International Journal of Applied Research 2017; 3(5): 521-526


**ISSN Print:** 2394-7500
**ISSN Online:** 2394-5869
**Impact Factor:** 5.2
IJAR 2017; 3(5): 521-526
www.allresearchjournal.com
Received: 18-03-2017
Accepted: 20-04-2017

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# Hepatic Biotransformation of Chlordecone and Induction of Hepatotoxicity in the Cichlid Fish, *Pseudetroplus maculatus* (Bloch, 1795)

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**Abstract**
Detoxification mechanism in hepatic tissue after exposure to one of the environmental contaminants, chlordecone, was investigated by analysing certain biotransformation biomarkers of phase I enzyme - hepatic 7-ethoxyresorufin-O-deethylase (EROD), and Phase II enzymes - UDP-glucuronosyltransferase (UGT), glutathione-S-transferase (GST) and gamma-glutamyl transpeptidase (GGT) in the cytosolic and microsomal fractions of liver tissue in the cichlid fish, *Pseudetroplus maculatus.* Two sublethal concentrations of chlordecone (3.5 and 7µg/L) were exposed to fish for short durations as 24, 72 and 96 h. At the end of treatment period, the activities of EROD, UGT and GGT were increased significantly ($P<0.05$) in concentration and time-dependent manner when compared to the control groups. The present observation clearly illustrates that chlordecone promoted the activities of biotransformation enzymes in liver in order to eliminate the toxic compound from the body of the animal. The activity of GST was decreased significantly ($P<0.05$) after chlordecone exposure, which reflects the inability of liver tissue to neutralize the oxygen free radicals generated. Thus the obtained data contribute to a better understanding of chlordecone-induced detoxification mechanism in liver of fish, which further resulted in hepatotoxicity in the cichlid fish, *Pseudetroplus maculatus*.

**Keywords:** 7-ethoxyresorufin-O-deethylase, UDP-glucuronosyltransferase, glutathione-S-transferase, γ-glutamyl transpeptidase, chlordecone, *Pseudetroplus maculatus*

## 1. Introduction
Over the past few decades, there is a great concern on the increased release of environmental contaminants into the aquatic ecosystems, which directly or indirectly affects the non-target organisms, including fish. As a result, the organisms in various aquatic environmental compartments are continuously and constantly exposed to pollutants. Recently, the population of fish is declining tremendously owing to the irreparable damages caused due to the exposure of pollutants (Helfrich *et al*., 1996) [1]. Man-made or artificial pollutants are called xenobiotics, which are chemicals or substances exogenous to the biological system. Xenobiotics includes environmental and industrial pollutants such as fertilizers, detergents, pesticides, heavy metals, plasticizers, surfactants, industrial effluents, drugs, drug metabolites etc which are toxic and poses significant risks to the environment and exposed organisms. Most of the xenobiotics in the aquatic environments are directly entered by drift during pesticide spraying, runoff from treated area and leaching through the soil. Besides, these contaminants also find the way to reach the aquatic bodies indirectly through heavy rainfall shortly after the application of pesticide to the wet soil, through transformation and mineralization by soil micro-organisms (Relyea and Hoverman, 2008) [2].
Chlordecone is a chlorinated polycyclic hydrocarbon insecticide, broadly used in the tropics for the control of banana root borer, leaf-cutting insects, Colorado potato beetle, wireworms and fire ants (ATSDR, 1995) [3]. It was initially produced in 1951 by Allied Signal Company and Life Sciences Product Company in Hopewell, Virginia and exported to Europe, Asia, Latin America, and Africa. Owing to its dangerous toxic effects in humans and other animals, the production and use of chlordecone has been ceased in most of the western countries, but still the use of chlordecone is more prominent in India. Currently, India stands the largest producer of pesticides in Asia and ranks twelfth in the world for the use of pesticides.

The average consumption of pesticides is far lower in India when compared to many other countries, but the problem of pesticide pollution is seriously high (Abhilash and Singh, 2009) [4]. Unfortunately, India is one among the few countries in the world still producing and using some of the chlorinated pesticides such as chlordecone.

The first severe intoxication of chlordecone has been reported among the employees of Hopewell Chemical Plant, USA and it lead to the ban of the production and use of chlordecone in 1976 (Kadhel *et al*., 2014) [5]. The symptoms of chlordecone intoxication include severe toxicity in the nervous system, liver, and testes (Reich and Spong, 1983) [6]. Chlordecone is persistent for several years in various environmental compartments and also reported as contaminant in food stuffs that caused several health problems such as male fertility, pregnancy complications, prostate cancer etc (Multigner *et al*., 2016) [7]. Chlordecone by means of its estrogenic properties is also known as an endocrine disruptor (Smeets *et al*., 1999) [8] and it is a persistent organic pollutant which has high degree of bioaccumulation and biomagnifications (ATSDR, 1995) [3]. Thus chlordecone targets all organisms, including wildlife, aquatic and terrestrial animals.

When a toxicant enters the body of an organism, the toxic effect of the compound is eliminated either by excreting the parent compound or by means of biotransformation into less toxic compound. Therefore, all organisms undergo certain structural and functional modifications or biotransformation in liver tissue in order to detoxify the parent toxic compound. Biotransformation is the conversion of more potent toxic compound into water soluble form which is catalysed by a series of highly specific enzymes and the resulting polar metabolites can be readily excreted from the body very easily than the parent compound (van der Oost *et al*., 2003) [9]. All animals possess two types of biotransformation pathways, namely phase I and phase II reactions. Phase I reactions involve oxidations, reductions and hydrolysis of xenobiotics, resulting in either more hydrophilic molecules or more reactive molecules. The hydrophilic molecules thus produced are readily excreted, while the second ones are conjugated with endogenous hydrophilic moieties such as glutathione, glucuronides, sulfate, or amino acids in phase II pathway. These two pathways are equipped with a series of enzymes and other compounds for detoxifying the toxic xenobiotics in the environment (Jancova *et al*., 2010) [10].

Phase I reactions are primarily catalyzed by the cytochrome P450 and flavin-containing monooxygensases. Phase II enzymes include glutathione S-transferase, glucuronosyl transferase, sulfotransferase, and acetyltransferase. These reactions reduce the xenobiotic reactivity with cellular proteins and facilitate its excretion outside the cell. Liver, the major detoxification organ, plays a crucial role in the metabolism and excretion of xenobiotic compounds with biochemical modifications and acts as main target of toxic substances (Hedayati, 2016) [11]. In addition to liver, the other major organs involved in xenobiotic metabolism in fish are gill, intestine, skin, and kidneys (Romanenko *et al*., 1986) [12].

For several reasons, fish are widely used as a laboratory model to evaluate the biological and biochemical responses to environmental contaminants. In the present study, the cichlid fish, *Pseudetroplus maculatus* was used to evaluate the biotransformation of chlordecone. It is one of the endemic and edible fishes of India, which inhabits both freshwater and brackish water habitats, commonly seen in rivers, ponds, streams and lakes. Fishes are competent to perform xenobiotic metabolism by microsomal oxidation, reduction and conjugation and the enzymes involved are similar to mammals, with some exceptions in physical conditions such as temperature (Anderson and Koivusaari, 1985) [13]. Despite the large number of toxicity studies of chlordecone there is no relevant data regarding the biotransformation of chlordecone in fish, *Pseudetroplus maculatus*. The present study was therefore undertaken to understand the hepatic biotransformation of chlordecone and the induction of hepatotoxicity in the fish.

## 2. Materials and Methods

### 2.1 Collection and maintenance of animal

The cichlid fish *P. maculatus,* weighing 7 ± 1 g and length 7 ± 1.5 cm were collected from the local fish farm, KKF Nursery, Manjeri, Vaniyambalam, Malappuram district, Kerala, India. Animal was transported to laboratory with least disturbance and were acclimatized for two weeks exposed to good lighting system along with constant supply of dechlorinated water in well-aerated tanks (40 L capacity).

### 2.2 Preliminary tests

Preliminary test were conducted by using standardized procedures as per American Public Health Association guidelines so that the water temperature was maintained as 28±2°C, oxygen saturation of water (70 and 100 %), and pH 6.5 to 7.5 (APHA, 1998) [14]. The standard physico-chemical features were maintained throughout the experiment in both control and treated groups.

### 2.3 Chemicals

Technical grade organophosphate insecticide, chlordecone (Kepone, decachlorooctahydro-1, 3, 4-metheno-2H-cyclobuta[cd]-pentalen-2-one, 99.9% pure) was obtained from Supelco, USA. 7-ethoxyresorufin, Triton X-100 and trichloroacetic acid were obtained from Sigma-Aldrich, USA. NADPH, reduced glutathione, para-nitrophenol, 1-chloro-2,4-dinitrobenzene and glycylglycine were obtained from Himedia Laboratories, Mumbai, India and L-γ-glutamyl-3-carboxy-4-nitroanilide was purchased from Carbosynth Limited, UK. All other chemicals were of analytical grade and obtained from local commercial sources.

### 2.4 Treatments

After two weeks of acclimatization, fishes were kept in different tanks for the experiment maintaining ten animals per group. Chlordecone was dissolved in 1% DMSO and therefore used as a solvent (vehicle) control in the experiment. Earlier studies from our laboratory determined the median lethal concentration ($LC_{50}$-96 h) of chlordecone in *P. maculatus* by using probit analysis as 35µg/ L (Asifa and Chitra, 2015) [15]. Two sublethal concentrations of chlordecone such as one-tenth (3.5µg/ L) one-fifth (7µg/ L) of $LC_{50}$-96 h was exposed for 24, 72 and 96 h durations maintaining negative and positive controls.

The first two groups of fishes were control groups (negative and vehicle controls). Third group was treated with chlordecone at 3.5 µg/ L and maintained for 24, 72 and 96 h, respectively. Fourth group of fishes were treated with chlordecone at 7µg/ L for 24, 72 and 96 h, respectively.

### 2.5 Killing of animals

At the end of each exposure period, fish was caught very gently using a small dip net, one at a time with least disturbance, killed and liver tissue was dissected out, and the tissue was processed immediately for biochemical analysis.

### 2.6 Preparation of hepatic cytosolic and microsomal fractions

Hepatic cytosolic and microsomal fractions were prepared as described by Bradford 1976 [16]. Briefly, after weighing, liver was chopped finely and 20% (w/v) homogenate was prepared in ice-cold 0.25 M sucrose with a Teflon homogenizer on crushed ice for a minute. Homogenate was centrifuged at 500 *g* for 15 min at 4 ºC, the fatty layer removed and the obtained supernatant was centrifuged at 12,000 *g* for 20 min at 4 ºC. The resulting supernatant was further centrifuged at 100,000 *g* for 60 min at 4 ºC in Beckman Ultracentrifuge to obtain the cytosolic and microsomal fractions. The resulting supernatant is called cytosolic fractions. The microsomal pellets were resuspended in 0.25M sucrose to get the microsomal fractions and both were stored at -80 ºC until used for the following biochemical analysis.

Protein was estimated by the method of Lowry *et al.* [17] both in the microsomal and cytosolic fractions of liver with BSA as the standard. Using hepatic microsomal fractions, the activities of 7-ethoxyresorufin O-deethylase (Fernandes *et al*., 2002) [18], UDP-glucuronosyltransferase (EC 2.4.1.17) (Zhivkov, 1970) [19] and gamma-glutamyl transpeptidase (EC 2.3.2.2) by the modified method of Christiansen *et al*., 1998 [20] were measured. Activity of glutathione S-transferase (EC 2.5.1.18) was assayed according to Fernandes *et al*., 2002 [18], in the cytosolic fractions of liver.

### 2.7 Statistical analyses

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test using statistical package SPSS 19.0. Differences were considered to be significant at $p<0.05$ against control groups. Data are presented as mean±SD for ten animals per group. All biochemical estimations were carried out in duplicate.

## 3. Results

In the present results, DMSO-treated fish (vehicle control) did not show any marked changes in all parameters tested and the data obtained are similar to the solvent-free control group. Activities of ethoxyresorufin-O-deethylase and UDP-glucuronosyltransferase were increased significantly at both concentrations of chlordecone in concentration-dependant manner in the hepatic microsomal fractions when compared to the control groups (Figures 1 and 2). The activity of gamma-glutamyl transpeptidase increased significantly ($P<0.05$) in the microsomal fractions of liver at both sublethal concentrations of chlordecone (Figure 3). Chlordecone administration significantly ($P<0.05$) decreased the activity of glutathione-S-transferase in concentration and time-dependant manner in the cytosolic fractions of liver when compared to the corresponding control groups (Figure 4).

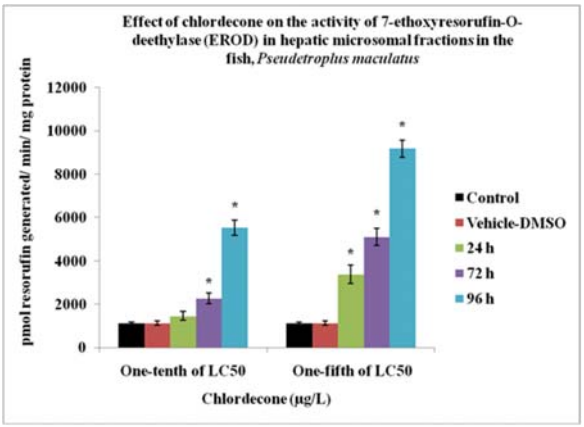


**Fig 1**

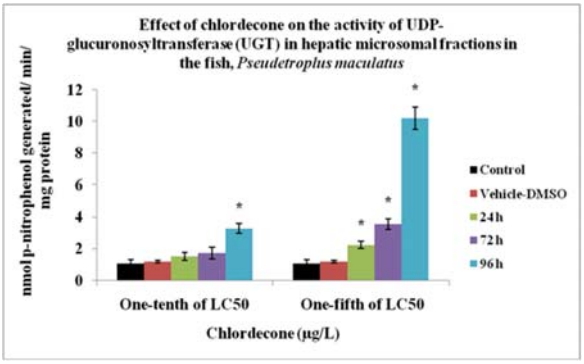


**Fig 2**

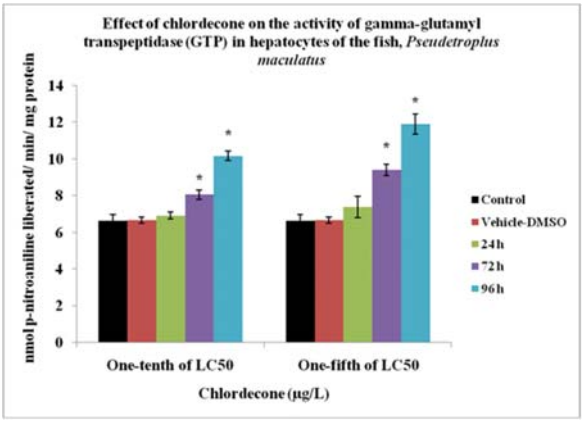


**Fig 3**

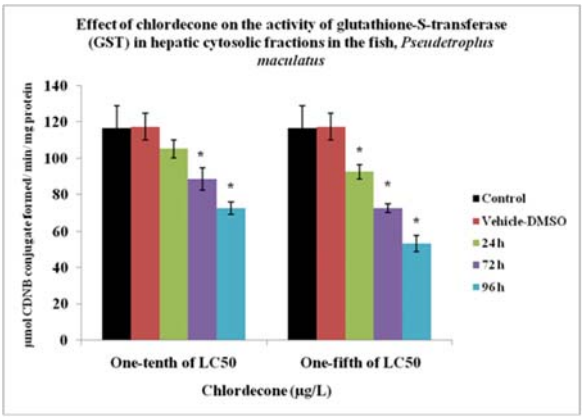


**Fig 4**

## 4. Discussion

India is primarily an agricultural country where pesticides have been continuously used for advanced farming and improving crop yields. Nowadays, there has been an increasing concern regarding pesticide-associated adverse health effects on both humans and animals. Most of the organochlorine pesticides are lipophilic in nature so that it has the ability to bioaccumulate and bioconcentrate in various tissues of animals leading to irreversible health impacts (Senthilkumar *et al*., 2001; Sethuraman *et al*., 2013) [21, 22]. Early report has demonstrated that chlordecone undergoes bioaccumulation in various tissues of rat with highest concentration in the liver tissue (Linder *et al*., 1983) [23]. However, the relevant information on the biotransformation of chlordecone in the cichlid fish, *Pseudetroplus maculatus* remains unclear. The present study, therefore, focused to evaluate the biotransformation of chlordecone in the fish by using biomarker enzymes such as ethoxyresorufin-O-deethylase, UDP-glucuronosyltransferase, glutathione-S-transferase and gamma glutamyl transpeptidase in the hepatic subcellular fractions of *P. maculatus*.

Ethoxyresorufin-O-deethylase (EROD) is one of the prime enzymes in the phase I reactions of biotransformation pathway that respond rapidly to various xenobiotics. In fish, EROD is a specific biomarker enzyme used to measure the activity of the cytochrome P450-dependent monooxygenase induction by the exposure to xenobiotic chemicals (Stegeman *et al*., 1997) [24]. Generally, cytochrome P450 catalyses the oxidation, reduction and hydrolysis reactions of phase I system. Thus, the enzyme introduces functional group to the toxic substrate, thereby making it more polar so that the toxin can be either easily excreted or further biotransformed into other water soluble products (Anzenbacher and Anzenbacherova, 2001) [25]. In fish, the CYP1A subfamily of isoenzyme cytochrome P450 family is mainly responsible for the biotransformation of a broad range of xenobiotics such as polycyclic aromatic hydrocarbons (PAHs) (Benedetti *et al*., 2007) [26]. Induction of EROD activity is an extremely sensitive indicator to the exposed contaminants in the environment and it is one of the initial quantitatively detectable responses to toxicant, naturally termed as early warning system (Payne *et al*., 1987) [27]. In the present study, chlordecone administration caused significant increase in the activity of ethoxyresorufin-O-deethylase in the microsomal fractions of liver at both sublethal concentrations in time-dependant manner. Similar observation as elevated activities of ethoxyresorufin- and ethoxycoumarin-O-deethylases and the content of cytochrome P-450 has been reported when chlordecone at 45 mg/kg was administered in mice (Carpenter and Curtis, 1991) [28].

In the phase II reactions of biotransformation pathway, the metabolites formed in phase I reactions bind to endogenous water-soluble compounds mediated by a series of enzymes. The microsomal UDP-glucuronosyltransferase enzyme catalyzes the conversion of hydrophobic xenobiotic substrates to more hydrophilic glucuronides as UDP-glucuronic acid. The glucuronide conjugates and other endogenous compounds formed as a result of glucuronidation are polar, water-soluble conjugates that are removed from the body in bile or urine based on the size of metabolite. In the present study, chlordecone exposure increased the activity of UDP-glucuronosyltransferase at both concentrations and about 9-fold increase was observed at 7µg/L concentration after 96 h of exposure. The present observation clearly demonstrates that the phase I metabolite of chlordecone is highly conjugated with endogenous hydrophilic moieties and are either ready to eliminate from the body or it is biotransformed to water-soluble and less toxic compound. The biotransformation of chlordecone to chlordecone alcohol occurs in humans, pigs and gerbils, where in humans chlordecone was excreted primarily as an unaltered compound (72%) and only 9% as conjugated-form with glucuronic acid (Fariss *et al*., 1980) [29]. Thus, the present results indicate that chlordecone forms glucuronide conjugates after 96h of exposure, evidenced by the elevated activity of UDP-glucuronosyltransferase in the microsomal fractions of fish liver. However, the present study also revealed that 100% of chlordecone is not metabolized to chlordecone metabolites in the body of animal exposed but the remaining contaminant may elicit toxic effect to the organism itself. It has been reported that certain biotransformation processes are accountable for the activation of exogenous chemicals to reactive intermediates that finally result in toxicity (van der Oost *et al*., 2003) [9]. It is evidenced by the previous study from our laboratory that chlordecone can induce genotoxicity, confirmed by the formation of micronucleus and other nuclear abnormalities such as blebbed, notched, lobed and irregular nuclei in the erythrocytes of *Etroplus maculatus* (Asifa *et al*., 2016) [30].

Glutathione S-transferases are one of the most studied enzyme of phase II reactions which catalyses the conjugation of xenobiotics having electrophilic functional groups to glutathione (Riol *et al*., 2001) [31]. Glutathione S-transferases has been shown to mediate cellular defences against toxic and reactive electrophiles such as reactive oxygen species, which are formed as a result of normal metabolic processes or exposure to various environmental contaminants (Jancova *et al*., 2010) [10]. In the present study, chlordecone decreased the activity of glutathione S-transferase in concentration and time-dependant manner in the cytosolic fractions of liver. The inhibited activity of glutathione S-transferase after chlordecone exposure could be due to the failure of hepatocytes to protect the tissue from oxidative damage. The present result was supported by one of the previous studies from our laboratory that chlordecone exposure induced oxidative stress in liver tissue of the fish, *Pseudetroplus maculatus* (Asifa and Chitra, 2017) [32].

Gamma-glutamyl transpeptidase is an enzyme involved in metabolism of glutathione and glutathionylated and electrophilic xenobiotics where it catalyses the glutamyl moiety to a variety of acceptor molecules including water, certain amino acids, and peptides, leaving the cysteine product to preserve intracellular homeostasis of oxidative stress (Zhang *et al*., 2005) [33]. GGT is concentrated in the liver, and its expression is detectable in biliary epithelium and in occasional periportal hepatocytes, besides it is also present in the gallbladder, spleen, pancreas, and kidneys (Lindros *et al*., 1989) [34]. The present study showed that chlordecone at both sublethal concentrations increased the activity of gamma-glutamyl transpeptidase in the hepatic microsomal fractions of *P. maculatus*. The elevated level of gamma-glutamyl transpeptidase is associated with lipid peroxidation in liver cells (Paolicchi *et al*., 1997) [35] and it was confirmed in our previous studies that chlordecone exposure altered antioxidant defence system and induced lipid peroxidations in gill, liver and brain tissues of *P.*

*maculatus* (Asifa and Chitra, 2017) [32]. The present data clearly indicate that gamma-glutamyl transpeptidase is directly involved in the generation of reactive oxygen species in liver tissue after chlordecone exposure.

## 5. Conclusion

Liver is the primary target organ for many toxicants and it is a detoxifying organ that accumulates, eliminates or biotransform the toxicants into less toxic metabolites. In view of the present study, chlordecone through phase I and phase II reactions has attempted to detoxify the substrate or its metabolites, however, its reactive intermediates finally resulted in hepatotoxicity in the cichlid fish, *Pseudetroplus maculatus*.

## Acknowledgment

Authors gratefully acknowledge the financial support from Kerala State Council for Science, Technology and Environment (KSCSTE), Thiruvananthapuram, Kerala, India to carry out this study.

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