



## Preliminary study on diminution level of RNA/DNA ratio in tissue of *Labeo rohita* by exposure to some endocrine disrupting compounds (EDCs)

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### ABSTRACT

Effects of EDCs particularly on RNA/DNA ratio are yet to be investigated to manage the effluents in natural waters. We investigated exposure effects of endocrine disrupting compounds (EDCs) phthalic acid ester (PAE) and hexachlorocyclohexane (HCH) on the RNA/DNA ratio in tissue of an Indian major carp *Labeo rohita*. Fish were exposed to pre-determined sublethal concentrations of phthalic acid ester (Di-methyl phthalate (DMP), di-butyl phthalate (DBP), and di-(2-ethylhexyl) phthalate (DEHP) and also HCH for determining the tissue RNA/DNA ratio after 30, 60 and 90 days of exposure in the doses of 0.2 mg L<sup>-1</sup>, 0.3 mg L<sup>-1</sup>, and 0.5 mg L<sup>-1</sup> respectively. All these tested chemicals significantly (P<0.05) inhibited RNA/DNA ratio. The ratio gradually significantly (P<0.05) decreased after DEHP where it was 1.9±0.51 F1, 18=15.8 P=0.014 n=19; in case of DBP it was 1.92±0.62 F1, 20=6.5 P=0.012 n=19 and for HCH it was 0.94±0.21 F1, 18=18.08 P=0.0012 n=19 at treatments concentrations of 0.3 mg L<sup>-1</sup> and 0.5 mg L<sup>-1</sup>, compared to control (2.9±0.2) after 90 days. However, there was no statistical significance (P>0.05) in RNA/DNA ratio after the DMP (F1, 20=2.4 P=0.15n=21) treatment.

**Keywords:** DMP, DEHP, DBP, HCH, endocrine disruptors, growth, reproduction

### INTRODUCTION

Currently, residual pharmaceutical compounds, agricultural runoff, domestic effluents, livestock waste, personal care products, industrial waste are reported to be present in aquatic environment and are generally recognized as a source of environmental pollutants (Gehring *et al.*, 2002; Ying *et al.*, 2002; Ankley *et al.*, 2003; Cespedes *et al.*, 2004; Harris *et al.*, 2005; Singh and Srivastava, 2013; Tijani *et al.*, 2013). Due to the presence of these compounds, there occurs a change in the chemical composition of natural aquatic environment which may affect the non-target aquatic organisms, particularly fish impairing with its reproductive physiology. Such contaminants can lead to interactions between the chemicals and biological systems, including physiological changes in fish (Schwaiger *et al.*, 2004). Many of these compounds are categorized as endocrine disrupting compound (EDCs). In our Indian River, presence of compounds like HCH, Bisphenol-A, polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides (OCPs) are reported plasticizers like phthalic acid ester (Singh *et al.*, 2005; Singh *et al.*, 2007; Srivastava *et al.*, 2010; Verma *et al.*, 2013).

The muscle of the fish contaminated with photochemical ozone creation potential (POCPs) (Organochlorine pesticide (OCPs) has been published to range between 2.58 and 22.56 ng g<sup>-1</sup> (Malik *et al.*, 2004; Singh *et al.*, 2005a). Although, these compounds are most frequently detected in rivers, its potential toxic effects on aquatic organisms (e.g., fish) remains principally unknown. It is well known that many environmental contaminants could produce severe damage in different organs of fishes and alter the activities of enzymes. Toxic pollutants interfere with energy yielding reactions indirectly inhibiting the synthesis of RNA, DNA and protein (Kim and Kang, 2004; Li *et al.*, 2010).

RNA/DNA ratio is useful indicators of natural or anthropogenic impacts in river, marine population and communities, such as upwelling or dredge fisheries (Chícharo and Chícharo, 2008). Ratio provides a measure of synthetic capacity of cell, but also could be a potential tool for reflecting environmental stress (Li *et al.*, 2009a; Li *et al.*, 2010) and nutritional level of cell (Chícharo and Chícharo, 2008). The objectives of this study were to investigate the effects of different doses of EDCs such as Phthalic acid ester (DMP, DBP, and DEHP) and HCH on the RNA/DNA ratio in muscle of *Labeo rohita*.

## MATERIALS AND METHODS

### Time and Location

Actively moving healthy immature *Labeo rohita* (total length, 22.5±2 cm, weight, 158±10.2 g, n=150) were collected from one of the culture ponds, Lucknow during the period from Dec 2013 to Dec 2015. Immediately after the collection, specimens were maintained in glass aquaria having 150 L water and they were acclimatized to the laboratory conditions for two weeks.

### Experimental Fish and Feed

After the acclimation period, 150 immature *Labeo rohita* of 158±10.2 g weight and 22.5±2 cm length were divided equally and kept into four glass aquaria containing 150 L water in triplicate. Fish of each aquarium were given 0.2 mg L<sup>-1</sup>, 0.3 mg L<sup>-1</sup> and 0.5 mg L<sup>-1</sup> of all Phthalic acid ester similarly, in other group of experiment fish of similar length and weight were maintained in triplicate and were exposed to 0.2 mg L<sup>-1</sup>, 0.3 mg L<sup>-1</sup>, 0.5 mg L<sup>-1</sup> HCH. Every 10<sup>th</sup> day, the water of each aquarium was replaced with fresh water containing similar dose of phthalic acid ester and HCH. The test chemical phthalic acid ester was diluted in carrier solvent DMSO (1%) whereas HCH carrier solvent used was acetone (Naciff *et al.*, 2005). These diluents were also mixed in control group as vehicle solution. After 90 days of exposure, length and weight of each group of fishes was recorded with the help of digital calipers scale and digital electronic balance respectively.

Fish were fed with pond collected zooplankton twice daily and also with commercially available fish food pellets (Tyio Pvt. Ltd. India). Twelve treatment groups were set up to study the effect of 99% pure Phthalic acid esters DMP, DBP and DEHP, and HCH (procured from Sigma Aldrich USA) on the RNA/DNA ratio in *Labeo rohita*.

### RNA/DNA Ratio Analysis

The muscle samples (100 mg ml<sup>-1</sup>, w/v) of each experimental group of fish was homogenized for 5 min in 5% trichloroacetic acid (TCA) at 90°C and then centrifuged at 5000 rpm for 20 min. For the determination of RNA, 2.0 ml of distilled water and 3.0 ml of orcinol reagent (1g orcinol +100 ml HCL+ 0.5g Ferric chloride) was added in 1.0 ml of supernatant. The reaction mixture was kept in boiling water bath for 20 min. The greenish-blue colour thus developed was read at 660 nm in a spectrophotometer. For DNA determination, 1.0 ml of distilled water and 4.0 ml of freshly prepared diphenylamine reagent (1 g Diphenylamine reagent + 100 ml glacial acetic acid + 2.75 ml con H<sub>2</sub>SO<sub>4</sub>) were added to 1.0 ml of the supernatant. The reaction mixture was kept on a boiling water bath for 10 min. The blue colour developed was measured with spectrophotometer at 600 nm. Standard curves for RNA and DNA were drawn using different concentrations of yeast RNA and calf thymus DNA, respectively. The values were expressed as µg 100 mg<sup>-100</sup> fish muscle tissue on dry basis. For the calculation of results, average of the duplicate readings for each standard, control and samples were obtained after subtracting the average zero standard optical density. Created a standard curve on log-log graph paper, with concentration on the y-axis and absorbance on the x-axis. Then drew the best fit straight line through the standard points and it was determined by regression analysis.

### Statistical Analysis

All data are reported as means ± standard deviation (SD). Differences between the control and each exposure treatment group were evaluated by one-way analysis of variance (ANOVA) and parametric multiple comparisons with group 1 as control performed by Dunnett test. P< 0.05 was considered statistically significant. All analyses were performed with SPSS 16.0 (SPSS, Chicago, IL, US).

## RESULTS AND DISCUSSION

The group of fish treated with Di-methyl phthalate (DMP) showed significant (P<0.05) decrease of RNA/DNA ratio in high dose during the experimental period when compared with the control (Figure 1a). However, DBP and DEHP treated groups showed significant decrease only in the dose of 0.3mg/L, 0.5mg/L when compared to control. (Figure 1b and Figure 2a). In control group RNA/DNA ratio ranged from 2.2±0.01 to 2.12±0.09, which decreased to 1.9±0.48 (F1, 20 =6.5 P=0.012) in DBP treated the dose of 0.3mg/L after 60 days of exposure, and 1.9±0.02 (F1, 20=7.4 P=0.01) after 90 days of exposure. In DEHP group the RNA/DNA ratio was 1.9± 0.14 (F 1, 20 =11 P= 0.07) after 60 days and 1.85±0.9 (F1, 20) 11.8P= 0.04) (P<0.05) after 90 days.

The experimental group of fish treated with HCH showed a gradual and significant decrease of RNA/DNA ratio in different exposure concentration (Figure 2b) RNA/DNA ratio ranged from  $2.6 \pm 0.09$  to  $2.74 \pm 0.05$  in control group and in HCH treated group it was significantly decreased in  $0.2 \text{ mg L}^{-1}$ ,  $0.03 \text{ mg L}^{-1}$ ,  $0.5 \text{ mg L}^{-1}$  it was  $1.9 \pm 0.13$  ( $F_{1,20}=5.8$   $P=0.02$ ,  $n=21$ )  $1.3 \pm 0.14$  ( $F_{1,20}=6.5$   $P=0.012$   $n=21$ )  $1.04 \pm 0.07$  ( $F_{1,20}=5.4$   $P=0.015$ ) after 30 days. It gradually decreased after long time exposure significantly ( $P < 0.05$ ) and the reduction was  $1.6 \pm 0.22$  ( $F_{1,18}=18.08$   $P=0.0012$ ), in  $0.02 \text{ mg L}^{-1}$ ,  $1.8 \pm 0.17$  ( $F_{1,18}=15.3$   $P=0.018$ )  $0.3 \text{ mg L}^{-1}$ ,  $1.45 \pm 0.07$  ( $F_{1,20}=8.5$   $P=0.01$ )  $0.5 \text{ mg L}^{-1}$  after 60 days. There was also significant ( $P > 0.01$  Dunnett test) reduction after 90 days where the values were  $1.9 \pm 0.13$  ( $F_{1,18}=4.41$   $P=0.05$ ),  $1.4 \pm 0.18$  ( $F_{1,20}=8.8$   $P=0.01$ ) and  $0.89 \pm 0.05$  ( $F_{1,20}=8.4$   $P=0.014$ ) after  $0.02 \text{ mg L}^{-1}$   $0.3 \text{ mg L}^{-1}$  and  $0.5 \text{ mg L}^{-1}$  exposures respectively.

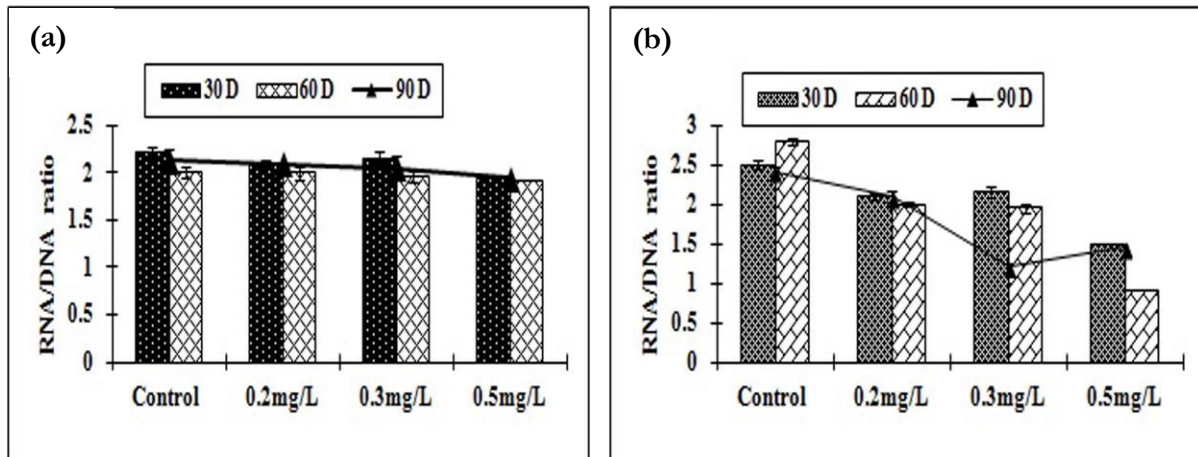


Figure 1. (a) Value of RNA/DNA ratio in *Labeo rohita* exposed to  $0.2 \text{ mg L}^{-1}$ ,  $0.3 \text{ mg L}^{-1}$  and  $0.5 \text{ mg L}^{-1}$  DMP for 90 days. (Y axis) represents the ratio, (b) Value of RNA/DNA ratio in *Labeo rohita* exposed to  $0.2 \text{ mg L}^{-1}$ ,  $0.3 \text{ mg L}^{-1}$  and  $0.5 \text{ mg L}^{-1}$  DBP for 90 days (Y axis) represents the ratio. The data are presented as the mean  $\pm$  standard deviation ( $n=20$ ) statistically significant differences according to a one-way ANOVA ( $P < 0.05$ ) Dunnett test compared to control.

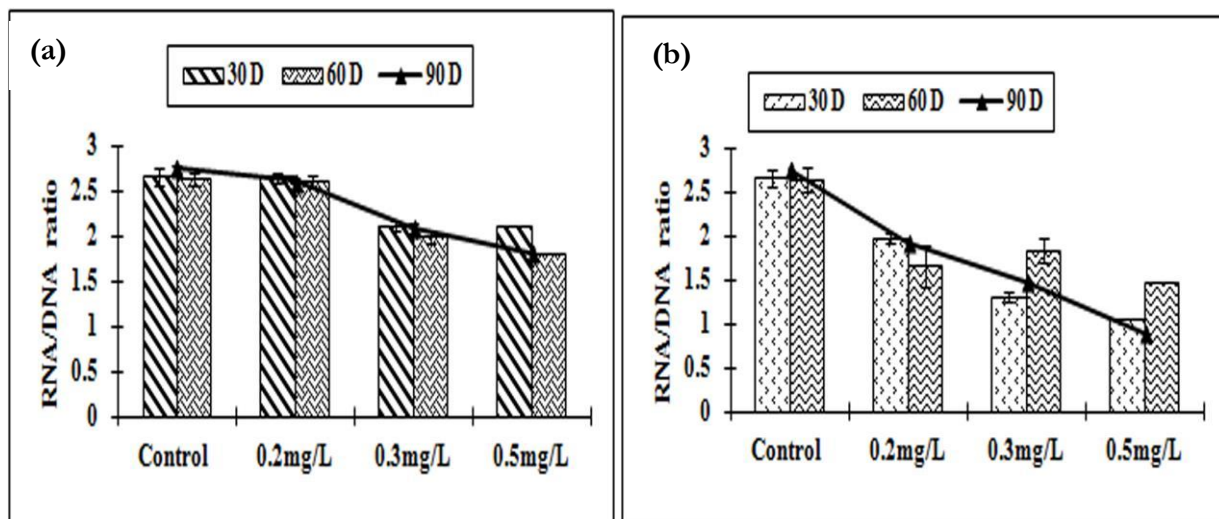


Figure 2. (a) Value of RNA/DNA ratio in *Labeo rohita* exposed to  $0.2 \text{ mg L}^{-1}$ ,  $0.3 \text{ mg L}^{-1}$  and  $0.5 \text{ mg L}^{-1}$  DEHP for 90 days (Y axis) represents the ratio, (b) Value of RNA/DNA ratio in *Labeo rohita* exposed to  $0.2 \text{ mg L}^{-1}$ ,  $0.3 \text{ mg L}^{-1}$  and  $0.5 \text{ mg L}^{-1}$  HCH for 90 days (Y axis) represents the ratio. The data are presented as the mean  $\pm$  standard deviation ( $n=20$ ) statistically significant differences according to a one-way ANOVA ( $P < 0.05$ ) Dunnett test compare to control.

The results of this study did not show any change in RNA/DNA ratio in DMP exposed group. Unlike the observations in this study, there are many earlier studies showing that several endocrine disrupting compound, phthalic acid ester and, HCH effect on GSI, cell death, sex differentiation,

reproductive defects, oxidative stress, steroid hormone concentration and RNA/DNA ratio in tissue (Tripathi *et al.*, 2000; Rakshekhar, 2012).

Similarly, environmentally relevant concentrations of organotin compounds (OTs) was found to trigger sex changes in marine invertebrates (An *et al.*, 2013) where it was reported that the RNA/DNA ratio was significantly ( $p < 0.05$ ) lower, and a slight increase in DNA damage was observed in females bringing about imposex individuals rather males. Parallel results were obtained by Li *et al.*, (2010), after long-term exposure to carbamazepine (CBZ) on the enzymatic alterations and RNA/DNA ratio in intestine tissue of rainbow trout. In an another study, the application of RAN/DNA ratio to evaluate the effects of toxicants on fish was reported, because a depressed RNA/DNA ratio was found in fish exposed to metal and organic contaminants (James and Sampath, 1999).

However, there are some studies which did not find any relation between the RNA/DNA ratio and the changes of environmental contaminants (Kim and Kang, 2004). Analogous revision done by Rathod and Kshirsagar *et al.* (2010) investigated the effect of sublethal concentration of two different kinds of pesticides Fenvalerate (synthetic pyrethroid) and Monocrotophos (organophosphate) for 24, 48, 72 and 96 hrs and observed declined level of DNA and RNA contents in selected tissues like gills, liver, kidney and muscle of freshwater fish *Puntius arenatus* (Day) due to exposure of different concentration of two different kinds of pesticides. Tripathi *et al.* (2003) also reported that fish exposed to Dimethoate (organophosphate) exhibited decreased nucleic acid (DNA and RNA) content. Related results was also reported by Wu and Or (2005), and the RNA:DNA ratio was most sensitive, and decreased significantly (by 50 to 86%) following exposure to 4.5 and 3.5 mg O<sub>2</sub> l<sup>-1</sup> for 1 week. Peakall (1992) reported that RNA/DNA ratios are useful but non-specific indicators of recent growth and general nutritional conditions in a variety of animals including mollusks, crustaceans and fish. Furthermore, RNA/DNA ratios were positively correlated with the somatic growth (Sambhu and Jayaprakas, 1997).

## CONCLUSIONS

From the above results and interpretation it can be concluded that the RNA/DNA ratio is precious indicator for the well being of the fishes i.e., growth and development, as it was observed that ratio was highly affected due to exposure of pesticides and endocrine disrupting compounds. The group of fish treated with DEHP showed significant decrease in RNA/DNA ratio in high dose. This needs to be explored further requiring a keen research to justify the relation of fish growth and the reproductive development in fishes after exposure of several EDCs compound. Moreover from the above study it can be concluded that due to exposure of toxic EDCs there is gradual loss in RNA/DNA content in muscle which alleviate the nutrition level of fishes.

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