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Cytochrome *P*450-dependent monooxygenase system mediated hydrocarbon metabolism and antioxidant enzyme responses in prawn, *Macrobrachium malcolmsonii*

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Abstract

We investigated the alteration of cytochrome *P*450-dependent monooxygenase enzymes and antioxidant enzymes in response to oil effluent in freshwater prawn, *Macrobrachium malcolmsonii*. The prawns were exposed to two sublethal (10% [0.91 ppt] and 25% [2.3 ppt] of 5-day median lethal concentration) concentrations of oil. After 30 days, treated prawns were transferred into untreated freshwater and depuration was followed for another 30 days. At 7-day intervals, hydrocarbons and detoxifying enzymes were analysed in the hepatopancreas. Accumulation of hydrocarbon in the tissues gradually increased when exposed to sublethal concentration of oil effluent associated with enhanced levels of cytochrome *P*450, NADPH cytochrome *c* reductase and cytochrome b_5. During depuration, the levels of accumulated hydrocarbons decreased due to the induction of these detoxifying enzymes. Oil derived hydrocarbon mediated oxyradical production would have occurred in *M. malcolmsonii*. This was confirmed by elevated levels of superoxide dismutase (SOD) and catalase (CAT). Thus, cytochrome *P*450-dependent monooxygenase enzymes and antioxidant enzymes in oil-exposed prawns demonstrate a well-established detoxifying mechanism in *M. malcolmsonii*.

Keywords: Cytochrome *P*450; Catalase; Glutathione-*S*-transferase; Glutathione peroxidase; NAD(P)H cytochrome *c* reductase; *Macrobrachium malcolmsonii*; Superoxide dismutase

1. Introduction

The increased exposure of contaminants in the aquatic environment has caused concern for the adverse effect of lipophilic compounds in the aquatic system. Uptake and accumulation of xenobiotics in the tissues of aquatic organisms occur from the sediment, contaminated water column and food chain (Livingstone et al., 1994) that cause deleterious effects. However, enzymatically, aquatic organisms possess some intrinsic detoxifying mechanisms to eliminate accumulated xenobiotics. In phase-I reaction, cytochrome *P*450 (CYP450) and its monooxygenase enzymes play a main role in the detoxification of xenobiotics (Livingstone, 1993; Arun and Subramanian, 2003). In phase II conjugation reaction, glutathione-*S*-transferases (GST) catalyse the conjugation of electrophilic substances with endogenously reduced glutathione and protect other nucleophilic centers such as protein and nucleic acids (George, 1994). During phase I metabolism, CYP and monooxygenase enzymes are involved in the generation of oxyradicals (Livingstone, 1991). In addition, studies have focused on contaminant-stimulated reactive oxygen species production and mechanism of oxidative damage in aquatic organisms (Livingstone, 2001).

Oxyradicals are capable of inducing oxidative tissue damage, lipid peroxidation, nucleic acid damage, enzyme inactivation and protein degradation (Borg and Schaich,

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1984). In order to prevent damage to cellular components, numerous enzymatic antioxidant defenses act to scavenge oxyradicals including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px). These biotransformation and antioxidant enzymes have been extensively studied in fishes (Goksøyr and Forlin, 1992; George, 1994; Arun and Subramanian, 2002) while fewer studies have been done with invertebrates (Livingstone, 1991; Lemaire and Livingstone, 1993; Livingstone and Goldfarb, 1998). In crustaceans, the principal activity of these enzymes, in response to the exposure to hydrocarbons, remains unexplained.

Therefore, cytochrome *P*450-dependent monooxygenase and antioxidant enzymes were measured in the hepatopancreas of freshwater prawn, *M. malcolmsonii*, following exposure to 0.91 and 2.3 ppt of oil effluent (10% and 25% of the 5-day LC_{50}) for 30 days. The aims of the study were to examine (1) alteration of biotransformation enzymes (CYP-dependent monooxygenase) and antioxidant enzymes in response to oil pollution and (2) whether these enzymes would serve as a biomarker to detect the accumulated hydrocarbons in this species.

2. Materials and methods

2.1. Animals

Subadult freshwater prawns, *M. malcolmsonii*, were collected from Cauvery River (Tiruchirappalli, India) and transported in aerated polyethylene bags to the laboratory. The prawns were acclimatized for 5 days before sampling and starved for 1 day prior to sacrifice.

2.2. Acute toxicity experiment

The aqueous oil effluent originated from the coal conversion plant turbine section and boiler units of Bharat Heavy Electrical Limited (BHEL), situated 14 km from Tiruchirappalli. It consisted mainly of aromatic hydrocarbons. Initial experiments were conducted to assess the minimum concentration of oil effluent to obtain maximum mortality, for *M. malcolmsonii*, over a 5-day exposure. After confirming the minimum concentration, ten *M. malcolmsonii* were placed in different tubs (each) and exposed to oil effluent, ranging from 4 ppt to 16 ppt for 5 days. In addition, a control was also maintained. The 5-day LC_{50} values with 95% confidence limits were calculated using National Crop Production Centre Technical Bulletin (1986) method.

2.3. Exposure experiment

Based on a 5-day LC_{50} value of oil effluent, sublethal concentration of 10% (0.9 ppt) and 25% (2.3 ppt) of LC_{50} were prepared and used for detoxification study. In this study, two sets of 10-l plastic tubs were used. In each tub, prawns were exposed to 0.9 or 2.3 ppt of oil. A control was also run simultaneously without the addition of oil. Every 7 days, prawns were sacrificed. After 30 days, the treated prawns were released into freshwater and a 30-day depuration study was conducted. Prawns were sampled at 7-day intervals. Two prawns were randomly chosen and removed from each of the two tubs ($n=4$) for dissection.

2.4. Detection of oil-derived hydrocarbons

Total hydrocarbons in oil and hepatopancreas were determined by the method of Law et al. (1988). Samples were extracted with hexane by using cylindrical glass separating funnel. The content of total hydrocarbon was measured with a florescence spectrophotometer (Ex=310 nm; Em=360 nm) using chrysene as a standard since the pure aromatic compound chrysene strongly absorbs UV fluorescence intensity at 310 nm. Results are expressed as μg/g tissue in terms of chrysene equivalents.

2.5. Biochemical analysis

Cytosol and microsomes were prepared according to Livingstone and Farrar (1984). All the preparation procedures were carried out at 4 °C. The pooled hepatopancreas (from four animals) was homogenized in 20 mM Tris–HCl pH 7.6 containing 0.25 M sucrose, 0.15 M KCl, 1 mM EDTA and 1 mM DTT. The homogenates were centrifuged at 500×*g* for 1 h. The pellets were discarded and supernatants were centrifuged at 12,000×*g* for 45 min. The supernatants were centrifuged at 100,000×*g* for 90 min and the resulting supernatant was used as the cytosolic fraction. Resuspension buffer consisting of 20 mM Tris–HCl, pH 7.6, 1 mM DTT and 1 mM EDTA was added into the remaining pellets. The resuspended pellets were used as a microsomal fraction.

Cytochrome *P*450-dependent monooxygenase enzymes were measured in microsomes. Antioxidant enzymes were measured in the cytosolic fraction as was suggested by Arun and Subramanian (1999). NADH and NAD(P)H cytochrome *c* reductase were measured by the reduction of cytochrome *c* at 550 nm (Shimakata et al., 1972). Total cytochrome *P*450 (CYP) content was measured by the carbon monoxide difference spectrum of sodium dithionate-reduced sample (Omura and Sato, 1964). Cytochrome b_5 content was measured by the reduction of NADH (Estabrook and Werringloer, 1978). GST activity was monitored by measuring the rate of conjugation of 1-chloro-2,4-dinitrobenzene with glutathione at 340 nm (Habig et al., 1974). The SOD assay was based on the auto-oxidation of epinephrine and measured at 340 nm (Sun and Zigman, 1978). CAT assay was based on the decomposition of H_2O_2 and measured by the decreased absorbance at 240 nm (Caliborne, 1985; Arun et al., 2003). GSH-Px assay was based on the reduction of hydroperoxides catalysed by GSH-Px using GSH (reduced glutathione) as a reducing agent. The activity was measured by coupling the oxidation of NADPH with reduction of GSSG (oxidised glutathione) catalysed by glutathione reductase (Lawrence and Burk, 1976; Arun et al., 1999). Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

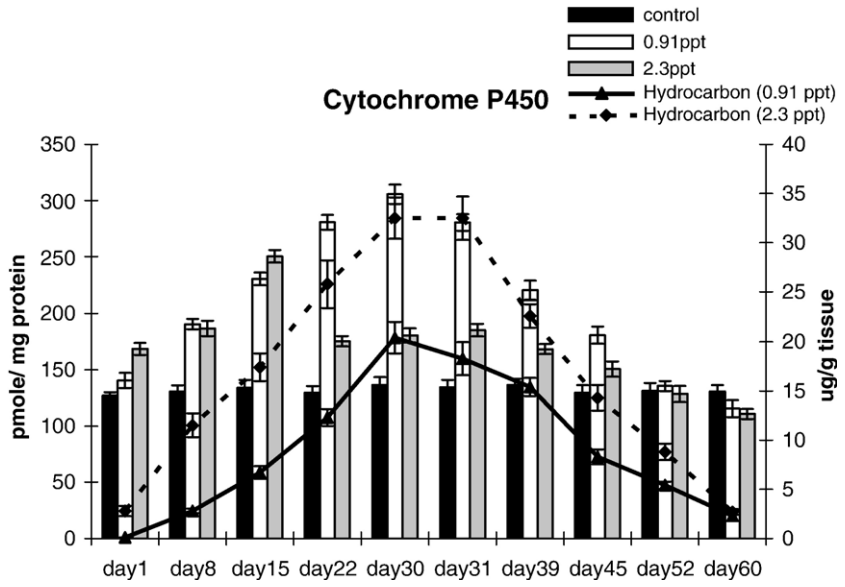

Fig. 1. Cytochrome *P*450 content in freshwater prawn, *M. malcolmsonii*, exposed to 0.91 and 2.3 ppt of oil effluent for a period of 30-day accumulation and 30-day depuration. Each value represents mean±S.E.M. for 4 determinations.

2.6. Statistical analysis

Values were presented as mean±S.E.M. and analysed by Student's *t*-test; $p<0.05$ was considered as statistically significant.

3. Results

3.1. Hydrocarbons

The 5-day median lethal concentration of oil effluent for the prawns was 9.12 ppt. Accumulation of hydrocarbons, in the hepatopancreas of prawns exposed to 0.9 and 2.3 ppt, increased gradually and attained maximum value at Day 30 (Fig. 1). Hydrocarbons accumulated on Day 30 were more than tenfold higher when compared to Day 1 or Day 8. During depuration phase, the hydrocarbon levels gradually decreased and attained a minimum value of 2.7±0.28 μg/g tissue and 2.3±0.52 μg/g tissue in the two groups on Day 60.

3.2. Cytochrome P450

Compared to control, the content of CYP increased by twofold on Day 15 on exposure to 2.3 ppt of oil effluent. Similarly CYP content was twofold higher on Day 22 when exposed to 0.91 ppt of oil effluent. Whereas the content of CYP decreased on Day 22 and Day 30 on exposure to 2.3 ppt of oil effluent when compared to Day 15 content. During depuration period, the content of CYP decreased gradually and reached a fairly constant level on Day 60 (Fig. 1).

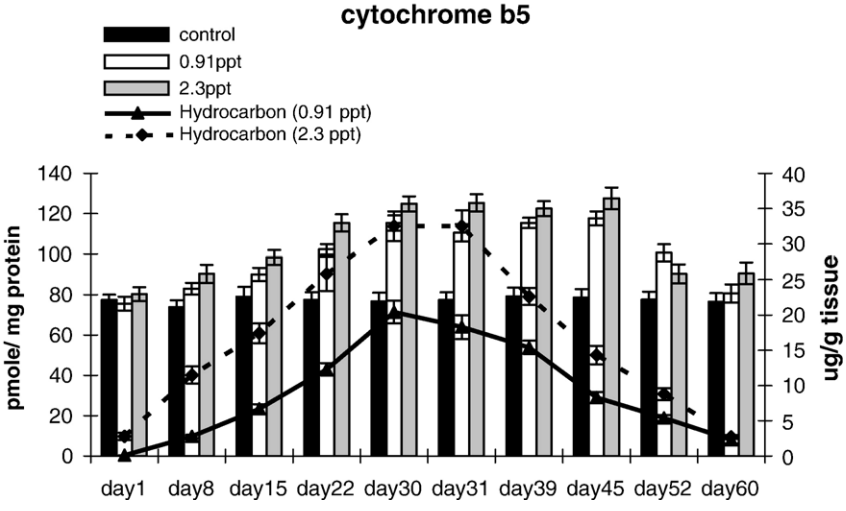

Fig. 2. Content of cytochrome b_5 in freshwater prawn, *M. malcolmsonii*, exposed to 0.91 and 2.3 ppt of oil effluent for a period of 30-day accumulation and 30-day depuration. Each value represents mean±S.E.M. for 4 determinations.

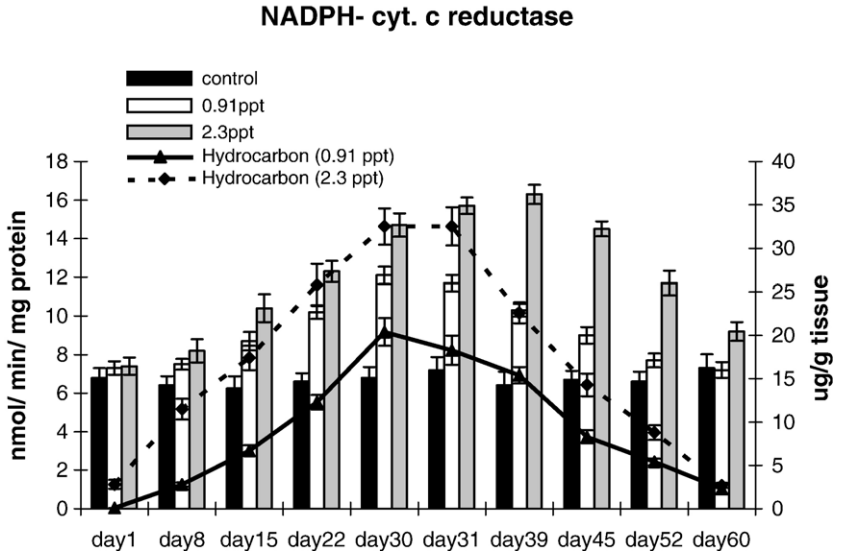

Fig. 3. NADPH cytochrome *c* reductase activity in freshwater prawn, *M. malcolmsonii*, exposed to 0.91 and 2.3 ppt of oil effluent for a period of 30-day accumulation and 30-day depuration. Each value represents mean±S.E.M. for 4 determinations.

3.3. Cytochrome b_5, NAD(P)H cytochrome c reductase and glutathione-S-transferases

When compared to control, the content of cytochrome b_5 and NADPH cytochrome *c* reductase increased significantly from Day 8, throughout the exposure period, on exposure to 0.91 ppt and 2.3 ppt of oil effluent. During the depuration phase, the contents decreased gradually and reached the control value (80.60±4.3 pmol/mg protein) on Day 30 in prawns which had been exposed to 0.91 ppt of oil extract (Figs. 2 and 3). No difference in the activity of NADH cytochrome *c* reductase was observed on exposure to both 0.91 ppt and 2.3 ppt of oil effluent. Significantly enhanced activity of GST was observed on Day 22 and Day 30 (Fig. 4).

3.4. Antioxidant enzymes

On exposure to oil effluent, SOD and CAT were increased several fold after Day 15. During depuration phase, no drop in both enzyme activities occurred on 2.3 ppt concentration. However, on 0.91 ppt concentration, SOD and CAT activities were slowly decreased from Day 45. No change in specific activity of Se-dep-GSH-Px level was observed on exposure to both concentrations of oil effluent. Similarly, during the depuration period, no significant change was measured (data not shown). In contrast, the activity of Se-indep-GSH-Px increased by 2 fold compared to the control level on Day 30. During the depuration period, the enhanced activity of Se-indep-GSH-Px was short-lived and the activity reached control level from Day 39 (Table 1).

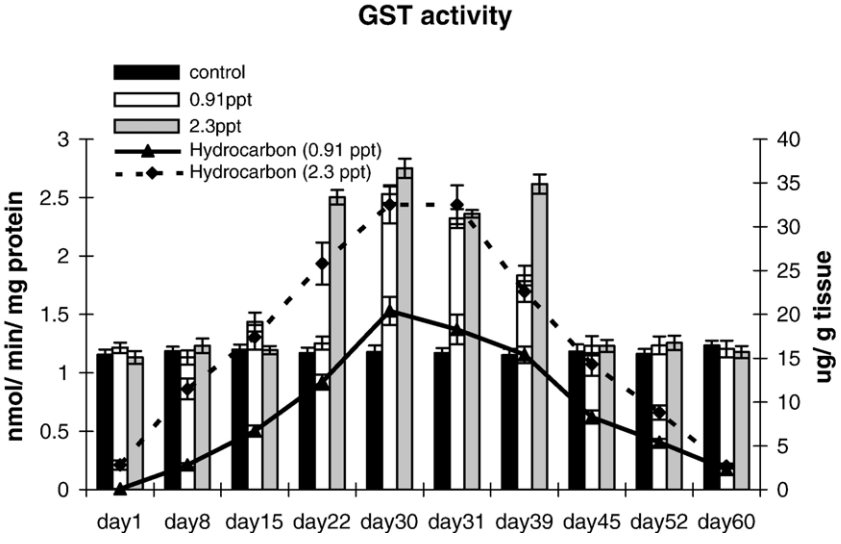

Fig. 4. Glutathione *S*-transferase activity in freshwater prawn, *M. malcolmsonii*, exposed to 0.91 and 2.3 ppt of oil effluent for a period of 30-day accumulation and 30-day depuration. Each value represents mean±S.E.M. for 4 determinations.

Table 1
Superoxide dismutase (SOD), catalase (CAT) and Se-independent glutathione peroxidase activities in freshwater prawn, *M. malcolmsonii*, exposed to 0.91 and 2.3 ppt of oil effluent for 30-day accumulation and 30-day depuration

		Day 1	Day 8	Day 15	Day 22	Day 30	Day 31	Day 38	Day 45	Day 52	Day 60
SOD (U/min/mg protein)	Control	32.68±0.68	33.28±0.85	33.88±0.73	32.15±0.85	31.85±0.66	33.64±0.50	34.12±0.76	32.68±0.64	30.95±0.77	31.48±0.65
	0.91 ppt	32.18±0.78	31.56±0.64	66.38±0.48	68.68±0.55	68.42±0.66	70.32±0.78	68.68±0.50	65.64±0.71	35.62±0.50	38.68±0.61
	2.3 ppt	30.49±0.75	32.54±0.73	74.68±0.79	96.34±0.77	110.48±0.84	96.48±0.66	98.68±0.66	64.75±0.55	65.38±0.66	63.84±0.56
CAT (μmol/min/mg protein)	Control	49.33±0.68	48.55±0.75	49.27±0.77	48.88±0.85	49.68±0.77	48.56±0.75	48.45±0.68	49.55±0.74	48.95±0.62	49.54±0.68
	0.91 ppt	47.82±1.2	50.28±1.35	105.68±0.95	110.28±1.15	108.63±0.95	115.32±0.88	112.68±0.72	112.75±0.85	47.68±0.82	48.24±0.55
	2.3 ppt	47.68±0.82	46.48±0.85	135.68±1.25	145.68±1.5	140.28±1.25	150.68±0.95	148.68±1.25	138.48±0.90	110.6±0.82	102.54±0.79
Se-indep-GPX (nmol/min/mg protein)	Control	9.3±0.15	9.45±0.23	9.28±0.17	9.38±0.10	9.38±0.15	9.35±0.25	9.45±0.28	9.35±0.17	9.35±0.2	9.37±0.15
	0.91 ppt	9.25±0.23	9.0±0.23	9.42±0.20	9.25±0.19	20.15±0.38	22.27±0.28	10.28±0.48	9.25±0.15	9.38±0.25	9.35±0.42
	2.3 ppt	9.58±0.12	9.25±0.18	9.47±0.21	9.15±0.20	30.5±0.38	28.54±0.32	9.21±0.25	9.0±0.20	9.45±0.18	9.37±0.25

Each value represents mean±S.E.M. for 4 determinations.

4. Discussion

Our data show that approximately 90% of the accumulated hydrocarbons are eliminated in 30 days. Similarly *Mytilus edulis* released 90% hydrocarbon after 35 days (Di Salvo et al., 1975) and hydrocarbons are undetectable after 45 days in PAH-exposed mussels (Dunn and Stich, 1976). Our results indicate that concentration of hydrocarbon, duration of hydrocarbon exposure and induction of CYP monooxygenase and antioxidant enzymes may influence patterns of accumulation and release of hydrocarbons in freshwater prawns.

Exposure of *M. malcolmsonii* to 0.91 ppt and 2.3 ppt sublethal concentrations of hydrocarbon-induced CYP. After exposure to 2.3 ppt of oil effluent, on Days 22 and 30, the content of CYP was lower than the Day 15 value. This could be attributed to the statement that high accumulation of hydrocarbons (more than 25.8 μg/g) in the tissue may impair the function of CYP or inhibit the content of CYP. Monosson and Stegeman (1994) also observed the inhibition of CYP content due to heavy accumulation of PCB in fish. During sublethal exposure, the uptake and accumulation of hydrocarbons were increased in the hepatopancreas of prawn. In addition, the CYP450 content also significantly ($p<0.05$) increased, in order to oxidise the accumulated hydrocarbon. This increased content of CYP was measured after 24 h, in prawn. Livingstone et al. (1985) also reported an increased level of CYP after 24 h of exposure to diesel oil. Similarly, Batel et al. (1988) measured an increased level of CYP in spiny crab when exposed to hydrocarbons. In prawn, the level of CYP was identified to increase during the exposure to oil effluent and returned to control levels on Day 60 of depuration phase. The accumulated hydrocarbons and induction of CYP in the hepatopancreas of prawns indicate that the response of the CYP is dose-dependent. Similar result was also obtained in *M. edulis* (Livingstone et al., 1985) that exerts an increased content of CYP after 24 h of exposure to hydrocarbons and returned to control concentration after 8 days of recovery.

An increased response of cytochrome b_5 content was associated with the increased accumulation of hydrocarbons. Maximum level of cytochrome b_5 was observed on Day 30, which was 1.5-fold greater than control level ($p<0.01$). Goksøyr et al. (1987) also measured an almost threefold higher cytochrome b_5 content in BNF-treated rainbow trout. Similarly Livingstone et al. (1985) also observed a twofold increase of cyt.b_5 in mussel when exposed to diesel oil. During the depuration period, prawns exert declined cyt.b_5 content along with the decreasing hydrocarbons and attained almost control level on Day 60. This decreased content of cytochrome b_5 may be due to the low detection of hydrocarbons. Livingstone et al. (1985) also observed similar findings in mussel, i.e., low accumulation of hydrocarbon induce low level of cyt.b_5 than high accumulation of hydrocarbon. In prawns, the activity of NADPH cytochrome *c* reductase was significantly increased ($p<0.05$) in all experimental days. Simultaneously during the recovery period, the activity was gradually decreased. Similar increase

in NADPH cytochrome *c* reductase activity of digestive gland has been observed with the exposure of Mytilids and gastropods to PAH and other hydrocarbons (Gilewicz et al., 1984; Livingstone et al., 1986; Woodin et al., 1997). In contrast to these results, there were no significant changes observed in NADH cytochrome *c* reductase in prawns when exposed to sublethal concentration of oil effluent. This result is in agreement with the notion that NADPH cytochrome *c* reductase is predominantly involved in donating electrons to cytochrome b_5 in prawn, rather than NADH cytochrome *c* reductase (Stegeman and Kaplan, 1981). Cytochrome b_5 donates electrons to CYP and the CYP involved in oxidation of hydrocarbons to produce reactive metabolites and/or water-soluble by-products. These reactive metabolites are toxic and further undergo phase II metabolism. Therefore, GST was analysed in prawns after exposure to 0.91 ppt and 2.3 ppt of oil effluent. No significant difference in GST activity was seen in prawn up to 15 days when exposed to 2.3 ppt of oil effluent.

An increased activity of GST observed in prawn on Day 30 may be due to high accumulation of hydrocarbons and subsequent higher induction of CYP-dependent mono-oxygenase enzymes in these organisms. Bioactivation of CYP450 would produce toxic reactive metabolites and hence to remove these metabolites higher GST would be produced on Day 30 in prawns. Similarly Woodin et al. (1997) observed the toxic consequences of reactive metabolites due to increased CYP in fish and suggested the increased GST may give protection from the toxic effect of reactive metabolites. During this biotransformation process, hydrocarbons such as quinones undergo cyclic univalent reduction by flavoprotein reductase, NAD(P)H cytochrome *c* reductase and produces $O_2^{\cdot-}$ (oxyradical). Similarly, the disturbance of electron flow between the flavoprotein reductase and cytochrome *P*450 by the induction of a xenobiotic compound produced the oxyradical (Lemaire and Livingstone, 1993). Antioxidant enzymes are involved in the removal of these oxyradicals. In prawns the activity of SOD was significantly ($p<0.001$) elevated upon 15 days of exposure to 0.91 ppt and 2.3 ppt of oil effluent. From the $O_2^{\cdot-}$-generating mechanism, it is known that high accumulation of hydrocarbons could mediate the generation of oxyradicals in prawn. Garcia Martinez and Livingstone (1995) also observed xenobiotic-stimulated oxyradical production in common mussel. Whereas, autooxidation of enhanced CYP and flavoprotein reductase would also be involved in the production of oxyradicals in freshwater prawns. Hence, the activity of SOD increased after 15 days to degenerate oxyradicals. Similarly *Limanda limanda* exerts an enhanced SOD activity after 80 days of exposure to PAH-contaminated sediments (Livingstone et al., 1993).

The activity of catalase in response to 0.91 and 2.3 ppt sublethal concentration of oil effluent was similar to the response of SOD. The enhanced activity of CAT suggests that CAT is mainly involved in the removal of H_2O_2 that was generated by superoxide dismutase. Hence the elevated trend of CAT was similar to superoxide dismutase pattern and this finding can be corroborated with the observation of Livingstone et al. (1993) who measured an enhanced SOD and CAT activity in limanda after 80 days of exposure to PAH-contaminated sediment.

The activity of Se-dep GSH-Px was not increased, but the activity of CAT was increased. Even though CAT and GSH-Px play a similar role in the removal of H_2O_2, CAT was thought to be more important in the removal of H_2O_2 (Arun and Subramanian, 1998). The induction response of GSH-Px was very small when compared to CAT exposed to oil effluent. Similar results were also observed in sea bass, dab and rainbow trout when exposed to various contaminated sediments (Lemaire et al., 1996; Livingstone et al., 1993).

Consequently from the above findings it was clearly revealed that, in freshwater prawns, microsomal electron transport component and monooxygenase enzymes (cytochrome b_5, NADPH-cytochrome *c* reductase and CYP) were involved in the removal of accumulated hydrocarbons. In addition, during depuration, alteration that occurred in these enzyme activities reflect the ability of detoxification. In freshwater prawn, during microsomal electron transport system, cytochrome b_5 received the electrons from NADPH-cytochrome *c* reductase and donates to CYP. Then this oxycytochrome *P*450 metabolises the hydrocarbons into their derivatives. This phase I metabolism was clearly noted in *M. malcolmsonii* by analyzing enhanced monooxygenase enzymes when exposed to sublethal concentration of oil effluent. Furthermore, phase II-metabolizing enzymes removed the metabolites of hydrocarbon. This reaction was confirmed by the increase of GST enzymes in *M. malcolmsonii* after 22 days of exposure to oil effluent, respectively. Xenobiotic-mediated oxyradical production also occurred in *M. malcolmsonii*. During phase I metabolism, autooxidation of CYP and flavoprotein reductase may generate oxyradicals in freshwater prawns. This was confirmed by measuring elevated level of SOD and CAT ($p<0.001$). No significant change occurred in Se-GSH-Px activity when exposed to oil effluent. This result would reveal that CAT enzyme alone was involved in the removal of H_2O_2. A marked increase in Se-independent GSH-Px activity was observed at 30-day exposure to oil effluent. This result indicates the enhanced activity involved in the removal of organic hydroperoxides in freshwater prawns. Thus, by analyzing phase I, phase II and antioxidant enzymes in oil-exposed prawns, the detoxification mechanisms were easily predictable in *M. malcolmsonii*. In addition, the enhanced activity of biotransformation enzymes in response to the exposure to oil effluent could be used as a biomarker for the early detection of oil pollution. The elevated level of antioxidant enzymes indicates the occurrence of reactive oxygen species that are generated from phase I metabolism. Thus, antioxidant enzymes could also be used as a biomarker for the detection of reactive oxygen species in freshwater prawn, *M. malcolmsonii*.

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