7.1 Introduction

The attachment and establishment of sessile organisms, ranging from single proteins to bacteria, algae and marine animals, on artificial structures submerged in the sea such as offshore platforms, jetties, harbours and ships generally designated as the biofouling (WHOI 1952; Abarzua and Jacobowsky 1995; Olsen 2007). It demands substantial financial implications to the marine engineering constructions. The problem is so critical that the worldwide expenditure obtained on antifouling measures alone is approximately US $ 6.5 billion a year (Bhadury and Wright 2004). Antifouling coatings have been a widely employed strategy for controlling fouling on underwater marine structures. Among the various coatings, Self-Polishing Copolymer antifouling paints (SPCs) with triorganotin (TOT) as biocide was mainly preferred (Chambers et al. 2006). Unfortunately, this most popular antifouling coating, having a life time up to five years, also turned out to be the most toxic. Heightened establishment of this biocide into the environment lead to shell thickening in oyster population and imposex in gastropods (His and Robert 1987; Alzieu 1991). Their build-up in the marine food chain during
bioaccumulation and biomagnifications engrossed supreme concern. The subsequent total global ban imposed on TOT-based coatings by the International Maritime Organization (IMO) led the antifouling paint industry into a very precarious situation (Champ 2000). After the ban of TOT compounds in antifouling formulation, shipping industry introduced Irgarol 1051, Chlorothalonil, Dichlofluanid etc. as new biocides. Irgarol 1051 is basically a triazine compound and the degradation of Irgarol 1051 is slow and expected to accumulate in the ecosystem (Callow and Willingham 1996). Irgarol 1051, like other triazinic herbicides, acts by blocking electron transport in chloroplasts during photosynthesis (Holts et al. 1993) and thus it is toxic to aquatic lives at low concentrations. Sediment contamination by Irgarol 1051 was also found to be relatively high than that in water column (Gough et al. 1994; Thomas et al. 2002). Dichlofluanid is relatively insoluble in water and Thomas et al (2002) suggested that it is easy to accumulate by the association with particulate matter. The global market share of non- organo tin products increased from 2% to 16% between 1988 and 1993 (Voulvoulis et al. 1999). In the ecosystem some booster biocides are present at measurable levels. The reported concentrations of such biocides in sediments are sufficient to damage microalgal communities (Dahl and Blank 1996), macroalgae (Scarlett et al. 1997), endosymbiotic corals, sea grasses and indirectly but ultimately to herbivorous mammals, such as dugongs (Scarlett et al. 1999).

Biomarkers allow an integrated measurement of contaminant’s bioavailability that can cause biochemical responses, providing early indicators of potential pollution. For coastal and marine pollution biomarkers have many advantages when compared to chemical analysis. Among the biological effects of pollutants, biochemical changes occur more quickly than
physiological responses and provide information on the sensitivity of organisms with regard to uptake, biotransformation and detoxification patterns (Galloway et al. 2002). The toxicity of a compound depends ultimately on its concentration, exposure duration and its bioaccumulation. The rate of degradation is important and can be influenced by many factors. The degradation depends on the chemical and physical properties of compounds and environmental parameters, such as the nature and concentration of microbial populations, dissolved and suspended material, temperature and light. However, persistence does not necessarily equate to a compound being toxic because it may not be bio available. Laboratory studies suggest that there are considerable differences in the degradation pattern of biocides. Acetylcholinesterase (AChE) has been reported as a responsive biomarker to neurotoxic compounds in biomonitoring studies that assess negative effects on aquatic organisms and environmental quality (Escartín and Porte 1997; Radenac et al. 1998; Mora et al. 1999; Galgani and Bocquené 2000; Dailianis et al. 2003). The enzyme acetylcholinesterase which catalyses the hydrolysis of acetylcholine is omnipresent in the animal kingdom. It is a well-characterized enzyme in the vertebrates because of its critical catalytic function at the cholinergic synapses. The enzyme acetylcholinesterase (EC. 3.1.1.7) hydrolyzes the neurotransmitter acetylcholine to acetic acid and choline at the cholinergic synapses, terminating nerve impulse transmission. AChE as a potential cell membrane marker enzyme was already approved ( Severson et al. 1972; Steck et al. 1974; Watts et al. 1978).

Bivalves are often used for ecotoxicological monitoring because they are filter-feeding organisms living in sandy-mud bottoms and can accumulate
contaminants in their tissues to relatively high levels. Bivalve enzyme activity including AChE has already been widely used in monitoring environmental pollution in laboratory and field conditions (Le Bris et al. 1995; Dellali et al. 2001; Doran et al. 2001; Alves et al. 2002; Mohamed et al. 2003). AChE activity of invertebrates, in particular bivalves, differs from vertebrates. The classifications, characteristics and tissue localization of cholinesterase (ChEs) in vertebrates are generally valid for invertebrates, but there are differences. ChE may exhibit broad substrate specificities and are able to cleave among acetylthiocholine (ATC), butarylthiocholine (BTC) or propionylthiocholine (PrTC) (Kristoff et al. 2006). The ChE activity of sample organisms can vary with biotic (class or species of organism, age, size, reproductive stage, and physiological conditions) and abiotic factors like temperature, pH, salinity, etc related to the habitat (Varela and Augspurger 1996; Fairbrother et al. 1989). Variations in the ChE activity of aquatic organisms may reflect various contaminants including heavy metals, PAHs, hydrocarbons, detergents, phytotoxin, and other industrial pollutants (Payne et al. 1996; Flammarion et al. 1996; Magni et al. 2006; Senger et al. 2006; Linde-Arias et al. 2008; Bervoets et al. 2009).

7.1.1 Bivalves as Pollution Indicators

Bioindicators are organisms, which are used to monitor environmental pollution. Bivalves are mainly used as bioindicators, because of its filter feeding habit. Najimi et al. (1997) and Bainy et al. (2006), evaluated the effect of heavy metal exposure on AChE activity in mussel. Most studies on acetyl cholinesterase inhibition in marine molluscs have focused on whole organism or muscle extracts. (Bocquene 1997, Radenac et al.1998; Bainy et al. 2006). *Villorita cyprinoides var Cochinensis* is a bivalve species, which is
commonly found in the Cochin estuary. The bivalves *Villorita cyprinoides var cochinensis* were sampled from freshwater region, near Vikkom an area deprived from industrial activities. It is a purely brackish water species and is capable of tolerating a wide range of salinity up to a maximum of 34 ppt. It is a cheap source of protein rich food for the habitants and used as a raw material for the manufacture of cement and lime. The animal abundant in those parts of the backwaters wherever the bottom deposit consists of sand and silt. Large organisms were obtained during December to April and young one during August and September when the salinity was comparatively low. The optimum habitat salinity of the clam has been reported to be parts per thousand.

### 7.1.2 Acetylcholine esterase as Indicator of Toxicity

Previous studies mainly concentrate on the effects of pollutants on individual organisms which have direct toxic effects. The classical toxicological approach of the toxic effects on individual animals is highly relevant. The most obvious effect of exposure to a pollutant is acute toxicity which leads to a rapid death and it is common practice to assess this type of toxicity studies by the LD$_{50}$, LC$_{50}$ measurements. These measures are used to assess the ecological impact of pollutants.

Earlier reports on Organophosphorus (OP) compounds which inhibit a whole range of esterase enzymes, and it is the consequences of inhibition of one of these enzymes, acetylcholinesterase (AChE), that produce the symptoms of acute poisoning. Acetylcholine (Ach), the natural substrate for AChE, is one of the principal known transmitters of impulses across synapses between adjacent nerve ending, and across neuromuscular junctions. Nerve impulses stimulate the release of Ach, which transmits the stimulus across the gap to the adjacent nerve or
muscle cell. The Ach is normally broken down rapidly, by hydrolysis, catalysed by the enzyme, AChE. Inhibition of AChE means that Ach persists much longer, normal nerve functions are grossly disturbed, and sufficiently serve disturbance ends in death (Eto 1974). In brief, the lethal lesion disturbs impulse transmission across synapses and neuromuscular junctions, many physiological processes are interrupted in consequence, and death, in vertebrates at least, usually results from paralysis of the respiratory system. Figure 7.1 shows the action of Acetylcholine esterase enzyme in nerves and table 7.1 displayed previous studies on toxicological studies around the world.

Figure 7.1 Action of Acetylcholine esterase enzyme in nerves
<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Area</th>
<th>Organotin</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>South and East Coast, Korea</td>
<td>12.2983 (ng/g dry wt)</td>
<td>Hang et al. 2002</td>
</tr>
<tr>
<td>2</td>
<td>Port of Osaka, Japan</td>
<td>19.953 (ng/g dry wt)</td>
<td>Harms et al. 1998</td>
</tr>
<tr>
<td>3</td>
<td>US coast</td>
<td>32.269 (ng/g dry wt)</td>
<td>O'Connor and Lawnstein 2006</td>
</tr>
<tr>
<td>4</td>
<td>Port of Genova, Italy</td>
<td>1784-14151 (ng/g dry wt)</td>
<td>Rivarol et al. 1999</td>
</tr>
<tr>
<td>5</td>
<td>Atlantic coast, Portugal</td>
<td>&lt;14-1195 (ng/g dry wt)</td>
<td>Deiz et al. 2005</td>
</tr>
<tr>
<td>6</td>
<td>Bay of Pian, Slovenia</td>
<td>1222-8554 (ng/g dry wt)</td>
<td>Nemani et al. 2002</td>
</tr>
</tbody>
</table>

**Table 7.1 Previous studies on toxicological studies around the world**

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Organotin</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Daphnia sp (Crustaceans)</td>
<td>96mg/l (24 h EC50)</td>
</tr>
<tr>
<td>2</td>
<td>Daphnia species (Algae)</td>
<td>2.63mg/l (48h LC50)</td>
</tr>
<tr>
<td>3</td>
<td>Fish (fresh water)</td>
<td>2.13 mg/l (96h LC50)</td>
</tr>
<tr>
<td>4</td>
<td>Fish (saline water)</td>
<td>1.33 mg/l (96h LC50)</td>
</tr>
<tr>
<td>5</td>
<td>Oncorhynchus mykiss (fish)</td>
<td>3.66 mg/l (96 h LC50)</td>
</tr>
<tr>
<td>6</td>
<td>Daphnia magna (Crustaceans)</td>
<td>16 mg/l (24 h LC50)</td>
</tr>
<tr>
<td>7</td>
<td>Reduced growth of E. interstitialis</td>
<td>&lt;500mg/l (EC 50)</td>
</tr>
<tr>
<td>8</td>
<td>Artemia salina (Crustaceans)</td>
<td>&gt;40mg/l (24 h LC50)</td>
</tr>
<tr>
<td>9</td>
<td>Daphnia pulex (Crustaceans)</td>
<td>5.7 mg/l (24 h LC50)</td>
</tr>
<tr>
<td>10</td>
<td>Thamnocephalus platyurus (Crustaceans)</td>
<td>12 mg/l (214 h LC50)</td>
</tr>
</tbody>
</table>

**Table 8.1 Organotin in bivalves**

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Organotin</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cancer magister</td>
<td>560μg/l</td>
</tr>
<tr>
<td>2</td>
<td>Penaeus Duorarum</td>
<td>3.162 mg/l</td>
</tr>
<tr>
<td>3</td>
<td>Mytilus edulis</td>
<td>5.94 mg/l</td>
</tr>
<tr>
<td>4</td>
<td>Crassostrea virginica</td>
<td>3.026mg/l</td>
</tr>
<tr>
<td>5</td>
<td>Cyprinodon variegatus</td>
<td>3.033mg/l</td>
</tr>
<tr>
<td>6</td>
<td>Galaxias aceratus</td>
<td>3.029mg/l</td>
</tr>
<tr>
<td>7</td>
<td>Galaxias maculates</td>
<td>23.7 μg/l</td>
</tr>
<tr>
<td>8</td>
<td>Galaxias trettaceus</td>
<td>3.019 mg/l</td>
</tr>
<tr>
<td>9</td>
<td>Leostinus zanthurus</td>
<td>3.032 mg/l</td>
</tr>
<tr>
<td>10</td>
<td>Pseudaphritis urvilii</td>
<td>3.2 μg/l</td>
</tr>
</tbody>
</table>
7.2 Result and Discussion

7.2.1 LC\textsubscript{50} experiment and enzyme assays of Biocides

In order to understand the toxic effect of organotin in the Cochin estuary, ecotoxicological studies were carried out. *Villorita Cyprinoides var Cochinensis* were selected as indicator organism for this investigation. The *Villoritta* species were collected an area which was considered as less human intervention in the Vembanadu lake and been used for the toxicity study. The bivalve were acclimatised for a week in the laboratory with appropriate suitable environment- habitat condition. For the feeding of bivalves, chlorella species were cultured in the laboratory and stock is maintained for this purpose. The LC\textsubscript{50} of each biocides were seperately determined. The sublethal concentration considered from the LC\textsubscript{50} values (1/5\textsuperscript{th}, 1/7\textsuperscript{th} and 1/10\textsuperscript{th} of LC\textsubscript{50}) and the inhibition rate of acetylcholinesterase studies were conducted at 96hr interval. The bivalves were not fed during the study period. On each day, water was changed and fresh toxins were added to maintain the same concentration throughout the experimental period. In the final day, enzyme inhibition activity experiments were conducted. Table 7.2 shows the LC\textsubscript{50} concentrations and its sublethal concentrations of biocides.

**Table 7.2 LC\textsubscript{50} values and sublethal concentrations of Biocides**

<table>
<thead>
<tr>
<th>Toxine</th>
<th>LC-50</th>
<th>1/5\textsuperscript{th} of LC-50</th>
<th>1/7\textsuperscript{th} of LC-50</th>
<th>1/10\textsuperscript{th} of LC-50</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPT</td>
<td>200 ppb</td>
<td>40 ppb</td>
<td>28.57 ppb</td>
<td>20 ppb</td>
</tr>
<tr>
<td>DPT</td>
<td>500 ppb</td>
<td>100 ppb</td>
<td>71.4 ppb</td>
<td>50 ppb</td>
</tr>
<tr>
<td>MPT</td>
<td>300 ppb</td>
<td>60 ppb</td>
<td>42.86 ppb</td>
<td>30 ppb</td>
</tr>
<tr>
<td>Irgarol-1051</td>
<td>200 ppb</td>
<td>40 ppb</td>
<td>28.57 ppb</td>
<td>20 ppb</td>
</tr>
<tr>
<td>Chlorothalonil</td>
<td>400 ppb</td>
<td>80 ppb</td>
<td>57.14 ppb</td>
<td>40 ppb</td>
</tr>
</tbody>
</table>
The LC$_{50}$ concentration of Monophenylnit (MPT) from the experiment is 300 ppb. The three sublethal concentrations of the above LC$_{50}$ were selected and experimental concentrations are 60 ppb, 42.86 ppb and 30 ppb. Acetone was used as solvent to dissolve MPT. Five tanks were maintained in the order; control, solvent control (acetone) and three experimental toxicant (30 ppb, 42.86 ppb and 60 ppb). The solvent control influenced to gives a little more activity than control (Figure 7.2). The three sublethal concentrations showed inhibition of acetylcholine esterase enzyme. So acetylcholine is unable to split at the nerve ganglion which later leads to death of organism. In the present study, a linear relationship between dose-response has been observed. The dose-response relationship established between monophenyl tin trichloride and acetylcholine esterase can be taken as an indicator for the extent of pollution.

In the case of Diphenyltin (DPT), the LC$_{50}$ concentration was found to be 500 ppb and the sublethal concentrations selected for the study were 100 ppb, 71.4 ppb and 50 ppb. The toxins were dissolved in milli-Q water which is used as solvent control and total five tanks were maintained for the experiment. The control and solvent control samples were showed normal physiological activity of the enzymes. The 50 ppb and 71.4 ppb also showed high enzyme activity when compared to control and solvent control (Figure 7.3). Whereas at 100 ppb, it was found that the inhibition of acetylcholine esterase enzyme. This is due to the activation of enzyme synthesis to nullify the effect of stress by increasing the neurotransmission. Hence the acetylcholine regeneration at the nerve ganglions start to diminish in 100ppb and this inhibition
increases as the concentration increases and finally it leads to paralysis and death of the organism.

Triphenyltin (TPT) belongs to a class of chemicals (organotins) known to be immunotoxic. TPT is considered to be a toxicant, although it is not considered to have mutagenic/genetic toxicity properties. It was carcinogenic both in the rat (inducing pituitary and testicular tumors) and in the mouse (inducing liver tumors). It is classified as a B2, possible human carcinogen by all routes of exposure (oral, dermal and inhalation).

The LC$_{50}$ of TPT from present experiment was found as 200 ppb. In order to understand the enzyme activity, three sublethal concentration (1/5$^{th}$, 1/7$^{th}$, and 1/10$^{th}$ of LC$_{50}$). They are 40 ppb, 28.5 ppb and 20 ppb respectively. In the present study, the solvent, ethanol was used as diluent for TPT and it activate the acetylcholin esterase at a very low rate (Figure 7.4). From the graph at 20 ppb (1/10$^{th}$) sublethal concentration, the biocide has almost no specific effect on acetylcholinesterase and the enzyme activity has been increased at 1/7$^{th}$ sublethal concentration. At 28.5 ppb (1/7$^{th}$) the inhibition of acetylcholine esterase is prominent. At higher concentration of biocide 40 ppb (1/5$^{th}$ of LC50) showed the minimum enzyme activity and gives an idea that the excess acetylcholine released from the ganglion were not split into acetyl and choline groups. Without the enzyme, muscles would continue to contract causing spasms in bivalve.
Ecotoxicological studies of Antifouling Biocides

Enzyme Activity of MPT

Specific activity

Concentration: control, control(Solvent), 50ppb MPT, 71.4ppb MPT, 100ppb MPT

Enzyme Activity of DPT

Specific activity

Concentration: control, control(Solvent), 30ppb DPT, 42.86ppb DPT, 60ppb DPT
Similar to OT’s the acetylcholine esterase study were conducted for Chlorothalonil and Irgarol 1051. From the toxicity study conducted the LC$_{50}$ value for Chlorothalonil was 400 ppb. It’s mode of action involves its combination with a molecule called glutathione. When these glutathione-Chlorothalonil derivatives form, they tie up all of the cells’ available glutathione, leaving enzymes glutathione-dependent unable to function. Several enzymes that are important in cellular respiration, the process by which large molecules are broken down and provide the cell with energy, are glutathione dependent (Tillman et al. 1973). In order to determine the effect of Chlorothalonil on acetylcholine esterase enzyme, sublethal concentration of the LC$_{50}$ values were taken. These are 1/5$^{th}$ - 80 ppb, 1/7$^{th}$ - 57.14 ppb and 1/10$^{th}$ -40 ppb. 96 hr experiments were conducted and experimental tanks were maintained for the three sublethal concentrations.
and one control and one solvent control. The solvent, acetone itself has an inhibitory effect on the enzyme and the combined action with Chlorothalonil and acetone increased the inhibitory action in the toxicity experiment (Figure 7.5). All the sublethal concentrations showed a decline in acetyl choline activity. According to EPA reports Chlorothalonil’s acute toxicity through ingestion is low and the median lethal dose ($LD_{50}$) for laboratory animals is found between 5 and 10 grams of Chlorothalonil per kilogram of body weight (US EPA 1993). The control without toxin and solvent showed high normal physiological activity as compared to other samples and the experimental animals were found health. Solvent control and three sub lethal concentrations showed less physiological activity when compared to control organisms. Although Chlorothalonil proponents refer to Chlorothalonil as “not genotoxic,” (toxic to genetic material) (Wilkinson and Killeen 1996), this fungicide has caused genetic damage in mammals in studies of both live animals and cell cultures (Lodovici et al. 1994) earlier.

![Figure 7.5 Graphs showing the AChE action against Chlorothalonil](image)

*Figure 7.5 Graphs showing the AChE action against Chlorothalonil*
Irgarol 1051 has been formulated as a more hydrophobic analogue of triazine herbicides (Ciba-Geigy 1995). The present toxicity study of Irgarol 1051 on marine organism is the first experiment performed in India. Similar to other biocides, the LC$_{50}$ and toxicity experiments were conducted. The LC$_{50}$ value obtained for Irgarol 1051 is 200 ppb. The sub lethal concentrations were 80 ppb, 57.14 ppb and 40 ppb. Irgarol 1051 possesses a combined inhibitory effect on acetylcholinesterase activity and due to the solvent acetone. A slight increase in the enzyme activity was observed in the concentration 1/10$^{th}$ (20 ppb) due to the synthesis of enzyme to overcome the stress caused by Irgarol 1051 (Figure 7.6). The control without toxins and solvent showed the normal high acetylcholinesterase activity showing the healthy condition of the organism. The solvent control showed less activity than the control. The three sub lethal concentrations showed very less activity than to the solvent control.
and control, and thus well illustrates the rate inhibition of choline formation in the ganglions. Irgarol 1051 significantly inhibited periphyton photosynthetic activity at 0.81 mg/l in short-term (h) tests, and with long-term (weeks) exposure, produced significant changes in the community structure at 0.25 mg/l (Dahl and Blanck. 1996). It has also been shown by Scarlett et al. (1997) that Irgarol significantly inhibited the growth of Enteromorpha intestinalis zoospores, a key reproductive stage of the early colonizing alga at its maximum concentration of 127 ng/l that found at Sutton Harbour marina. These toxic levels are within the threshold limit concentration reported for environmental samples and may cause damage to the biota in later stages. The minimum inhibition concentration reported for algae (i.e. Enteromorpha intestinalis) and several diatoms (i.e. Navicula, Nitzschia, Amphora and Achnanthes) is 10 mg/l (Readman et al.1993). In addition, it is toxic to several microalgae with 50% effective concentration (EC50) values ranging from 0.45 to 2.12 mg/l (Toth et al. 1996). Its phytotoxicity to Selenastrum capricornutum and Skeletonema costatum has been reported at 1.26 mg/L (120 h EC50) and 0.45 mg/l, respectively. Acute toxicity to the crustacean mysid shrimp is 400 mg/l (96 h LC50) and 3200 mg/l to oyster larvae Crassostrea virginica (48 h EC50). Theoretically, Irgarol 1051 action mechanism should not affect organisms that consume algae. Nevertheless, some of the long-lived herbivores, dugong and green turtle, are prone to show toxicity (Hall et al. 1999 and Scarlett et al. 1997).

7.3 Conclusion

The present study gives an insight the study of antifouling biocides used in shipping industry and its toxicological effects on bivalves. The results outweighs that the pollution caused by antifouling biocides in the
Cochin estuary is intense when compared to other shipping channels and ports. This may be due to the indiscriminate use of organotin compounds in paints. Many countries banned paints with organotin compounds from the year 2003 onwards. Lack of legislation is the major reason behind this high concentration. The toxicological studies confirm the toxic effects of organotin. Acetylcholinesterase inhibition occurs at different concentration of three phenyltin derivatives. The residual levels of organotin estimated in the various samples collected from the Cochin estuary is found below the LC\textsubscript{50} values. But the long term exposure of this system may cause lethality to the aquatic organisms life in the estuary. A reduction in the density of bivalves in and around the Cochin estuary clearly give this evidence. The residues of Irgarol 1051 and Chlorothalonil were also estimated in the study area which points the intense use of these three compounds in the shipping industry. Detailed study with the biota residing in the estuary would give a clear picture of the extensive use of antifouling biocide in the Cochin estuary.
Reference


Chapter -7


Ecotoxicological studies of Antifouling Biocides


Ecotoxicological studies of Antifouling Biocides


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