Available online at www.elixirpublishers.com (Elixir International Journal)

Pollution

Elixir Pollution 43 (2012) 6859-6864

ISSN: 2229-712X

Predicting NOEC through *Mugil cephalus* exposed to heavy metal concentrations with special reference to Ennore creek, Tamilnadu, India

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ARTICLE INFO

Article history:
Received: 29 December 2011;
Received in revised form:
30 January 2012;
Accepted: 11 February 2012;

Keywords
Mugil cephalus,
Heavy metals,
No Observed Effect Concentration (NOEC),
Ennore creek,
Acute-to-chronic estimation.

ABSTRACT

The present investigation was carried in the experimental test organism, *Mugil cephalus* exposed to cadmium, copper, lead and zinc in acute and chronic toxicity test. The raw survival data from the acute toxicity test were utilized for the prediction of No Observed Effect Concentration (NOEC). The data obtained from the laboratory and the predicted endpoints were then compared with the heavy metal concentrations in the Ennore creek. Acute toxicity tests revealed that fingerlings were sensitive to copper, followed by cadmium, lead and zinc, and the 96-hour LC_{50} values were 4.29 mg/l Cd, 2.29 mg/l Cu, 6.90 mg/l Pb and 7.92 mg/l Zn. Experimental NOEC had strong correlation with the predicted NOEC at P=0.0001 and P=0.005. Correlations were significant at P<0.01 (2-tailed), (α =0.05). The predicted and the experimental NOEC values were lower than the heavy metal concentrations in the Ennore creek. The concentrations of heavy metals in the Ennore creek were relatively higher than the NOEC values for juvenile marine organisms tested in the laboratory. Hence, there exists threat for survival and an urgent need for the enactment and enforcement of stringent laws to control the heavy metal pollution in the Ennore creek as well as to protect estuarine and marine bio-resources.

Introduction

Coastlines, estuaries, creeks, brackish water, lagoons and lakes are the richest resources of India. Many major rivers drain into the Bay of Bengal and are rich in marine fauna of the southeast coast of India. The Chennai coastal region is a typical example of numerous recreational and commercial activities that degrade the quality of coastal water, decrease in coastal resource and destruction of natural defence structures puts the marine biota and man into serious health risk (Palanisamy *et al.*, 2006). Aquatic pollution started long back but intensified during the last few decades, and currently the situation has become alarming in India (Girija *et al.*, 2007). Metals are natural components of the aquatic environment, but their levels have increased due to anthropogenic activities (Shanthi and Gajendran, 2009). Balancing the ecosystem structure and functions, needs several directives over time to protect estuaries and coasts. The environmental quality standards rely on the concentrations of contaminants as quality objectives for comparing the state of sites. The ecological integrity is judged using water in toxicity tests (Tueros *et al.*, 2009). The toxicity tests measure the integrated responses to the possible chronic effects of contaminants, on these processes. Chronic toxicity tests data are generally more reliable and have higher ecological relevance when dealing with a key species for the ecosystem at risk. If biological tests are to be ecologically relevant, the species should be widespread and easily available (Watts and Pascoe, 2000). Fish is an integral component of the aquatic ecosystems. In addition to being a source of protein to man, they play an important role in energy flow, nutrient cycling and maintaining community balances in aquatic ecosystem. Thus utility of fish for assessing environmental conditions in aquatic ecosystem has gained prominence. In recent years fish are considered to act as suitable biomonitors for environmental pollution as they are exposed to the heavy metals *in vitro* and the effect of metals on fish is studied (Altindag and Yigit, 2005; Chari and Abbasi, 2005; Lugowska, 2007; Sikorska and Wolnicki, 2010). A reason for interest in heavy metals and behavior in aquatic communities is that heavy metals may have different behavioral effects at concentrations much less, than at which they have lethal effects, suggesting that regulatory pollution limits based upon standard toxicological studies may be too high to prevent damage to aquatic communities through the sublethal behavioural effects (Klaschka, 2008).

The NOEC, determined statistically by hypothesis testing based on chronic test results is the effect level currently used for environmental protection control (Chapman, 1996). The estimated effect level should be biologically significant; it should protect a high proportion of all species and be predictive of a contaminant concentration that produces adverse effect in the receiving water (Grothe *et al.*, 1996). Modeling is a valuable tool for the prediction of effects resulting from chronic exposure to sub-lethal concentrations of contaminants (Raimondo and McKenney, 2006). Therefore, in the presence study, an attempt was made to evaluate the chronic endpoints through laboratory test and prediction from acute toxicity test with acute to Chronic estimation software (ACE) and to compare with the heavy metal concentrations in the Ennore creek with NOEC values.

Materials and Methods

Study area and sampling methods

Ennore creek (13°13'54.48" N, 80°19' 26.60" E) (Fig. 1) is located in the northeast coast of metropolitan Chennai city, Tamil Nadu, India. Ennore comprises of lagoons, with salt

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marshes and backwaters, and form an arm of the sea opening in to the Bay of Bengal. The total area of the creek is 2.25 sq km and is nearly 400 m wide. Its channels connect it with Pulicat lake to the north and to the Kortalaiyar river in the south (Kannan *et al.,* 2007). Ennore coast receives untreated sewage from Royapuram sewage outfall, untreated / treated industrial effluents from Manali industrial belt, which houses many chemical industries (Palanisamy *et al.,* 2006). In Ennore creek, four sampling stations were selected, Station 1 (bar mouth (13°14'02.31" N, 80°19'49.47" E)), Station 2 (creek (13°13'52.54" N, 80°19' 24.26" E)), Station 3 (Buckingham canal (north towards Pulicat lake (13°14'02.72" N, 80°18' 54.18" E)) and Station 4 (down the railway bridge (13°13'30.39" N, 80°19' 02.30" E)).

Fig. 1. Ennore creek

*Map was designed from ARCGIS 9.0; not to scale

The water samples for trace metal analysis were collected using 1 L polyethylene-tereftalate (PET) bottles and was immediately acidified with 3ml of concentrated nitric acid. The preserved samples transported to the laboratory were filtered through Millipore vacuum pump using 0.45 µm filter paper (HA-Millipore). The filtered samples were acidified using 1.5 ml of suprapure nitric acid analytical quality (Merck) until the pH was adjusted to 2. These samples were stored at 4°C until analysis following internationally recommended protocols (APHA, 1998). The dissolved metals in seawater were extracted according to the method described by El-Moselhy and Gabal (2004) using ammonium 1-pyrrolidinedithiocarbamate (APDC), methyl isobutyl ketone (MIBK), and back extraction with suprapure nitric acid and distilled water. Trace metal concentrations (cadmium, copper, lead and zinc) were measured using Varian SpectraAA 220FS Atomic absorption spectrophotometer (AAS). Suitable internal metal standards (Merck Chemicals, Germany) were used to calibrate the instrument. All the reagents used were analytical grade of high purity.

Test animal and toxicity tests

Juvenile specimens of *Mugil cephalus* collected from Ennore creek (13°13'54.48" N, 80°19' 26.60" E, Tamilnadu, India) were immediately transported to the laboratory in air-filled plastic bags. Test organisms were acclimatized in glass aquaria with aerated natural filtered seawater for a period of 8 days with 28 ppt salinity, temperature of 29 ±2 °C, dissolved oxygen of 5.5 mg/l and pH of 7.9. Captured wild organisms were quarantined immediately with oxytetracycline. After a day of acclimatization, *M.cephalus* was fed with pellets of rice bran and oil cake. The dead animals were removed immediately and remaining detritus were removed by siphoning (USEPA, 1996).

Prior to toxicity tests and stock solution preparations, all the glassware's were washed in 10 per cent nitric acid and rinsed with deionized water. Stock solutions of cadmium, copper, lead and zinc were freshly prepared by dissolving the proper metal salts ($CdCl_2 .2.5 H_2O$ for Cd, $CuCl_2$ for Cu, $Pb (NO_3)_2$ for Pb and $ZnSO_4.7H_2O$ for Zn in deionized (double distilled) water with glass standard flasks. Stock solutions were acidified by the addition of 0.1 ml of concentrated nitric acid per litre of stock solution (Chapman, 1978). Fresh stock solutions were prepared daily. These solutions were serially diluted to get the experimental concentration for the toxicity test.

The experimental method includes static renewal (24-hour renewal) test by following the method of USEPA (2002a). Five concentrations in a geometric series including control were prepared for the test for 30 days for chronic toxicity test (USEPA, 2002b). Toxicant and seawater were replaced on daily basis. Each series of test chambers consisted of triplicates with 10 animals in a 10 L glass trough. Test chambers were loosely covered to reduce evaporation, to prevent loss of test animals. All the experiments were conducted at salinity of 29 PSU, temperature of 29 ±2 °C, dissolved oxygen of 5.6 mg/l and pH of 7.8 with gentle aeration. Water samples for the determination of metal concentrations from different experiment were collected from central point within the test vessel daily from aquarium containing metal concentration in acid-washed, rinsed containers. Test animals were fed regularly three times a day during the test. Each toxicity test lasted 4 days for acute and 30 days for short-term chronic. Daily observations were recorded for survival and mortality. The criterion for determining death was the absence of movement when the animals were gently stimulated. Dead animals were removed at each observation and survivors were counted. Maximum-allowable control mortality was 10 per cent for a 96-hour period of testing and 20 per cent for 30 days for chronic (USEPA, 2002b).

The survival data was transferred to GraphPad prism version 5.00 where, one-way ANOVA (Multiple comparison method: Dunnett (P<0.05)) was performed. Final 'q' value was compared with the Dunnett's critical value table (α = 0.01) (USEPA, 2002b). For 11 degrees of freedom and 6 concentrations, the Dunnett's critical value (α = 0.01) was 3.56. The value of the highest concentration which was significantly different from control and greater than Dunnett's critical value (α = 0.01) was taken as No-observed effect concentration (NOEC) and the lowest concentrations 'q' value which was not significantly different from control and lesser than the Dunnett's critical value (α = 0.01) was taken as Low-observed effect concentration (LOEC). Maximum acceptable tolerance concentration (MATC) was calculated as the geometric mean of NOEC and LOEC, also known as Threshold observed effect concentration (TOEC) or Chronic value (ChV). A computerized Probit analysis program (USEPA Probit analysis program version 1.5) (Probit Program version 1.5) was carried out for the calculations of LC_{50} values at the termination of each test and upper and lower 95 per cent confidence levels were also calculated. PRIMER (Plymouth routines in multivariate ecological research) version 6 (6.1.7) was used to study the parameters through draftsman plot (Clarke and Gorley, 2006). PASW (2009) (Predictive analytical software) (SPSS) version 18 was used to study the correlation statistics.

Acute to chronic estimation (ACE)

ACE is a software model version 2.0, which runs on raw acute toxicity test data to predict the NOEC of the chronic test. This model runs on three methods - Accelerated life testing (ALT), Multifactor probit analysis (MPA), and Linear regression analysis (LRA) (Ellersieck *et al.,* 2003).

Results and Discussion

Dissolved heavy metals in Ennore creek

Heavy metal contamination of the environment is a serious pollution problem and is capable of exerting considerable biological effects even at low levels because of their pervasiveness and persistence nature (Singh and Chandel, 2006). Concentrations of cadmium, copper, lead and zinc in the surface waters of the Ennore creek varied significantly (P<0.001) with respect to stations. One-way ANOVA performed showed a high statistical significance indicating that all measured concentrations in all the stations (P <0.0001, 0.0001, 0.001 and 0.005) were relatively high in Ennore creek. The chief source of cadmium, copper, lead and zinc was found in stations 3 and 4, which was distributed throughout the creek correlating with a high significance in all the stations (Table 1). Studies in Pulicat lake (Tamilnadu, India) recorded an elevated level of heavy metal concentrations, especially iron, cadmium and mercury (Kannan and Krishnamoorthy, 2006). Cadmium concentration in water samples was 0.01 mg/l during premonsoon and postmonsoon. Since Pulicat lake is being considered as an important source of fishery in Tamil nadu, India, the presence of toxic heavy metals in water and sediments would be the primary source for biomagnification of metals in fish, invertebrates and other aquatic plants and animals and will cause ill effects to those who consume contaminated fish. Rajathy and Azariah (1996) reported that the levels of zinc and copper in water and sediment samples showed seasonal fluctuations in the Ennore and Adayar estuary. The evident values of dissolved lead, sustained that a continuous source of this metal occurs within Ennore creek. Dissolved lead concentrations from Ennore creek as presented in this study were higher when compared with those reported by Vazquez *et al.* (1998) for Alvarado lagoon water. In Mexico (0.009-0.063 mg/l), by Kraepiel *et al.* (1997) for the Gironde estuary in France (0.0003 mg/l), and Nayar *et al.* (2004) for the Ponggol estuary in Singapore (0.020 mg/l). Goody *et al.* (2002) reported 0.0002-0.0038 mg/l Pb in UK and Man *et al.* (2004) reported 0.0002-0.185 mg/l Pb in China. The levels of dissolved cadmium in Ennore water presented in this study was in the same magnitude to those reported by Mucha *et al.* (2004) at Douro estuary, in Portugal, as well as those concerned with Jayaprakash *et al.* (2005) was considered as anthropogenically stressed but not strongly polluted systems. The values presented here were much lower than those reported for strongly polluted estuaries, such as the Rio Tinto Ria Huelva on southwest Spain (Achterberg *et al.*, 2003), where the values were 0.045 mg/l Cd. Goody *et al.* (2002) reported cadmium concentrations from the Chalk system, at Salisbury (Wiltshire, UK) of 0.0001-0.0027 mg/l, and values were lower when compared with the present study. All the above literature values were lower, when compared with the concentrations of cadmium in Ennore creek, which clearly indicates that the creek is polluted with cadmium. Hall *et al.* (1996) reported lead concentration in the dissolved phase was between 0.0009-0.0002 mg/l in Scottish estuaries with low anthropogenic input. Dissolved lead concentration in station 1 decreased with increasing salinity, but in station 4 with lower salinities and a complex behaviour exists with high concentration of lead in the downstream direction (Dauby *et al.*, 1994). Increase in dissolved lead in Ennore creek is linked to the high industrialization of the surrounding areas, which produces large amounts of lead-based effluents. Moreover, the shallow depth of the river favours re-suspension of sedimentary matter that can contain a significant level of heavy metal, easily scavenged at the sediment-water interface (Dauby *et al.*, 1994). In the present study conducted in Ennore creek, the heavy metal concentrations were higher than the literature values, indicating pollution of heavy metals in the aquatic environment.

Acute and chronic toxicity test and prediction with ACE

Water quality is a crucial facet for the survival and well-being of the living resources, especially in the coastal and estuarine areas. Assessing the toxicity of contaminants on aquatic life has been a long-standing practice (Smithwick *et al.*, 2005). The fingerlings of *M.cephalus* were sensitive to copper and tolerant to zinc concentrations. The sensitivity of mullet juveniles to metals are in the order of Cu>Cd>Pb>Zn. The 96-hour LC_{50} values were used for the calculation of range for conducting the short-term chronic toxicity test (Table 2). *M.cephalus* were exposed to 10, 20, 40, 80 and 160 µg/l of cadmium and copper; 51, 76, 114, 171 and 256 µg/l of lead; and 29, 46, 74, 118 and 188 µg/l of zinc in static renewal chronic toxicity test. During the toxicity test, temperature was maintained at 28 °C ±0.3, salinity at 28 ±1.2 ppt, 7.78 pH, and dissolved oxygen at 4.9 mg/l. The total hardness varied from 1550 to 1786 ±11.3 mg/l. The recovery of the concentrations in the test chamber ranged from 96.4 to 118 per cent of the nominal concentrations. The chronic endpoints derived with laboratory tests and the predicted are presented in Table 2. Correlation statistics was analyzed between the experimental NOEC and predicted NOEC (ALT, LRA and MPA). Correlation was significant at P<0.05 (α=0.05) (2-tailed) (Fig. 2).

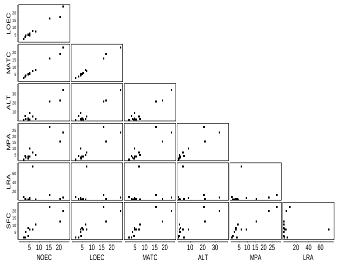

Fig. 2. Draftsman plot, correlation plotted against the experimentally derived and predicted chronic endpoints (r^2=0.88, 0.74, 0.001 (LRA) and 0.72), correlation was significant at P<0.05 (2-tailed) (α=0.05)

Experimental NOEC had strong correlation with predicted NOEC (ALT and MPA), not with the NOEC predicted by LRA method. The experimental and predicted NOEC values flowed in the same line, except for some deviation with LRA. The higher rates of mortality were observed with *M. cephalus* when exposed to copper than lead and zinc in the acute toxicity test (4 days). Taylor *et al.* (1985) reported that the mullet, *Chelon labrosus* showed LC_{50} value of 1.4 mg/l for copper, >4.5 mg/l for lead and 21.5 mg/l for zinc in Continuous flow through system (CFTS) for 4 days and *C.labrosus* was sensitive to copper and gave the sensitivity in the order of Cu>Pb>Zn. Dab, *Limanda limanda* an estuarine fish showed a 96 h LC_{50} of 0.3 mg/l for copper and no value was derived for lead and zinc and Taylor *et al.* (1985) summarized that *L.limanda* was sensitive to copper than lead and zinc. These results correlate the present study that *M.cephalus* was sensitive to copper than lead and zinc.

Mohapatra and Rengarajan (1997) reported that the mullet, *Liza parsia* exposed to lead, copper and zinc in acute toxicity test revealed 96-hour LC_{50} values of 64.7, 21.8 and 13.7 mg/l. Copper was sensitive to *L. parsia* than zinc and lead (Cu>Zn>Pb). Bioassays conducted for 12, 24 and 72 hours with *Tilapia nilotica* by Somsiri (1982) and was reported that the copper is toxic than zinc. Based on 96 hour LC_{50}, copper was found to be toxic to *M.cephalus* than cadmium, lead and zinc in the present study. Concentrations of cadmium and copper used in the present study are regarded to be high for *M.cephalus*. Such concentrations do not occur permanently in surface waters. However, due to accidental industrial discharges of heavy metals into the aquatic environment, fish may have shorter or longer contact with such concentrations of heavy metals. This may be dangerous for fish, especially for larvae that are considered more vulnerable to intoxication caused by heavy metals than embryos or older individuals (Akan *et al.*, 2008). Data on the chronic toxicity of copper to tropical marine organisms were very limited. Coglianese and Martin (1981) reported a rapid increase in the percentage of oyster embryos, which developed abnormally when exposed to more than 6 μg/l copper. Abnormalities included retarded shell growth, stunting and extremely erratic swimming behaviour. The long-term effects on rainbow trout fry and fingerlings exposed to various concentrations of lead for 19 months in hard and soft water, (60 to 100 per cent) developed spinal deformities in hard water at measured lead concentrations of 850 μg/l Pb (USEPA, 1980). However, during the soft water exposure most trout (44 to 97 per cent) developed spinal deformities in measured lead concentrations as low as 31 μg/l Pb. These results strongly demonstrate that lead is more chronically toxic in soft water than in hard water (USEPA, 1980). The reported values for the invertebrates and the fishes were high when compared with the present study. *M.cephalus* was sensitive to copper, followed by zinc, cadmium and lead. Lead was most tolerated by *M.cephalus*. Acute toxicity raw data was used for the prediction of chronic NOEC value and all the predicted NOEC values were high for *M.cephalus*. Accelerated life testing (ALT), Multiple probit analysis (MPA) and Linear regression analysis (LRA) were the three methods used for prediction of chronic NOEC. The correlation between the experimental NOEC and the predicted NOEC by the three methods reveal that the ALT and MPA were significant at P=0.0001 for ALT, P=0.005 for MPA and P=0.91 for LRA. The LRA were not significant with the experimental NOEC, hence this method may not be applied for the prediction of chronic NOEC. Various acute to chronic mortality data extrapolation methods have been proposed (Mayer *et al.,* 1994; Lee *et al.,* 1995; Sun *et al.,* 1995; Warne, 1998). The models included here are more comprehensive approaches to predicting chronic toxicity, both toxicologically and statistically. The acute toxicity value represents only one point in time (96 hour LC_{50}), and the relationship of degree of response with duration of exposure should be essential when chronic toxicity is predicted from acute toxicity data (Sun *et al.*, 1995). A time to response approach gives a better understanding of the progression of toxic effects over time, and survival time modeling has shown great applicability in toxicological studies (Crane *et al.,* 2002). In the present study, the predicted (ALT) and the experimental NOEC values were lower than the heavy metal concentrations in the Ennore creek, hence the fingerlings thriving in the creek would have created the impact on the growth and life cycle in particular.

Conclusion

Estuaries are highly sensitive zones subjected to heavy industrialization and overpopulation. Heavy metal contamination of the environment, which has been recognized as a serious pollution problem, is capable of exerting considerable biological effects even at low levels because of their pervasiveness and persistence nature. Ideally, these assessment tools promote the sustainability of ecosystems and pinpoint early symptoms of exposure in order to prevent the progression of environmental degradation whilst conditions are still reversible. Protection can be accomplished if causes associated with effects are quantifiable and can be used to generate preventive guidelines. The guidelines should be protective for as many species as possible and should be flexible to ensure the generation of new data that would provide maximum protection. The challenge that remains for ecologists and ecotoxicologists is the definition of what effects on the ecosystem are acceptable or unacceptable in relation to the most sensitive endpoints on the species level. Thus in the present study, an attempt was made to establish an certified programme to predict the chronic endpoints from acute toxicity test, which would largely reduce the usage of test animals and laboratory infrastructure for conducting chronic toxicity test and developments in risk assessment models should focus on the translation from laboratory species to field communities.

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Table 1. Heavy metals concentrations in the Ennore creek at four stations

Heavy metals	Station 1	Station 2	Station 3	Station 4
Cadmium (Cd)	0.012 ±0.13	0.013 ±0.19	0.017 ±0.07	0.024 ±0.15
Copper (Cu)	0.065 ±2.56	0.118 ±0.34	0.131 ±2.70	0.142 ±3.55
Lead (Pb)	0.078 ±2.98	0.198 ±1.04	0.122 ±1.97	0.226 ±1.63
Zinc (Zn)	0.198 ±2.93	0.223 ±1.47	0.256 ±2.27	0.372 ±3.57

*Values were significant at $P<0.05$ (one way ANOVA), $P<0.001$ (n= 4), Correlation was significant at $\alpha=0.05$ ($P< 0.05$) when compared between station 1 and 2, 3, 4 Values are mean and standard deviation each n=3

Table 2. *Mugil cephalus* exposed to cadmium, copper, lead and zinc in acute and chronic toxicity test with the predicted NOEC

Heavy metals	96 hour LC_{50} mg/l (95%LCL-UCL)	Experimental chronic end points (µg/l)			Predicted chronic end points (µg/l)		
		NOEC	LOEC	MATC	ALT	MPA	LRA
Cadmium (Cd)	4.29 (3.29 – 5.56)	160	80	113	119	216	27
Copper (Cu)	2.29 (1.91 – 2.95)	40	20	28	45	133	141
Lead (Pb)	6.90 (5.34 – 8.89)	171	114	140	183	324	188
Zinc (Zn)	7.92 (6.33 – 9.84)	118	74	93	115	388	439

Correlations were significant at $P<0.01$ (2-tailed), (α =0.05), except LRA, when correlated experimental (NOEC) and predicted chronic endpoints (ALT, MPA and LRA), except LRA all the values were significant ($P<0.0001$, $P=0.0003$; 0.91 and 0.005, $r^2=0.88$, 0.74, 0.001 and 0.72 for ALT, MPA and LRA); NOEC-No-Observed Effect Concentration, LOEC-Low-Observed Effect Concentration, MATC-Maximum Acceptable Tolerance Concentration, ALT-Accelerated Life Testing, MPA-Multifactor Probit Analysis, LRA- Linear regression analysis