifos through skin of earthworm (*Eisenia foetida*) resulted in inhibition of AChE activity at 0.0158 µg/l for 12 h (Rao et al., 2003).

Cytochromes P450

Cytochromes P450 are a super family of monooxygenase enzymes with the major function to detoxify xenobiotic substances. There have been reports about induction of cytochrome P450 by xenobiotics and usage of the enzymes as biomarker (Fossi et al., 1998; David et al. 2003; Rewitz et al., 2003; Dauphin-Villemant et al., 1999). Chorpyrifos has been reported to affect the cytochrome P450 level. Preweanling rat cytochromeP450 was activated by chlorpyrifos and the metabolic capacity related to the increasing of age (Timchalk et al., 2006). Examination of *cytochrome P450* induction in German Cockroach *Blattella germanica* exposed to residue 2.6 ug/cm^2 chlorpyrifos for 72 hr indicate the increase in total cytochrome P450 (Scharf et al., 1998). Cytochrome P450 induction of *Daphnia pulex* was reported to be a sensitive biomarker of polyphenol rich area in the subalpine temporary aquatic habitats in France. Using semiquantitative PCR followed by southern blot hybridization, the expression level of *CYP4C32* of animal from polyphenol-poor habitat was 6 time higher than that from polyphenol-rich habitat (David et al., 2003).

Cytochrome P450 of *P. monodon* was present in both haemocyte and hepatopancreas but not in gill. In this study, 2 forms of Cytochrome P450 were isolated from *P. monodon* by degenerate PCR and mRND DDRT-PCR.

Cytochrome P450 obtained from degenerate PCR provided nucleotide sequence that was similar to CYP4C39 reported in *Carcinus maenas* while product from mRND DDRT-PCR (UBC119A-650-F-5) provide nucleotide sequence that was similar to *CYP330A1* reported in *Carcinus maenas* enzyme ($3x10^{-53}$).

Rewitz, et al. (2003) revealed induction of *CYP330A1* mRNA level in green crab (recently moulted) *Carcinus maenas* using Northern blot analysis. Crabs Receiving ecdysteroids including ponasterone (3.8 µg/individual) and ecdysone (10 µg/individual) and xenobiotics, including phenobarbital (0.04mg/individual) and benzo(a)pyrene (1.2 mg/individual) showed significant induction of *CYP330A1* mRNA levels. However, moult stage was involved with in induction of *CYP330A1*. Induction of mRNA level of *CYP330A1* found in green crab receiving ecdysone (10 µg/individual) and phenobarbital (0.04mg/individual) was not detected in red crab (having moulted less recently than green crab). For *CYP4C39*, induction was not detected using the same condition.

The result from this study show no evidence of induction of both nucleotide sequences of *CYP3301* and *CYP4C39* gene homologue in hepatopancreas of *P. monodon*. Although CYP4 enzymes in insect was believed to involve in detoxification process (Rewitz, et al. 2003), for example in *CYP4D10* of Drosophilla with 17.4-fold over expression from isoquinoline alkaloids exposure and *CYP4G8* of *Helicoverpa armigera* with 2-fold over expression found in a pyrethroid-resistant strain, various *CYP4* were not expressed in the same direction (*CYP4S2*, *CYP4S1*, *CYP4G9*, *CYP4G10*, and *CYP4M4*) (Pittendrigh et al., 1997; Danielson, et al., 1998).

Glutathione-s-transferase

Glutathione-s-transferase plays a key role in the detoxification of carcinogens, therapeutic drugs, and products of oxidative stress substances. It acts by catalyzing the reaction of glutathione with an acceptor molecule to form an S-substituted glutathione (Medicine Net Inc., 2006).

Glutathione-s-transferase activity has been evaluated as biomarker for organophosphorus pesticide in Tasmanian lacewing (*Micromus tasmaniae*). Glutathione-s-transferase activity was reduced by exposure to chlorpyrifos led to a significant reduction compared to the water controls (Hodge et al., 2000). Chlorpyrifos was also found to inhibited glutathione-s-transferase expression in *Boophilus microplus* (Vaz et al., 2004). Rat received oral chlorpyrifos at the concentration of 13.5 mg/kg body weight for 8 weeks shown a significant inhibition of liver glutathione-*S*-transferase activities (Goel, et al. 2005). Chlorpyrifos decreased

glutathione-*S*-transferase activities of amphipod *Hyalella azteca* after exposed to 0.4 nM for 48 hr (Steevens and Benson, 1999).

The result indicated that glutathione-s-transferase of *P. monodon* was expressed mainly in hepatopancreas. Glutathione-s-transferase obtained from RACE-PCR provided nucleotide sequence that was similar to glutathione-s-transferase 1-1 (GST class-theta) reported in *Tribolium castaneum* (1e-67). From the study of Fu and Xie (2006) showed that glutathione-s-transferase (theta) mRNA expression was not changed after intraperitoneal injection with microcystinsa, a hepatotoxcin (100 μg /kg body weight). In tarnished plant bug, however, *Lygus lineolaris*, total glutathione-s-transferase mRNA expression levels were compared between malathion susceptible strain and resistant strain using real-time PCR. Results showed that the resistant strain had 1.3-fold higher glutathione-s-transferase expression levels than the susceptible strain (Zhu et al., 2007).

Beta glucuronidase

Beta glucuronidase was reported to be a very sensitive biomarker of acute organophosphorus insecticide exposure. After administrated by chlorpyrifos, plasma glucuronidase activity increased in approximately 100-fold the control level in rats (Fujigawa et al. 2005). In *P. monodon*, it was found that the expression of this enzyme could be obtained in all 3 main tissues, however, the expression level in hepatopancreas was not induced by low level chlorpyrifos exposure.

Heat shock protein

The expression levels of heat shock protein 70 and heat shock protein 90 were investigated in the chlorpyrifos-exposed shrimp. The presences of both genes were detected in all 3 tissues of the shrimp indicated the potential use of these tissue for study of gene expression. However, the induction of these 2 genes in hepatopancreas shrimp exposed to chlorpyrifos was not detectable. Gill and haemocyte would be the alternative target for the study of gene expression.

Synthesis of heat shock protein 90 was enhanced by chlorpyrifos in cultured neuroactive PC-12 cells of female rats after 24 h of exposure to 50 mM chlorpyrifos (Bagchi et al., 1996). Expression of heat shock protein 70 in the third-instar larval

tissues of transgenic *Drosophila melanogaster* (heat shock protein 70 -*lacZ*) following dietary exposure to chlorpyrifos for various time intervals was investigated. A significant increase (61%) in heat shock protein 70 expression was observed in the higher concentration of the toxicant after 6 h exposure when compared with that of the lowest dose.

Vitellogenin

Organochlorine pesticide e.g. DDT, and metabolites, chlordecone, methoxychlor has been recognized as endocrine-disrupting, based on their ability to bind with estrogen receptors and leaded to the expression of vitellogenin (Mills and Chichester, 2005). However, report on the endocrine disruption and induction of vitellogenin by organophosphorus pesticide was not found. To examine the induction of vitellogenin by chlorpyrifos at the concentration of 0-27.24 µg/l, the positive result was not detected. The study from this part can be concluded that at the experimental concentration of 0-27.24 µg/l chlorpyrifos did not showed xenoestrogenic property to the shrimp. However, the result indicated that, at the juvenile stage, the gene was mainly expressed in hepatopancreas in comparison with haemocyte and gill.

Group II

Genes from the second group consists of the genes obtained from the differential display analysis in the hepatopancreas of chlorpyrifos-exposed shrimp. A total of 44 differential displayed transcripts, including 20 up-regulated and 24 down-regulated PCR products were obtained. Twenty-two transcripts were identified as known genes (16 up-regulated and 6 down-regulated) and 22 were unknown genes (8 up-regulated and 14 down-regulated). Five of the known genes (*CYP330A1*, esterase, LDL receptor member LR3, ubiquitin-like-7, and leucine zipper protein 5) and one of unknown gene were primarily chosen for the analyses of tissue distribution and the transcriptional levels in chlorpyrifos-exposed shrimp. Results showed expression of the genes in hepatopancreas of shrimp.

Similar results were obtained from the expression of the genes in this group. The absence of differences in transcriptional levels of these genes between controls and treatments in this study might be associated with the relatively low concentrations of chlorpyrifos used.

In addition, the effect of chlorpyrifos exposure at the translational level was needed due to there are evidences on some genes e.g. acetylcholinesterase, cytochrome P450 aromatase that were regulated at the translational level.

For example, after exposing neuroblasma cells of rat to $1x10^{-10}$M and $5x10^{-10}$M soman, an acetylcholinesterase irreversible inhibitor, for 2 h, there was the detectable of the acetylcholinesterase activity reduction ($P<0.05$). However, the difference of gene expression analyzed using RT-PCR was not detected (Perrier et al., 2005).

For cytochrome P450 aromatase (*CYP19*), the enzyme activity was significantly increased 2.4-fold compared to control after exposed to 50 ng/l of ethynylestradiol for 7 days but the induction of gene expression was not detected using real-time PCR (Lyssimachou et al., 2006).

5.7 Application of acetylcholinesterase activity as biomarker of chlorpyrifos exposure

Field study conducted at Vilaine River estuary, France, reported the use of acetylcholinesterase activity in copepods (*Tigriopus brevicornis*) as biomarker for detecting the contamination of triazines, amids, oxadiazoles, piperidines, morpholines, phenylamids, oxazolidine derivative, and methylparathion. Correlation between inhibition of enzyme activity and atrazine concentration was performed due to atrazine (triazine) was detected as the highest level contaminant. The result showed that enzyme activity of copepods inhabited the area contaminated with 170 ng/l atrazine was 80% lower than that of the copepod inhabited the area contaminated with 23 ng/l atrazine (Reference site) (Forget et al., 2003).

The use of acetylcholinesterase activity as biomarker was also reported in fish species. *In situ* exposure of rainbow trout, *Oncorhynchus mykiss* to contaminated area of Lourens River, South Africa with 0.01 and 74.4 µg/l chlorpyrifos for water and suspended particle and 0.14 and 57.9 µg/l azinphos-methyl for water and suspended particle resulted in the 40% reduction of the enzyme activity in comparison with that

of the fish exposed to the uncontaminated area (the pesticide can not be detected) (Sturm et al., 2007).

The result of this study showed that the inhibition of acetylcholinesterase activity was clearly detectable in shrimp exposed to very low concentration of chlorpyrifos (0.681 µg/l). In terms of environmental monitoring and risk assessment, the high sensitivities of acetylcholinesterase activity to the effects of chlorpyrifos found in *P.monodon* indicate a potential used of the enzyme as biomarker of chlorpyrifos exposure.

From the lethal concentration reports, the results showed different levels of the lethal concentrations of chlorpyrifos among species of organisms. The 72 h LC_{50} value of *P. monodon* was 23.64 µg/l which was much more sensitive than Nile tilapia (*Oreochromis niloticus*), an economic fish species found to inhabit natural water of Thailand, of which the 72 h LC_{50} was 151 µg/l (Herzberg, 1987). This can be suggested that the determination of maximum contamination level and pesticide zoning program should included data from several species to prevent error from deviation among species.

Based on the reports of pesticide contamination in surface water of Thailand which revealed that chlorpyrifos concentrations were found between 0.02-1.5 µg/l (Nayavon, 1996; Ciglasch 2003), the shrimp cultured in the contaminated areas can be at risk. In USA, the maximum contaminant level of chlorpyrifos allowed in freshwater and saltwater was generated by US EPA (1986) as criteria maximum concentration (CMC) and criteria continuous concentration (CCC). The CMC and CCC of chlorpyrifos were 0.083 and 0.041 µg/l, respectively for freshwater and were 0.011 and 0.0056 µg/l, respectively for saltwater. According to the information from the department of water standard for surface water from pollution control department, maximum contaminant level or maximum permissible level of a contaminant in water which is delivered to any user of a public water system for organophosphorus pesticide in Thailand has not been established. The effects of chlorpyrifos obtained from this study will provide basic information that can be used as criteria for pesticide controlling program in Thailand and for shrimp farming management.