Chapter 4

GENERAL DISCUSSION

4.0 General discussion.

OPs are currently among the most commonly used insecticides. OPs have been replacing organochlorine insecticides as they are less persistent in the various environmental compartments (Eto, 1974). However, due to their toxic effects routine applications of OPs may adversely affect nontarget organisms.

Animals have been exposed to plant and microbial secondary metabolites which they cannot metabolize or utilize for their own life processes. Therefore, enzyme systems in animals have evolved to enable detoxication and removal of xenobiotic compounds from the body. In the 20th century, more xenobiotic compounds have been produced as a result of exploitation of petroleum and the ultimate basin for many of them is the aquatic environment (George, 1994).

The present study looks into the effects of OP insecticides on a nontarget aquatic species, *T. trichopterus* which is a commonly found fresh water fish in Malaysia.

In this research, AChE activity in brain and EROD activities in liver of fish were examined when the fish were subjected to sublethal concentrations of the insecticide for 28 days. The recovery of these enzymes were also examined upon 24 hr exposure followed by transfer into clean water for 21 days. Finally a pulse exposure experiment in which fish were exposed to the insecticides with intermittent 21 days recovery period, were correlated with the activity of AChE and EROD activity.

The I_{50} values for chlorpyrifos and diazinon were found to be 3.9×10^{-1} M and 4.6×10^{-4} M respectively; clearly an indication of the greater AChE inhibiting potential

of diazinon compared to chlorpyrifos.

Brain AChE inhibition in Tilapia was observed when subjected to as low as 0.25 mg/l dichlorvos which indicated the sensitivity of the species to OPs (Rath and Misra, 1981). The same study showed that the degree of enzyme inhibition was positively correlated with insecticide concentration and the period of exposure. At the end of 15 days exposure, fish exposed to 1.25 mg/l dichlorvos exhibited a maximum enzyme inhibition of 68%.

Rath and Misra (1981) also demonstrated that fish brain exhibited a higher degree of the AChE enzyme inhibition compared to than in the liver. Thus the extent of environmental toxicity by OPs could be assessed by measurable change of AChE activity in the brain.

In the semistatic exposure experiment, the AChE activity inhibition of the test species exposed to chlorpyrifos increased with increasing concentrations of the insecticide. It was also observed that the AChE inhibition increased with period of exposure. After 3 days of exposure to chlorpyrifos, test species exposed to 1.0 mg/l of the insecticide showed about 60% inhibition compared to control and remained significantly inhibited throughout the experiment while fish exposed to 0.1 mg/l chlorpyrifos exhibited 24% and 48% AChE inhibition compared to control after 3 and 14 days exposure respectively. At the end of the experiment (28 days), AChE inhibition was 67%.

On the other hand, this trend was observed only in the test species exposed to diazinon at a concentration of 1.0 mg/l. After 3 days exposure to 1.0 mg/l diazinon, test species exhibited 36% AChE activity inhibition compared to control and remained

significantly inhibited throughout the experiment. However, test species exposed to concentrations lower than 1.0 mg/l did not show AChE inhibition. The reduction of AChE activity observed in the test species after 7 days exposure to the lower concentrations was not significant. More over, after 28 days exposure to 0.01 mg/l and 0.001 mg/l diazinon, test species exhibited about 45% higher AChE activity compared to control.

The immediate effect of high concentrations of insecticide as observed above has also been reported in previous studies. In a study on carp, Balint *et al.* (1995) reported that carp exposed to 2 mg/l methidathion for 5 days resulted in more than 80% decrease in brain AChE activity. Manila clams exposed to 1.0 and 0.1 mg/l dichlorvos for 6hr exhibited 70% and 40% AChE inhibition respectively (Le Bris *et al.*, 1995).

In the present study, test species also exhibited stimulating effects of AChE activity in fish exposed to 0.01 mg/l and 0.001 mg/l chlorpyrifos as well as 0.01 mg/l and 0.1 mg/l diazinon. Higher AChE (10% - 25% higher than control) activities were observed at these concentrations in the initial 7 days exposure.

Stimulating effects observed in AChE activity due to initial exposure to insecticides was reported by Moulton *et al.* (1996). In their experiment on freshwater mussel, test species were exposed to acephate at various concentrations (0- 10 mg/l) for 96 hr. ChE activity was significantly inhibited at acephate concentrations of $\geq$ 1.3 mg/l. 94-96% depression of ChE activity compared to control was observed after 96 hr but concentrations of 0.01 mg/l and 0.1 mg/l of acephate seemed to stimulate ChE activity (60% - 74% higher than control). In the present study, AChE activity at 74%

higher than control was only observed in test species exposed to 0.01 mg/l diazinon after 21 days exposure. This activity might had been attributed to synthesis of new enzyme.

Based on the semistatic exposure experiment, in which a concentration of 1.0 mg/l was not found to be lethal to the test species for an exposure period of 28 days, this concentration was chosen in the recovery experiment. The initial 24 hr exposure to 1.0 mg/l chlorpyrifos resulted in 65% AChE inhibition compared to control. Following 24 hr in clean water, a recovery of about 10% AChE activity was observed. Similarly, after 24 hr exposure to 1.0 mg/l diazinon, AChE activity in test species exhibited about 54% inhibition compared to control. A 20% recovery in AChE activity which approached that of control fish was observed after 24 hr in clean water. However, unlike exposure to chlorpyrifos, subsequent samplings showed that AChE activity in test species exposed to diazinon was observed to be inhibited. The different period of recovery exhibited by the test species exposed to different insecticides were also observed in previous studies.

Mussells exposed to 5 mg/l acephate for 96hr did not exhibit recovery of ChE activity until days 12 to 24 (Moulton *et al.*, 1996). Nile tilapia exposed to 1 mg/l monocrotophos for 96 hr recovered 95% of AChE activity after 4 weeks transfer to toxicant-free water (Thangnipon, 1995).

Based on the result of the recovery experiment, the pulse exposure experiment adapted 21 days as the intermittent recovery period for the test species.

The initial 24 hr exposure to 1 mg/l chlorpyrifos resulted in a 46% inhibition of brain AChE activity compared to control at 1.85 mM/hr/mg. The second 24 hr

exposure caused a 51% AChE inhibition compared to control while the third exposure caused 63% AChE inhibition compared to control. The decrease in AChE activity following the 2nd and 3rd 24 hr exposures to the insecticide suggested that previous exposure to chlorpyrifos affected the sensitivity of fish to subsequent exposure to the same insecticide since a complete recovery was observed at the end of each 21 day stay in clean water.

The initial 24 hr exposure to 1 mg/l diazinon resulted in a 35% AChE activity inhibition compared to control. The second 24 hr exposure however, resulted in a 26% AChE inhibition compared to control while the third 24 hr exposure exhibited 44% AChE inhibition compared to control. However, rate of recovery of AChE activity during both the 21 d recovery phase were similar, indicating that the second exposure to diazinon did not influence the recovery period of AChE.

In both experiments using the two different insecticides, it was observed that previous exposures to insecticide was followed by a period of almost complete recovery at the end of 21 days. Higher AChE activity observed during the second recovery period might have been caused by the synthesis of new enzyme after the first exposure to insecticide which was not completely inhibited by the second exposure. As a result, the new enzyme synthesized due to the second exposure, together with the existing enzyme produced higher AChE activity.

The cytochrome P-450 system in fish, though less diverged than the mammalian counterpart, shows significant similarities with that in mammals and has been described in detailed in recent reviews (Andersson and Forlin, 1992; Goksoyr and Forlin, 1992; Stegeman, 1993; Stegeman and Hanh, 1994). Description of P-450

systems in fish has focused primarily on the liver, for the simple reason that the organ is the primary site of xenobiotic metabolism and elimination (Stegeman and Hahn, 1994). Previous studies showed that MFO activity could be measured as EROD activity (Palace *et al.*, 1996). EROD activity is most commonly associated with forms of cytochrome P-450 which are induced by 3-MC and other similar inducers (Klotz *et al.*, 1984).

The semistatic exposure experiment in which test species were exposed to various concentrations of insecticide clearly demonstrated the induction of EROD activities in fish liver. However, the levels of induction were observed to be dependent on the concentration of the insecticide though higher concentration did not necessarily result in higher EROD activity induction.

In the semistatic exposure experiment, it was observed that after 3 days of exposure to 1.0 mg/l chlorpyrifos, test species exhibited about the same EROD activity as control fish. The highest EROD activity following 3 days exposure was observed in fish exposed to 0.1 mg/l insecticide which was 3.2 times higher than control.

At the end of 7 days exposure, test species exposed to 0.01 mg/l chlorpyrifos exhibited approximately 5 fold increase in EROD activity which was also the highest observed during this experiment. After 14 days exposure, the highest EROD activity was observed in test species exposed to 0.1 mg/l chlorpyrifos which exhibited a 42% increase in EROD activity.

At the end of 21 days exposure, EROD activities in test species exposed to 1.0 mg/l chlorpyrifos remained the lowest. After 28 days exposure test species exposed to 0.01 mg/l chlorpyrifos exhibited the highest EROD activity which was 1.6 times higher

than control followed by test species exposed to 0.001 mg/l chlorpyrifos which exhibited 1.5 times higher EROD activity compared to control.

Fish exposed to 0.1 mg/l diazinon exhibited the highest EROD activity at approximately 3.4 times higher than control at the end of 3 days exposure.

At the end of 7 days exposure fish exposed to 0.1 mg/l diazinon exhibited a 64% reduction in EROD activity, approaching that of control while test species exposed to 0.01 mg/l diazinon exhibited 1.6 fold increase in EROD activity.

After 14 days exposure, test species exposed to 0.01 mg/l diazinon exhibited the highest EROD activity at 31% higher than control while test species exposed to 0.1 mg/l diazinon exhibited further 28% reduction in EROD activity

At the end of 21 days exposure EROD activity in test species exposed to 0.01 mg/l diazinon remained to be the highest at about 13 times higher than control. However, at the end of 28 days exposure, test species exposed to concentrations 0.01 and 0.1 mg/l diazinon exhibited 65% and 38% reduction in EROD activities respectively but still remained higher than control.

In the semistatic exposure to chlorpyrifos, it was observed that after 7 days exposure to the insecticide, test species in all concentrations exhibited higher EROD activity compared to control. However in subsequent samplings, test species exhibited fluctuating EROD activities. At the end of the 21 days of the experiment only test species exposed to 1.0 mg/l chlorpyrifos exhibited low EROD activity, at about 56% less than control.

A similar pattern was also noted with diazinon. However, only the test species exposed to 1.0 mg/l diazinon exhibited low EROD activity throughout the period of

exposure with even the highest activity observed being 13% lower than control. Induction of EROD activity was observed in test species exposed to other concentrations as early as 3 days exposure.

In both experiments, it was observed that high EROD activities in fish liver induced by exposure to 0.01 mg/l chlorpyrifos and 0.1 mg/l diazinon were also followed by significant reductions in EROD activities.

Toxicity is dependent not only on dose and duration of exposure, but also on the route by which exposure occurs (Bloomquist, 1992). The present study used organophosphate insecticides to induce EROD activity in *T. trichopterus*.

There appears to be a paucity of research into the induction of EROD activity as a reaction to OP insecticides. However recent study by Escartin and Porte (1996) involving fenitrothion showed that EROD activity in *P. clarkii* exposed to 20 µg/l fenitrothion increased 5.2 fold after 48 hr exposure. In the present study some amount of induction was observed. However, the induction appeared to be unrelated to the concentration of the chemical or the length of exposure.

In addition to using other inducers such as 3-methylcolanthrene (3-MC) and BNF, most toxicity studies also used i.p injection as a mean of exposure. The present study however, looked at EROD activity in fish exposed to insecticides, introduced in the aqueous medium.

In a study by Arinc and Sen (1994), benzo(a)pyrene which is a member of PAHs, was injected into gilthead seabream at a rate of 25 mg/kg for 5 consecutive days. As a result, a 2 to 3 fold increase in EROD activity of the fish liver was observed. In another study by Murphy and Gooch (1997), channel catfish were given i.p injection

of benzo(a)pyrene at a rate of 20 mg/kg body weight. After 4 days, EROD activity in the fish liver averaged more than 5 times the control values.

Blue striped grunt subjected to a single i.p injection of 50 mg/kg body weight exhibited approximately 55 fold induction of EROD activity 3 days after treatment (Stegeman *et al.*, 1997).

In the recovery experiment, the initial 24hr exposure to 1.0 mg/l chlorpyrifos resulted in 11 times higher EROD activity compared to control. At the end of 24 hr in clean water a further 21% increase in EROD activity in test species was observed which resulted in a higher activity than control by as much as 3 fold.

EROD activity in test species was 75% less than control after the initial 24 hr exposure to 1.0 mg/l diazinon. Following 24hr in clean water, a 8.5 times increase in EROD activity was observed. Results from the recovery of EROD activity after 24 hr exposure to 1 mg/l chlorpyrifos indicated the inability of the EROD activity in the test species to return to basal levels at the end of 21 days. However, test species subjected to diazinon clearly showed maximum EROD activity induction, which was followed by an abrupt reduction approaching that of control levels at the end of the recovery period.

It should be noted that the changes in EROD activities observed in this study was expressed in percent of EROD reduction in sampled fish based on control fish sampled on the same day. The zero values of EROD activity recorded might had been due to non detectable activity.

In the pulse exposure experiment, the initial exposure to 1 mg/l chlorpyrifos resulted in approximately 5 times higher EROD activity in the test species compared to

that of control. The second 24 hr exposure to chlorpyrifos resulted in only 28% increase in EROD activity which was about 89% of control.

It was observed that the second exposure to the insecticide did not seem to induce EROD activity in test species as did the first exposure and the subsequent recovery period did not appear to result in any significant differences in EROD activity level. These results showed that the EROD activity in test species was fully induced by the first exposure to chlorpyrifos and that subsequent exposures, even after allowing the activity to return to basal level, were not capable to induce further EROD activity.

A significantly different pattern of EROD activity was exhibited by test species exposed to diazinon after 24 hr in insecticide-free water. Induction of EROD activities were observed during the first 24 hr in clean water subsequent to further exposures to the insecticide. At the end of each 21 days recovery phase, the EROD activity returned to basal level.

The present study showed that prolonged exposure of the test species to the insecticides did not result in higher EROD activity induction, suggesting that prior exposure to the insecticides may be the reason for the non-inducibility observed. Previous studies by Celander and Forlin (1995) reported that prolonged exposure to PCB seemed to impair the inducibility of the CYP1A1 system in rainbow trout, in agreement with Wirgin *et al.* (1992) who proposed that prior exposure to PCB may be the reason for the non-inducibility observed in Atlantic tomcod collected from a PCB-contaminated area.

The results in the present study showed that there appeared to be no relationship between elevated liver EROD activity in fish and the inhibition of brain

AChE acitvity in the test species observed subsequent to exposure to the insecticides. Some insecticides must be metabolically activated in order to interact with their molecular targets; a prominent example is a class of organophosphorous insecticides, the phosphorothionates, which must be activated by cytochrome P-450 to their oxon (phosphate) metabolites to become potent anticholinesterases (Chambers and Carr, 1995). Escartin and Porte (1996) also reported that an important biotransformation pathway for OPs is oxidation by the cytochrome P-450 monooxygenase system into the corresponding oxon forms that are able to irreversibly inhibit the enzyme AChE.

In a previous study on guppies exposed to diazinon, Keizer *et al.* (1991) suggested that inhibition of AChE was afforded by the oxons which resulted from oxidative transformation of diazinon by MFO. A study on channel catfish by Strauss and Chambers (1995) also reported that chlorpyrifos can be metabolized to a very potent and effective ChE inhibitors. Therefore, the biotransformation reactions might play an important role in the bioconcentration and toxicology of the insecticides. However, the inducibility of *T. trichopterus* MFO system in the present study does not appear to indicate the level of exposure to chlorpyrifos and diazinon.

Escartin and Porte (1996) reported that *P. clarkii* exposed to 20 µg/l fenitrothion for 48 hr exhibited significant (38%) AChE inhibition on the first day of treatment and maximum (54%) inhibition was reached 2 days after the organisms were transferred to clean water. It was suggested that since fenitrothion is a poor AChE inhibitor, it could have been metabolically activated *in vivo* by the cytochrome P-450 monooxygenase system to the oxygen analog (fenitrooxon), which is a potent inhibitor. Biotransformation enzymes catalyzes the bioactivation of xenobiotics leading to the

production of metabolites more toxic than the parent compounds and some members of the cytochrome P-450 superfamily are particularly involved in toxication pathways (Monod *et al.*, 1996).

In a study by Chambers and Carr (1995), channel catfish were exposed to concentrations of parathion and chlorpyrifos, and their respective oxons which yielded greater than 90% brain AChE inhibition but not mortality. It was observed that the inhibition of brain AChE in catfish was more persistent following chlorpyrifos exposure than parathion.

The onset of EROD activity induction in the present study differ according to concentrations of the insecticides. Except for the test species exposed to 1.0 mg/l of the insecticide, which is also the highest concentration used in this study, test species exposed to other lower concentrations of insecticide exhibited higher EROD activity compared to control as early as 3 days after exposure to the insecticide. Though not fully induced, it should be noted that EROD activity might had been induced almost as soon as the test species were exposed to the insecticides. This is further supported by the results obtained from the recovery experiments in which test species exhibited significant EROD induction after only 24 hr in insecticide-free water subsequent to 24 hr exposure to the insecticides. Andersson (1985) reported that EROD activity in rainbow trout which were given i.p injection of BNF (100 mg/kg body weight) increased almost 170 fold compared to control. EROD activity in control fish was reported to be 0.022 ± 0.006 nmole/ min/ mg protein. Stegeman *et al.* (1981) reported that EROD activity in scup and trout given i.p injection of 3-MC (20 mg/kg fish) exhibited approximately 15 and 40 fold induction compared to control respectively, 36

hr after treatment.