Ecotoxicology and Environmental Safety 148 (2018) 327–335

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

# Biomarker and histopathological responses of *Lates calcarifer* on exposure to sub lethal concentrations of chlorpyrifos

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ARTICLE INFO

*Keywords:*
Fish fingerlings
Bioassay
Chlorpyrifos
Biomarker
Histopathology

ABSTRACT

Bioassay tests on fingerlings (3.0 ± 0.5 cm) of euryhyaline fish *Lates calcarifer* were conducted using customized continuous flow through system and derived 96 h acute toxicity value for chlorpyrifos (CPF). Based on the measured concentration of CPF mean median lethal concentration ($LC_{50}$) of 1.07 μg/l with lower and upper 95% confidence limits (0.95 and 1.19 μg/l). No observed Effect Concentration (NOEC), Lowest Observed Effect Concentration (LOEC) and chronic values were found to be 0.4, 0.09 and 0.07 μg/l respectively. Key biomarker enzyme activities such as esterase, superoxide dismutase and malate dehydrogenase were measured in whole body tissues of the fish fingerlings on exposure to sublethal toxicity of CPF resulting in inhibition of enzyme activities. Native gel electrophoresis revealed single isoform of SOD and MDH enzyme activities exhibiting time and concentration dependent inhibition. Interestingly, three isoforms of esterase activity were witnessed, two isoforms didn't show changes and one isoform was completely inhibited. The observed changes indicated continuous production of reactive oxygen species (ROS) in cells, affecting the integrity and function of cell membrane. Decreased MDH activity indicates reduction of ATP production in the mitochondria leading to susceptibility of fish fingerlings due to the imposed CPF toxicity. Histopathological changes are evident as physiological signatures of chemical interactions in the cell and are prominently used for the evaluation of toxic effects. Gills and eye tissues were selected considering the possible effects on respiratory surfaces and vision impairment. Their tissue sections were observed for changes in primary & secondary lamellae, and retina of the eye respectively. Prominent pathological lesions of gills and retina of the eye include degeneration of cells, fusion, lifting of epithelium and increased cellular space, detachment of pigment epithelium, fusion of photoreceptor cells, respectively on exposure to 30 days of sub lethal concentrations. CPF was found to be highly toxic, affecting the vital functions of respiration, vision and cellular activities leading to susceptibility of fish fingerlings.

## 1. Introduction

Coastal and marine ecosystems are under ever increasing stress from human activities leaving behind signatures of pollution in the biotic and abiotic compartments. This is evident by the observations of hypoxia in coastal waters and also by decrease in biodiversity (Gu and Wang, 2015). Many synthetic organic chemicals including organophosphates are of growing environmental concern (Sharma and Sanghi, 2012). Since, the coastal environment consists of critical habitats comprising unique ecosystems supporting rich biological diversity and valuable assortment of natural resources. These resources would be adversely affected by pesticide residues transported from inland waterways into the coastal waters via rivers, estuaries through flooding, sewage, aerial deposition and rainfall (Rathore and Nollet, 2012). CPF [O, O-diethyl O-(3, 5, 6-trichloro-2-pyridinyl) - phosphorothioate] is a broad spectrum organophosphate insecticide used extensively in both agriculture and residential environments. It is widely used for urban and domestic pest control, livestock, ornamentals, turf maintenance, treating wood products, and termiticidal barrier in and around or under buildings (Christensen et al., 2009). Chlorpyrifos is a very highly toxic to aquatic invertebrates, freshwater fish, estuarine and marine organisms (Kamrin, 1997). Acute toxicity tests on aquatic invertebrates and fish are considered as gold standards, useful for ecotoxicological evaluation and to derive safe levels for protection of aquatic organisms. Fishes are generally regarded as sentinel organisms and are frequently used for monitoring both environmental quality and adaptation of organisms, considering their ability of rapid responses to change in the water quality. The fish, *Lates calcarifer* inhabits in coastal waters, often enters lagoons, estuaries, and harbors that are frequently subjected to pollution. It is a commercially important fish, widely distributed in great abundance throughout Asia Pacific including Indian coastal waters. However, acute toxicity information on tropical marine fish species are

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http://dx.doi.org/10.1016/j.ecoenv.2017.10.026
Received 19 June 2017; Received in revised form 7 October 2017; Accepted 10 October 2017

scarce for CPF. Hence, toxicity bioassay tests were attempted on sea bass to derive the median lethal concentration of CPF.

In recent years biomarkers are recognized as tools for the assessment of impacts of pollution on the marine environment, and they are already incorporated in environmental monitoring programmes (Viarengo et al., 2007). Biomarkers can be characterized as functional measures of exposure to stressors, which are usually expressed at the subcellular level of biologic organization (Adams et al., 2001). It is expected that, interactions among the xenobiotics and biological systems may give rise to biochemical changes, resulting in altered physiological conditions and exert their physiological signatures on the cells and tissues, which can be assessed by means of histopathology. As a result, wide range of histo-cytological alterations in fish and bivalves have been developed and recommended as biomarkers for monitoring the effects of pollution (Au, 2004). It is reported that, CPF affect the cells including generation of reactive oxygen species (ROS) and intracellular oxidative stress (Bebe and Panemangalore, 2003). ROS are formed as a natural byproduct of the normal metabolism of oxygen and have an important role in cell signaling and homeostasis. However during the pesticide stress ROS levels can increase dramatically (Devasagayam et al., 2004). This may result in significant damage to cell structures leading to cumulative effect known as oxidative stress, which reflects an imbalance between the systemic manifestations of reactive oxygen species. In this context present study was designed to address response of key biomarker enzyme activities like superoxide dismutase, malate dehydrogenase and esterase along with effects on gills and eye tissues through histopathological observation. Since, gills are the foremost organs of fish which are in intimate contact with external medium, while eye tissues were considered as organophosphorous pesticides produce abnormal sudden rapid movements, and loss of equilibrium in fish as reported in previous studies (Fulton and Key, 2001; Sandahl et al., 2005). Moreover, sublethal concentrations are relevant in the context of environmental bioavailability of CPF. Hence, both gill and eye tissues were studied for histopathological response evaluation at sub lethal concentrations of CPF.

## 2. Material and methods

### 2.1. Collection and maintenance of experimental organisms

Fish fingerlings (size: 3.0–3.5 cm in length) of Sea bass *Lates calcarifer* (Sea bass) were procured from Rajiv Gandhi Centre for Aquaculture (RGCA), Sirkali, Tamil Nadu, India. They were transported to the laboratory in 10 L Polythene bags filled with seawater enriched with about 80–90% of oxygen saturation. The fishes were acclimatized in 300 L Fiberglass Reinforced Plastic (FRP) tanks with pre-aerated and filtered seawater for about 10 days in a temperature controlled room (26 ± 1 °C) in homogenous salinity (30 psu) and pH (8.0 ± 0.2).

During acclimatization and chronic toxicity testing, fish fingerlings were fed with rice bran and oil cake mixed diet prepared in the laboratory. The fingerlings were not fed during acute toxicity exposure as prescribed in the standard protocol that was followed throughout the study period. During exposure, seawater was exchanged at every 12 h with fresh filtered seawater in order to remove the nitrogenous wastes. Seawater was prepared by filtering through sand filter, charcoal filter, 10 μm size filter and then finally passed through UV treatment unit to remove pathogenic organisms. A homogenous photoperiod of 12 h Light and 12 h dark was maintained in the room during acclimation and test exposure periods.

### 2.2. Seawater quality

Water quality parameters like salinity, pH, water temperature, and dissolved oxygen were measured prior to start of the exposure and during the test period by using a pre-calibrated water quality probe 'Hydrolab' (Make: Quanta). In addition to the above parameters, background concentration of CPF in seawater collected from bar mouth of Ennore estuary of Chennai was monitored throughout the study period.

### 2.3. Test solution and treatment

The stock solution of CPF (1000 mg/l) was prepared by dissolving 25 mg active ingredient of CPF 20% EC (HILBAN, Hindustan Insecticides Limited) in 25 ml of analytical grade acetone. Desired test concentrations were prepared in acetone from the stock solution using variable micropipette.

### 2.4. Acute and chronic toxicity bioassay tests

Bioassay experiments viz., acute and chronic tests were conducted using customized flow through test method. The bioassay for acute and chronic toxicity tests were conducted by using required life stage of fish selected as described by Sprague (1973) and the U.S. Environmental Protection Agency (USEPA, 2002). For acute toxicity tests, healthy fish fingerlings were selected from the storage tanks and divided into six groups viz., control, 1.0, 1.2, 1.4, 1.7 and 2.1 μg/l of CPF treatment for the five definitive tests. Control received a volume of acetone equivalent to the volume of solvent used at maximum concentration in the test and was found to be non toxic. 10 fish fingerlings were maintained in each test chamber with replicate for treatment and control groups. The acute exposure period of 96 h and totally 3 successive flow through tests were performed for reproducibility in duplicate groups of control and treatments. Mortality responses were recorded every 24 h interval regularly for 96 h. Similarly, for chronic tests five different sublethal concentrations viz., 0.05, 0.10, 0.18, 0.34 and 0.65 μg/l of CPF were selected along with control based on acute toxicity values. The chronic toxicity test was performed for 30 d of exposure in duplicate groups for each concentration and it was repeated once for confirmation. During the chronic toxicity test survival/mortality end points were recorded every 24 h interval regularly for 30 d. Fish fingerlings were fed twice a day and the uneaten feed was removed from test chambers at regular intervals. All other environmental conditions were maintained same during all the experiments as those used during acclimatization of the test organisms.

### 2.5. Determination of CPF concentration in seawater samples of test chambers

Dissolved CPF in seawater used in test chambers were measured at 24 h intervals (i.e. 0, 24, 48, 72 and 96 h) for acute toxicity tests and between 10-d intervals (i.e.10th, 20th and 30th day) for chronic tests. Seawater samples were extracted according to standard method (USEPA, 1986). 1 L of the seawater sample was taken in a separating funnel of 2 L capacity, 50 ml of dichloromethane (DCM) was added into the separating funnel. Sample was extracted using mechanical shaker at 225 RPM for 10 min and kept undisturbed for separation. The lower DCM layer was collected in a conical flask and extraction was repeated twice. Sodium sulphate (7. 3 g of anhydrous baked at 150 °C overnight in hot air oven) was added and kept undisturbed for 20 min. Sample was cleaned with florisil column elution and sample was condensed to about 1 ml by using rotary evaporator (Make: IKA, Germany, Model: RE 10 Control, Cyberlab). The final extract was collected in a glass vial and stored at 4 °C until analysis. Dissolved concentration of CPF was determined using an Agilent 6890 N Gas Chromatography fitted with Flame Photometric Detector (GC/FPD). Chromatographic separation of CPF was achieved with HP-5 fused silica capillary column (30 m length, 0.32 mm i.d., 0.25 μm film thickness, USA). Nitrogen 99.999% purity was used as the carrier gas and makeup gas at a flow rate of 7.1 ml/min. The GC temperature was programmed as follows: 75 °C (0 min), 40 °C/min ramp to 220 °C, 10 °C/min ramp to 250 °C (1 min hold) and 40 °C/min ramp to 290 °C in a splitless mode with 9.6 psi pressure.