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Metabolic Enzymes as Biochemical Markers of Effect Following Exposure of Fish to Sodium Pentachlorophenate (Na-PCP)

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The Swan and Canning Rivers run through suburban areas of Perth, Western Australia's capital city. Most of the population, approximately 1.4 million, lives on the shores of the rivers in the Swan Coastal Plains and along the coast. The extensive anthropogenic activities surrounding the shores cause the waterways to be contaminated by a variety of xenobiotics from agricultural lands, industrial sites, landfills and water run offs. Sodium pentachlorophenate (Na-PCP) used as a wood preservative for a multitude of private and commercial jetties is of special concern (Department of Environmental Protection 1996). Na-PCP is also commonly used for domestic, agriculture and industrial purpose due to its potent biocide properties. Trace concentrations of PCP compounds have the potential to cause adverse effects in aquatic organisms (Bostrom and Johansson 1972). Exposure of the fish *Notopterus notopterus* to Na-PCP induced metabolic alterations and caused impairments of gill function, which affected respiratory exchange (Verma et al. 1982). Impaired liver metabolism was also observed after 4 days of PCP treatment in the yellow eel (*Anguilla anguilla*) (Bostrom and Johansson 1972).

Metabolic imbalances related to Na-PCP exposure have been observed in a limited number of studies. Impairments to metabolism can be measured in various tissues via the activity of metabolic. The aerobic metabolic capacity of a tissue can be determined by the activity of citrate synthase (CS), the first enzyme of the Krebs cycle, located within the mitochondria (Pelletier et al. 1995). Cytochrome C oxidase (CCO) is the terminal enzyme of the electron transport system; its activity also informs on the aerobic capacity of tissues (Bostrom and Johansson 1972). Lactate dehydrogenase (LDH) activity is a good indicator of the anaerobic capacity of a tissue (Dickson et al. 1993). The aim of the present study was to investigate the effects of Na-PCP on a Western Australia native fish, pink snapper (*Pagrus auratus*). Juvenile fish were injected with high dose of Na-PCP to trigger maximum biochemical reactions. CS, CCO, and LDH activities were determined in white muscle and liver tissues.

MATERIALS AND METHODS

Sodium pentachlorophenate (Na-PCP) salt was obtained from Fluka Chemika,

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Switzerland. All other chemicals for enzymatic analysis were purchased from Sigma Chemical Co., USA. All chemicals were of the highest commercial purities.

A preliminary trial determined that for juvenile pink snapper, the lethal dose of i.p. (intra peritoneal) injection of Na-PCP (96 hr LD_{50}) was 25 mg/kg fish weight. Therefore doses of 0, 5, 10, and 20 mg Na-PCP/kg of fish weight were i.p. injected to eighty (80) juvenile pink snapper (20 fish/treatment, average weight mean ± SEM : 30.24±6.48g) obtained from Fremantle TAFE (Fremantle, Western Australia). Although these doses are not environmentally realistic, they aimed at triggering maximum biochemical response in the fish. Similar or higher doses were used by Kishino and Kobayshi (1996) and by Roche and Boge (2000). Fish were acclimatized to laboratory conditions in 100-L aquariums for two weeks, using seawater collected at the aquaculture site. Eighty percent water renewal was performed everyday, which also eliminated fecal wastes. Fish were immersed in an anesthetic solution of 70 mg per liter of tricaine methanesulfonate (MS-222) for about 3 minutes prior to injections. Treatments were performed in replicate (10 fish/aquarium). During the acclimation and experimental period the fish were fed twice daily with commercial salmon pellets at a rate of 1 % of body weight per day. A 12 hours light/12 hours dark regime was used, and temperature was kept at 21° C. Constant aeration of the tanks ensured 100% oxygen saturation at all times. Water parameters (pH, temperature, ammonia, and salinity) were measured daily during acclimation and experimental periods to ensure constant conditions. Treatment of animals was in accordance with the Curtin University's Animal Experimentation Ethics Approval number N 17-99.

Fish were sacrificed 10 days post-injection. Fish were killed by a blow on the head and the liver was excised and weighed. Liver somatic index (LSI) was calculated as follow: (weight of liver X 100)/weight of fish. Livers were washed with ice-cold potassium chloride (KCl) 0.15M. A sample of white muscle was collected in front of the dorsal fin, and it was ensured that the muscle was free from skin and red muscle tissue. The muscle and liver samples were immediately frozen in liquid nitrogen and stored in a -80°C freezer until enzymatic activity analyzed. A sample tissues were prepared by homogenizing one unit of liver or muscle tissue with 9 units of 50 mM imadizole-HCl buffer, pH 8.0 for about 20 seconds using a Diax 900 homogenizer. The homogenized tissues were centrifuged at 3000 rpm for 10 minutes using Orbital 400 centrifuge, and the supernatant was used in the assays. The reaction media for analysis of enzymatic activities are as described in Pelletier et al. (1993). The experimental conditions were as follows: Citrate synthase (CS): 100 mM Tris/HCl; 0.1mM 5,5'-Dithio –bis (2-Nitrobenzoic Acid) (DTNB); 0.2 mM acetyl CoA; 0.3 mM oxaloacetate in 50 mM imidazole buffer pH 8.0. Cytochrome C oxidase (CCO): 100mM potassium phosphate and 70 μM reduced cytochrome C, imidazole buffer pH 8.0. The reduction of cytochrome C was carried out by the addition of sodium hydrosulphite. The excess reducing agent was removed by gently bubbling for 60 seconds using an air pump. Reactions were run against a control of 70 μM

Table 1. Fish mortality during the experimental period.

Na-PCP (mg/kg)	Days									
	1	2	3	4	5	6	7	8	9	10
0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0
10	0	1	0	0	1	0	2	0	0	0
20	0	3	0	0	0	2	1	2	0	0

N = 20

cytochrome-C oxidized with 0.33% (w/v) potassium ferricyanide. Lactate dehydrogenase (LDH): 100 mM potassium phosphate, 0.15 mM NaOH; 0.8 mM pyruvate, imidazole buffer pH 7.4. The enzymatic activities were assayed spectrophotometrically following the rate of reduction of DTNB for CS, the rate of oxidation of cytochrome C for CCO, and the rate of conversion of NADH to NAD for LDH. The changes of absorbance were observed at 412 nm for CS, 550 nm for CCO and 340 nm for LDH using a LBK-Biochrome 4060 spectrophotometer. The extinction coefficients used were 13.6 for DTNB, 29.5 for reduced cytochrome C and 6.22 for NADH. All the enzymatic activities were performed at 20°C, and measurements were run in duplicate. Protein content of supernatant was analysed according to Lowry et al. (1951). The enzymatic activities were expressed in International Unit per mg protein. One International unit (U) is defined as that amount of the enzyme that will convert 1 μmol of substrate into product per minute.

Normality of data was tested using an Anderson-Darling Normality Test (α=0.05). All data were log transformed to achieve normality. The data were analyzed using a one-way analysis of variance (ANOVA) with replication. When significant differences between groups were found ($\alpha \leq 0.05$), a least significant difference (LSD) test was used to locate differences between the means (Zar 1984). The statistical analysis was performed using SPSS version 9.05.

RESULTS AND DISCUSSION

Water quality parameters as measured daily were stable during 17 acclimation days and 10 experimental days with means ± SEM: 7.35±0.02 for pH, 20.59±0.21 for temperature (^{0}C), 35.05±0.02 for salinity (ppt) and 0.03±0.00 for ammonia (mg/L). The fish mortalities occurred at doses of 10 and 20 mg/kg only, as described in Table 1. The remaining fish were not exhibiting any sign of stress or toxicity, and were swimming in a normal fashion. LSI was slightly higher in treated fish (5, 10 and 20 mg/kg) relative to control, but the increase was not significantly different (p=0.590).

No difference (p>0.05) in enzymatic activities between replicate treatments were identified, therefore replicates were pooled for graphical presentation. In white muscle CS and CCO activities were not affected by Na-PCP treatment at any dose (p = 0.981 and p = 0.524, respectively). However, LDH activity was significantly induced only at the highest dose of 20 mg/kg (p=0.035). In liver CCO and LDH

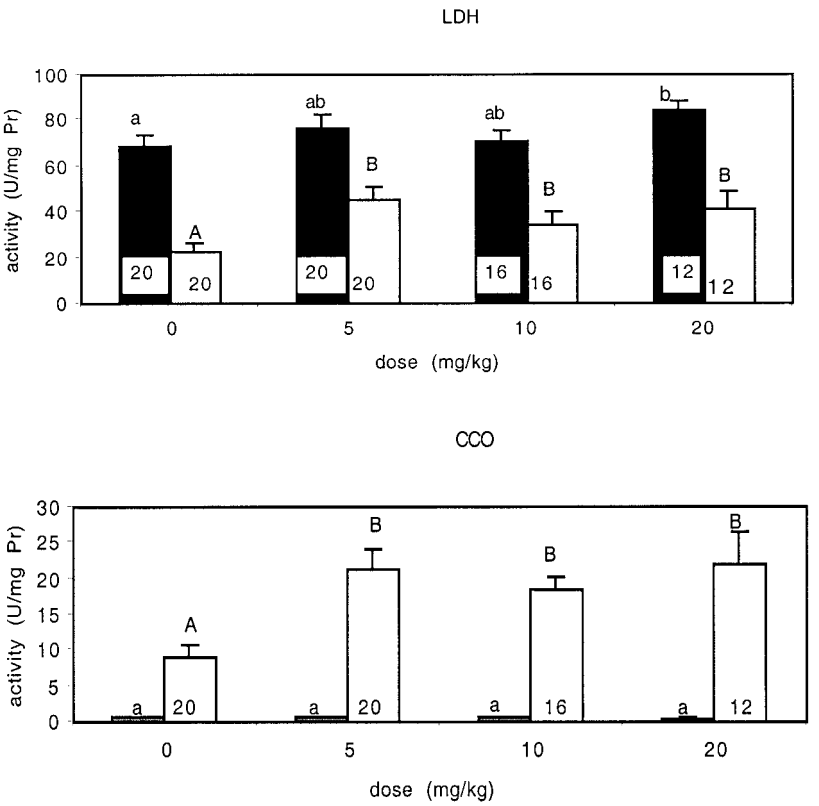

Figure 1. Enzymatic analysis (U/mg protein; means±SEM) of pink snapper i.p. injected by Na-PCP (mg/kg of fish weight). ■ : muscle tissue, □ : liver tissue. Number of fish (Ns) in each treatment is indicated at the base of each bar. Different letters indicate statistical differences amongst treatments ($p \leq 0.05$).

activities were significantly stimulated at all treatments ($p=0.001$ and $p=0.019$, respectively) (Fig.1), while CS activity remained unchanged.

Organic compounds such as pentachlorophenol possess the ability to partition into lipid membranes of mitochondria, and consequently are able to translocate protons across the mitochondrial membrane (Shannon et al. 1991). This suggests that the uncoupling of oxidative phosphorylation may be the major mechanism by which chlorophenols cause metabolic disturbances at the cellular level (Shannon et al. 1991). Uncoupling agents such as pentachlorophenols have the ability to stimulate mitochondrial respiration (Bostrom and Johansson 1972). In the present study, the increased oxygen requirement is reflected in the higher CCO activity in the liver of the poisoned fish. Increased CCO activity in aerobically functioning tissues such as the liver has been demonstrated to be a persistent response, which is related to the capacity of pentachlorophenols to bind very strongly to mitochondrial membranes (Bostrom and Johansson 1972). The lack of response in

the activity levels of aerobic enzyme CS and CCO in the white muscle is most likely related to the fact that this organ works predominantly under anaerobic conditions (Webb 1998).
Increased LDH activity in muscle and liver tissues indicates metabolic changes in chemically stressed fish. In studies of large numbers of teleost fish species, correlations of muscle metabolic enzyme activities with metabolic rate and locomotor activity have been established (Dickson et al. 1993). In the intoxicated juvenile pink snapper, the catabolism of glycogen and glucose appears to have shifted towards the formation of lactate, which may have adverse effects on the animal (Szegletes et al. 1995). Accumulation of lactate may lead to metabolic acidosis and subsequent muscle fatigue (Mosse 1980), with implications on foraging and escape capacities in wild animals. In the present study, the fish were chemically challenged at close to maximum levels, as shown by the mortality rate at high doses of Na-PCP. It is not expected that fish would be exposed to such high concentrations under real-life situations, but rather fish would be subjected to low-level, chronic exposure resulting in effects probably not as profound as those described above.

CS activity does not appear to have been tested in studies investigating toxicant-induced metabolic alterations. The results of the present experiment indicate that there are no effects of Na-PCP intoxication on CS activity in the liver and white muscle of juvenile pink snapper. It will be worthwhile, however, to test the CS enzymatic response to toxicants in other organs such as gills, since CS activity in the gills has been shown to vary with the metabolic rate in the crucian carp (*Carassius carassius*) (Lind 1992).

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