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Effects of endocrine disruption by 4-nonylphenol ethoxylate on the growth performance and immune response of female and male immature koi carp *(Cyprinus carpio carpio)*

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Abstract

Nonylphenol (NP) is an endocrine disrupting chemical which has been shown to be able tomodulate endocrine system of various organisms by different mechanisms. The objective of this study was to investigate the potential effects of 4-NP and 17-β -estradiol (E2) on the immune parameters (IgM levels and lysozyme activity) of the teleost Koi carp (*Cyprinus carpio carpio*) for a better understanding of the immune-reproductive system interactions. The experimental fishes were injected with ascending doses (10, 50,100 $\mu g g^{-1}$ body weight) of 4- nonylphenol (4-NP) and (2 $\mu g g^{-1}$ body mass) of 17-β-estradiol (E2) or vehicle during 3 weeks. After 21 days, the fishes (180) were anesthetizedand their blood samples were collected from caudal vein, then they were dissected and sexually separated by gonad characters. The measurement of immune parameters in plasma showed that 4-NP induced significant increase in the IgM levels and lysozyme activity at dose of 50 $\mu g g^{-1}$ while the levels of these parameters in the higher doses (100 $\mu g g^{-1}$) decreased compared with the control group (p<0.05). In addition the treatment, with 2 $\mu g g^{-1}$ E2 significantly decreased both the IgM levels and lysozyme activity after 21 days of injection. These results indicated that 4-NP and E2 could lead to disturb the balance of immune system with potential consequences for immature koi carp.

Keywords: 4-nonylphenol, 17-β-estradiol, Immune-reproductivesystem, Immunoglobulin IgM, Lysozyme, Koi carp *(Cyprinus carpio carpio)*

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Introduction

Over the past two decades there has been increasing awareness that contaminants can act through the endocrine system to have impacts on humans and wildlife (Colburn *et al.*, 1997; Harries *et al.*,1997). In the past years much concern have been raised about the Alkylphenols (APs), because they represent one of the most important categories of Endocrine Disrupting Chemicals (EDCs) (Monosson, 2000; Sumpter, 1995). They are the degradation products of alkylphenol, alkylphenol polyethoxylates (APEOs), an important group of nonionic surfactants commonly used in many formulated products for industrial, agricultural, and domestic applications (Tyler *et al.*,1998). About 60% of APEOs end up in the aquatic environment, they are incompletely degraded to alkylphenols (Aps) such as nonyl-phenol (NP) and 4-tertoctylphenol (t-OP), stable hydrophobic substances that tend to bioaccumulate in tissues of aquatic organisms (Jobling *et al.,* 1996). Alkylphenol polyethoxylates (APEs), and one of their degradation products, 4-nonylphenol (4-NP), are compounds of significant environmental concern due to their estrogenic effects (Cravedi and Zalko, 2005). NP is the major by-product of nonylphenol ethoxylates (NPEs), a large group of nonionic surfactants employed in lubricating oils, emulsifiers, synthetic rubber, plasti, paints, household and industrial detergents, paper and textile products (Vazquez-Duhalt *et al.,* 2005; Soares *et al*., 2008). Exposure to sub lethal levels of these compounds has been noted to evoke a variety of lesions such as gill necrosis degenerative changes in the muscles, and various inflammatory degenerative and necrotic change in heart, liver and spleen (McCormick *et al*., 2005). It has been demonstrated that the NPEs, can mimic the natural endogenous hormone estrogen and thus have the ability to interact with the endocrine system of fish (Jobling and Sumpter, 1993). Exposu-re to estrogen or EDCs is also known to modulate immune responses and reproductive performance of fish (Hoeger *et al*., 2005; Liney *et al*., 2006; Ziari *et al.*, 2015). In sparids, enhancement of gilthead seabream serum complement and agglutinating activities (Herna'ndez and Tort, 2003) coincided with the post-spawning period, when both E2 and T peaks have been reported (Chaves-Pozo *et al.*, 2005). In vivo exposure to estradiol has been shown to modulate the immune response to the hemoflagellate (*Trypanosoma danilewski*) (Wang and Belosevic, 1999). Additionally Yamaguchi *et al*. (2001) obtained similar results when they used physiological concentrations of in vitro administered estradiol and primary leucocytes from carp (Yamaguchi *et al*., 2001). Recent work by Cuesta *et al.* (2007) has also demonstrated the modulatory effects of estradiol on the complementary activity, serum peroxidase activity and IgM in sea bream. The present study was designed to assess the effect of three different concentrations of a

xenoestrogen, 4-NP on the levels of total immunoglobulin M (IgM) and lysozime activity in immature koi carp (*Cyprinus carpio carpio).*

Materials and methods

Fish

One hundred and eighty immature koi carp (*C. carpio carpio*) of both sexes, measuring (14±0.35 cm mean length, and mean body weight 55±0.5g n=90 female) and (15±0.39 cm mean length and mean body weigh 54±0.7 g n=90 male) respectively, were obtained from a local hatchery of ornamental fish in Tehran city in April 2015. Fish transferred immediately to the fishery laboratories at Faculty of Marine Sciences in Ollom Tahghighat University.

In the laboratory the fish were randomly selected, weighed, measured, then divided into six groups. They were kept in 18 glass aquariums (100×30×50 cm^3) (10 fish per aquarium), filled with de-chlorinated water. Rearing water was aerated and filtered through activated carbon before being added into the aquariums. The water temperature was maintained at 24±1 °C. The pH was 7.5±0.3, light intensity was 1000 lux and the photo period was set at (12D: 12L). Prior to the experimental period, the fish were acclimatized to the laboratory condition for 15 days. During the acclimatization fishes were fed with carp commercial dry pellets at 2% of bodyweight twice per day at 9:00 am and 7:00 pm. All animal care procedures were performed in accordance with the standards set forth in the guidelines for the care and use of experimental animals by the committee for the purpose of control and supervision of experiments on animals (CPCSEA) and the National Institutes of Health (NIH) (http://icmr.nic). They were fasted for 24 h before injection and throughout the experiment. Fecal particles were removed from bottom of the aquarium with siphon during water exchange every day.

Exposure to NP

Branched 4–nonylphenol (CAS No. 84852-15-3, 95.3% pure) was obtained from Schenectad International (Schenectady, NY, USA). 17β–estradiol was obtained from Sigma –Aldrich, Germ (E2, Sigma 98.5% pure) and used as the test xenoestrogen. These compounds were mixed with coconut oil at the appropriate amounts to achieve five treatment doses of NP (10, 50 and 100 $\mu g g^{-1}$ body mass) and one treatment dose of E_2(2 $\mu g g^{-1}$ body mass). Doses of 4-NP were based on fish studies that reported disrupting effects after intraperitoneal (i.p) injection of this compound (Arukwe *et al.*, 1997; Christensen, 1999; Yadetie *et al*., 1999; Casini *et al*., 2002). A total of 90 female koi carp and 90 male koi carp were used in the experiment. There were six exposure groups (30 individuals per group) with three replicates of each. Ten randomly chosen male or female fish, were placed in each treatment aquarium. At the start of experiment the fishes were anesthetized with 2-

phenoxyethanol 0.1% (Merck Germany) and their length and weight were recorded, and then the fishes of each treatment were injected intraperitoneally with vehicle only the corresponding concentrations of 4-nonyphenol (4-NP 5, 10, 50 and 100 μg g^{-1} body mass), and, 17-β-estradiol (E2 2 μg g^{-1} body mass), respectively, while the control group II (Positive control-C2) received the vehicle (50μL of coconut oil+ 50μL Ethanol) only.

Control group I, removed intact and not injected served as control (Sampling and providing plasma after 21 days-C1). Control group III, considered as initial blood sampling group (Sampling and provided plasma at zero time–C0). Fishes were injected on day 7, 14 and 21 after the initiation of the experiment. No mortality was observed during the experiment.

Sampling

On day 22, the fishes were anaesthetized, dissected and separated by sex gonad characters and total length and weight were measured (Ahmadnezhad *et al.*, 2013). Then blood was collected from the caudal vein using heparinized syringes and transferred into ice chilled vials (all samples were collected between the hours of 8 and 10 am). Additionally as initial blood (sampling and provided plasma at zero time-C0). Plasma was separated by centrifugation at 3000 RPM for 10 min and frozen at 80°C until analysis.

Immunological assays

Detection of immunoglobulin M (IgM)

Total IgM was determined following the method of Siwicki and Anderson (1993). The assay was based on the measurement of total protein content in plasma using a micro protein determination method (C-690; Sigma) prior to and after precipitating down the IgM molecules employing a 12% (w/v) solution of polyethylene glycol (Sigma). The difference in the protein contents was considered as the IgM content (Siwicki *et al*., 1994).

Lysozyme level

Lysozyme level in plasma was determined by the turbidimetric assay in microplates according to the method of Ellis (1990). Results were expressed in units of lysozyme per mm plasma. One unit is defined as the amount of sample causing a decrease in absorbance of 0.001 min at 450 nm (Ellis, 1990).

Statistical analysis

The statistical analyses were carried out using SPSS Version 16.0 for windows (SPSS Inc USA). Data were checked for normal distribution (Shapiro-Wilk's test) and homogeneity of variances (Levene's test) using P–P plot analysis. Data was evaluated by one way analysis of variance (ANOVA) followed by the NP exposure for each endpoint relative Duncan's test to examine the effects of the control group. The level of significance was set at (p<0.05) (Zar, 1999).

Results

Physicochemical characteristics

The mean values of the physic-chemical parameters (DO, total alkalinity, temperature and pH) of water in the experimental aquarium were within the conducive range during the experimental period (Table1).

Biological parameters

No mortality occurred during the treatment period. Body mass remained similar to initial experimental values (50–60g body mass) with no differences among the groups (Tables 2 and 3). Furthermore, there were no significant differences in the size, length and weight of fish among treatment aquariums (p>0.05) (Figs. 1 and 2).

Table 1: Physicochemical characteristics of water in experimental aquarium over a 3-week exposure period.

Parameter	Week 1	Week 2	Week 3
DO (mg L^{-1})	5.03±0.15	5.03±0.05	5.00±0.10
Temp (ºC)	25.66±0.57	27.33±0.57	26.66±0.57
TA(mg L^{-1})	34.66±1.15	35.33±1.15	34.00±0.0
pH	7.1±0.17	7.03±0.05	7.1±0.0

DO: dissolved oxygen; TA: total alkalinity.
Data are presented as mean±SD.

Table 2: Biological parameters of female koi carp injected with different doses of 4-NP for 21 days.

Parameters	Treatment				
	Control 1	Control 2	10 µg 4NP g^{-1}	50 µg 4NP g^{-1}	100µg 4NPg^{-1}
Initial weight (g)	54.66 ±4.50	54±4.10	53.55±2.92	53.77±2.48	52.77±3.11
Final weight (g)	56.66±4.50	56.66±4.50	56.16±2.78	55.33 ±2.23	53.88 ±3.15
Initial length (Cm)	15±2.0	15.16±1.89	14.66±1.56	14.44 ±1.26	14.33 ±1.27
Final length (Cm)	15±2.0	15.16±1.89	14.66±1.56	14.44 ±1.26	14.33 ±1.27

Data in the same row no significantly different (p>0.05).
Data are presented as mean±SD.

Table 3: Biological parameters of male koi carp injected with different doses of 4-NP for 21 days.

Parameters	Treatment				
	Control 1	Control 2	10 µg 4NP g^{-1}	50 µg 4NP g^{-1}	100µg 4NPg^{-1}
Initial weight (g)	53.66 ±2.51	54.66±2.51	53.33 ±2.51	53±2.64	53.33 ±2.51
Final weight (g)	53.66±2.51	55.83 ±2.84	54.83 ±2.75	55±2.64	54.83 ±2.56
Initial length (cm)	14.83±1.25	14.66±1.89	15.16±1.60	15.66 ±1.52	15.33 ±1.75
Final length (Cm)	14.83±1.25	14.66±1.89	15.16±1.60	15.66 ±1.52	15.33 ±1.75

Data in the same row no significantly different (p>0.05).
Data are presented as mean±SD.

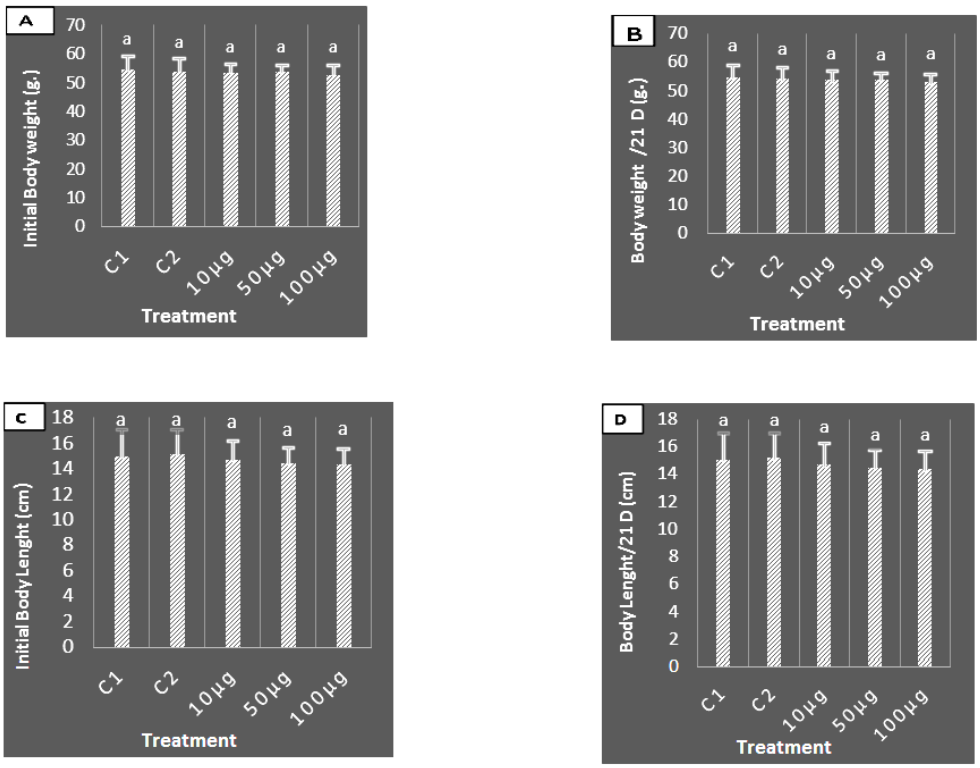

Figure 1: Average of initial body weight (A) and body weights 21 days after the initiation of the experiment (B) of immature female koi carp (mean±SD), injected with different doses of 4-NP. Results are the mean of 15 fish per treatment. Obtained results indicated that, fish growth was similar in all treatments and no significant differences were observed compared with control groups (p>0.05).

Average of initial length (C) and body length 21 days after the initiation of the experiment (D) of immature female koi carp (mean±SD), injected with different doses of 4-NP. Results are the mean of 15 fish per treatment. No significant differences were observed between the treatments and control groups (p>0.05).

Average of initial Length (C) and body length 21 days after the initiation of experiment (D) of immature male koi carp (mean±SD), injected with different dose of 4-NP. Results are the mean of 15 fish per treatment. No significant differences were observed between the treatments and control group (p>0.05).

Immune responses

The effects of the 4-NP on the immune responses of immature koi carp are shown in Tables 4 and 5. Immune responses measured (IgM levels and lysozyme activity) were significantly increased (p<0.05) in response to the median employed dose of 4-NP (50 µg 4NP g^{-1}) 21 days after the commencement of the experiment, whereas the treatment with higher doses of 4-NP (100 µg 4NP g^{-1}) and fishes treated with (2 µg E2 g^{-1}), showed a significant decrease in immune response compared with treatments 1 and 2 (p<0.05).

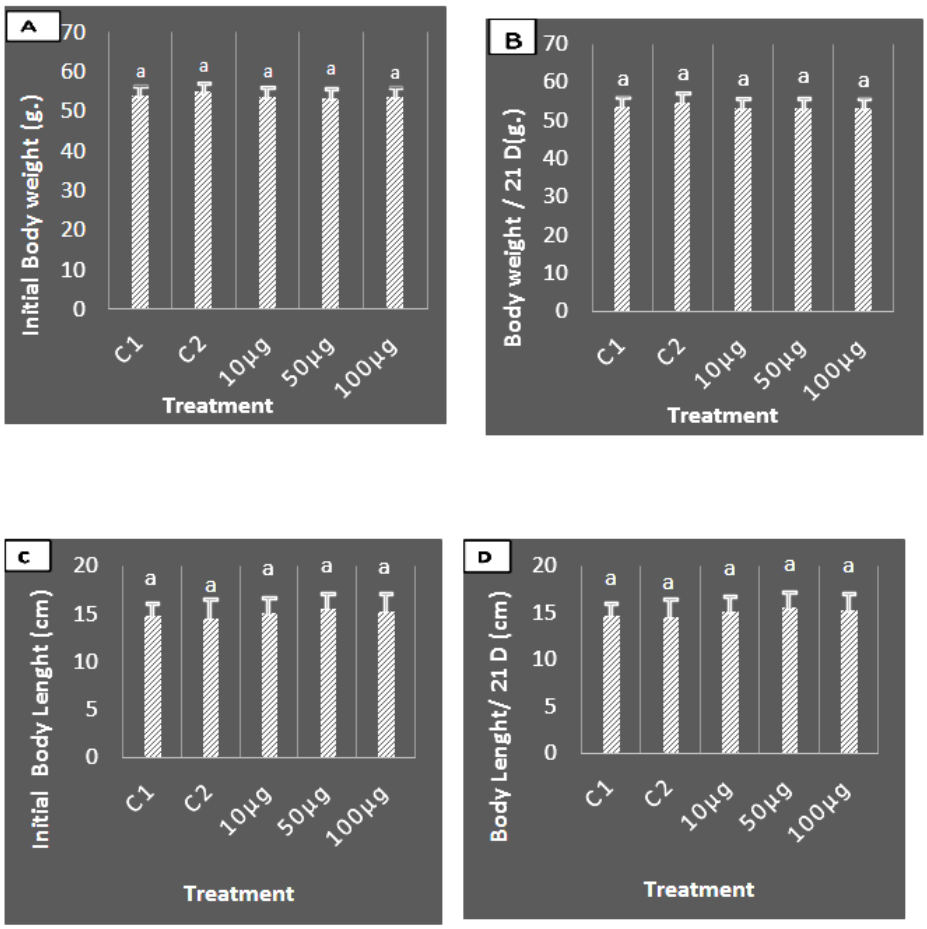

Figure 2: Average of initial body weight (A) and body weights 21 days after the initiation of experiment (B) of immature male koi carp (mean±SD), injected with different doses of 4-NP. Results are the mean of 15 fish per treatment. Obtained results indicated that, fish growth was similar in all treatments and no significant differences were observed among the treatments and control groups (p>0.05).

Table 4: Immune responses of female koi carp injected with different doses of 4-NP for 21 days.

Parameters	Treatment						
	Control 1	Control 2	Control 0	10µg4NPg $^{-1}$	50 µg 4NP g $^{-1}$	100 µg 4NPg^{-1}	2µgE2g^{-1}
IgM (ng mL^{-1})	41.33±3.51	41.66 ±4.16	32±2.02	53±5.01	55.66 ±2.51	37.66 ±1.25	45±4.01
Lysozym (ng mL^{-1})	24.66±1.52	25.66 ±2.08	22±2.02	51±2.02	64.33 ±2.08	29.66 ±6.11	33 ±5.01

Data in the same row by the same letters no significantly different (p>0.05).
Data are presented as mean±SD.

Table 5: Immune responses of male koi carp injected with different dose of 4-NP for 21 days.

Parameters	Treatment						
	Control 1	Control 2	Control 0	10µg4NPg $^{-1}$	50 µg 4NP g $^{-1}$	100 µg 4NPg^{-1}	2µgE2g^{-1}
IgM (ng mL^{-1})	41.66±5.03	42±3.6	30±2.08	55.33±3.05	59±2.01	42.66 ±5.03	45±2.30
Lysozym (ng mL^{-1})	30.66±1.52	32±2.02	21±2.01	53±4.58	62.66±2.51	39.33 ±1.52	34 ±2.05

Data in the same row by the same letters no significantly different (p>0.05).
Data are presented as mean±SD.

Furthermore the control II group that only received coconut oil did not exhibit any significant changes in comparison to the other control groups (p>0.05).

IgM levels

As Fig. 3 shows 4-NP had a notable influence on the plasma IgM levels of female (A) and male (B) koi carp. IgM levels were significantly higher in treatments 1 and 2 and peaked at 50 µg 4NP g^{-1} in treatment 2. The concentration of immunoglobulin IgM in treatment 2 with the amounts of (55.66±2.51 ng mL^{-1} female) and (59±2.01 ng/mL male) compared to the control groups (41.33±3.51 ng mL^{-1} female) and (41.66 ±5.03 ng mL^{-1} male), was significantly different (p<0.05). Plasma IgM levels showed a significant decrease in fish treated with 100 µg g^{-1} of 4-NP with the amounts of (37.66±1.25 ng mL^{-1} Female) and (42.66±5.03 ng mL^{-1} male) and fish treated with 2 µg E2 g^{-1} with the amount of (45±4.01 ng mL^{-1} female) and (45±2.30 ng mL^{-1} male) compared with treatments 1 and 2 (p<0.05). However, the control group II which only received coconut oil did not significantly change by 4-NP treatment during the experiment period (p>0.05). Furthermore, plasma IgM levels in the control group III, significantly changed compared with control groups (p<0.05).

Lysozyme activity

The effects of 4-NP on the lysozyme activity of plasma in female (A) and male (B) koi carp are shown in Fig.4. Significantly elevated lysozyme activity was observed in fish treated with 50 µg g^{-1} of 4NP after 21days of the start of the experiment (p<0.05). The concentrations of lysozyme activity in treatment 2 with the amount of (64.33±2.08 ng mL^{-1} female) and (62.66±2.51 ng mL^{-1} male), were significantly different (p<0.05) compared to the control group (41.33±3.51 ng mL^{-1} female) and (41.66±5.03 ng mL^{-1} male), whereas the treatments with the higher doses (100 µg 4NP g^{-1}) with the amount of (29.66±6.11 ng mL^{-1} female) and (39.33±1.52 ng mL^{-1} male) and the treatment with (2 µg E2 g^{-1}) with the amounts of (33±5.01 ng mL^{-1} female) and (34±2.0.5 ng mL^{-1} male) resulted in a considerable decrease compared with treatment 2 (p<0.05). However, the control II group that only received coconut oil did not significantly change by 4-NP treatment during the experiment. Furthermore, plasma lysozyme activity in the control group III, significantly changed compared with the other control groups (p<0.05).

Discussion

The results of this study indicate that 4-NP can substantially change the IgM levels and lysozyme activity in *C. carpio carpio*. Plasma IgM levels and lysozyme activity were clearly elevated in response to the median dose (50 µg g^{-1} bw) of 4-NP 21 days after the commencement of the experiment (Figs. 3 and 4).

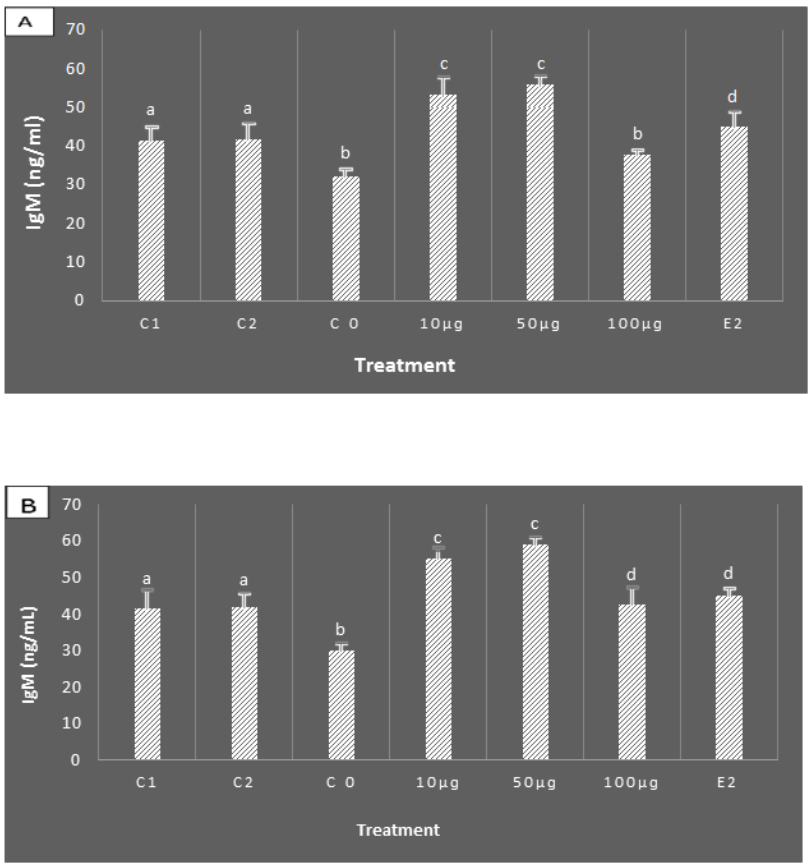

Figure 3: Effects of 4-NP on the total serum IgM level of immature female koi carp (A) and male koi carp (B) 21 days after the start of the experiment. Fish received intra-peritoneal injection with different doses of 4-NP. Values are expressed as means±SD (n=15 for each treatment). Different letters stand for statistically significant differences from control groups (p<0.05).

Whereas the treatments with the higher doses of 4-NP (100 µg g^{-1} bw) and fish treated with (2 µg E2 g^{-1}) showed a significant decrease compared with treatments 1 and 2 (p<0.05). Furthermore the control II group that only received coconut oil did not exhibit any significant changes compared with the control group (p>0.05). The results obtained in this study demonstrated the high estrogenicity of 4-NP on the fish which can effectively suppress, both the plasma IgM levels and the lysozyme activity in koi carp (*C. carpio carpio*). In fish, Ig are the major component of the adaptive humoral immune response. Fish were thought to have only one immunoglobulin isoform, the IgM. The fish IgM is tetrameric instead of pentameric as it occurs in mammals. Both membrane and soluble forms are observed by alternative processing of the mRNA (Wilson *et al*., 1995). In fish cellular and humoral non-specific and specific immune mechanism are present.

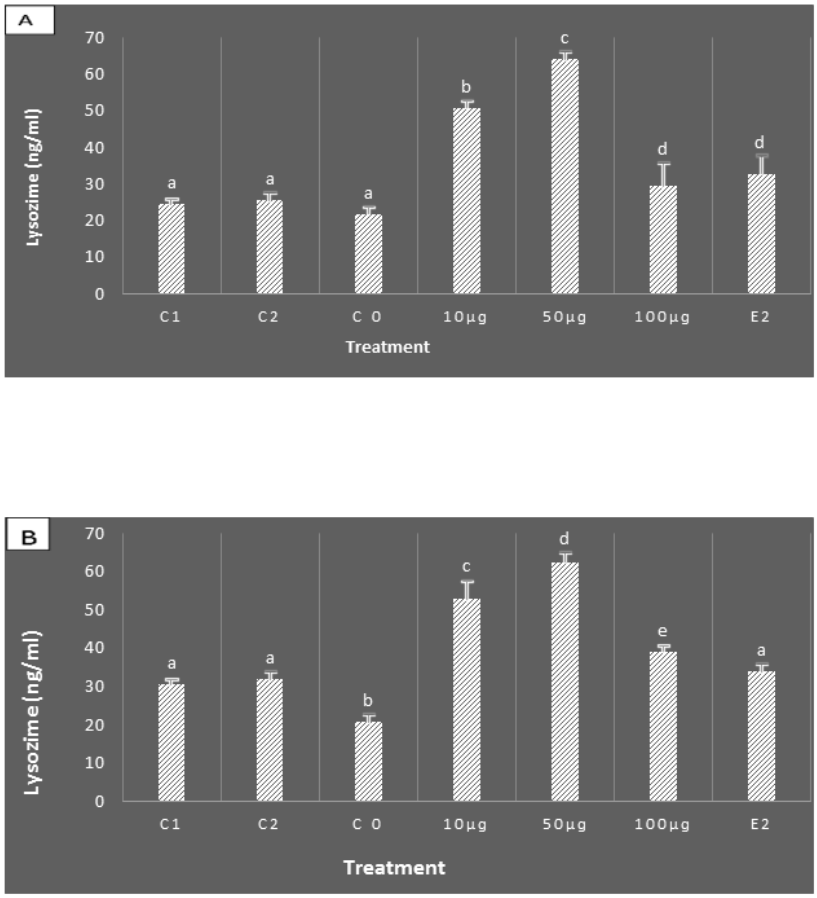

Figure 4: Effects of 4-NP on the lysozyme activity of the serum in immature female koi carp (A) and male koi carp (B), 21 days after the start of the experiment. Fishes received intra-peritoneal injection with different doses of 4-NP. Values are expressed as means±SD (n=15 for each treatment). Different letters stand for statistically significant differences from the control groups (p<0.05).

Fish immune response may serve as an alternate or additional model for predicting the immunotoxicity of the environmental contaminants as shown by many researchers (Tavares-Dias and Moraes, 2007; Witeska, 2010). NP is known to inhibit LPS-induced NO (granulocyte associated) and TNFα (Cytokine receptor-tumor necrosis factor-α) production which is attributed to an ER (estrogen receptor–Era and ERb) dependent inhibition of NF- κB (Cytokine receptor enhance the immune/inflammatory response by activating the NFkB signaling pathway) transactivation (You *et al*., 2002). Besides the estrogens play a role in the hematological homeostasis by mediating lymphocyte proliferation. Reduced mitogen induced T-cell and B-cell proliferation associated with elevated EDCs of blood levels were observed in several species (Luebke *et al*., 1997), indicated that Polycyclic aromatic hydrocarbons (PAHs) exposure reduced the lymphoproliferative response in medaka and deeper analysis led the authors to suggest that the targets were

the T-cells, since neither the LPS-induced B-cell proliferation and antibody–forming cells were unaffected (Luebke *et al.*, 1997). By contrast liquid creosote (3-10 µL L^{-1}),containing PAHs, exposure of rainbow trout produced decreased respiratory burst of head–kidney leucocytes but increased phagocytic activity and percentage of Ig+cells at short exposition times. However, after 28 days, respiratory burst and phagocytic activity returned to control levels while the count of B cells remained decreased (Karrow *et al.*, 2001). Moreover the treatment of rainbow trout with 10-70% sewage plant effluents (containing PAHs among other contaminants), also reduced the number of circulating lymphocytes but increased their in vitro proliferation capacity. Strikingly this effluent failed to alter any other immune functions such as respiratory burst phagocytosis, lysozyme activity, leucocyte populations other than lymphocytes and *Aeromonas salmonicida* specific IgM production (Hoeger *et al.*, 2005). By contrast intra-peritoneal (ip) injection of diesel oil based drilling mud extracts produced no effect on the IgM levels and complement activity, suppression of the serum lysozyme, and elevated head–kidney lymphocyte proliferation in response to phytohemaglutinin (Tahir and Secombes, 1995). The modulatory effects of estradiol on complement activity, serum peroxidase activity and IgM in gilthead sea bream by Cuesta *et al*. (2007) had also demonstrated that intra-peritoneal (ip) injection of E2 treatment enhanced the complement activity 1 day post-injection and peroxidase after 3 and 7 days. Concomitantly, E2 treatment suppressed complement activity and production of IgM at the latest experimental time points (Cuesta *et al.*, 2008). These results coincide with the suppression of IgM synthesis and inhibition of IgM producing cells in rainbow trout (Hou *et al.*,1999). 17-β- estradiol (E2) has been shown to induce lymphocyte proliferation and IgM production in some studies (Cook, 1994; Suzuki *et al*., 1997;Thilagam *et al.*, 2009), but other studies led to opposite conclusions (Wang and Belosevic, 1994) (Suzuki *et al*, 1996; Hou *et al.*, 1999; Hou and Han, 2001; Cuesta, 2007). Moreover androgens are negatively correlated with plasma IgM during the reproductive cycle in rainbow trout (Suzuki *et al.*, 1997). In accordance with these physiological impacts, the AR agonist TBT causes a decrease in lymphocyte numbers and inhibits lymphocyte proliferation. The relationship between sex steroids and the number of leukocytes underscores the difficulty of maintaining immune homeostasis in maturing fish (Misumi *et al*., 2004; Harford *et al.*, 2005). Taken together, these studies indicate that estrogen-like EDCs depress the immune proteins.

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known to inhibit LPS-induced NO (granulocyte associated) and TNFα (Cytokine receptor-tumor necrosis factor-α) production which is attributed to an ER (estrogen receptor–Era and ERb) dependent inhibition of NF- κB (Cytokine receptor enhance the immune/inflammatory response by activating the NFkB signaling pathway) transactivation (You *et al*., 2002). Besides the estrogens play a role in the hematological homeostasis by mediating lymphocyte proliferation. Reduced mitogen induced T-cell and B-cell proliferation associated with elevated EDCs of blood levels were observed in several species (Luebke *et al*., 1997), indicated that Polycyclic aromatic hydrocarbons (PAHs) exposure reduced the lymphoproliferative response in medaka and deeper analysis led the authors to suggest that the targets were the T-cells, since neither the LPS-induced B-cell proliferation and antibody–forming cells were unaffected (Luebke *et al.*, 1997). By contrast liquid creosote (3-10 µL L^{-1}),containing PAHs, exposure of rainbow trout produced decreased respiratory burst of head–kidney leucocytes but increased phagocytic activity and percentage of Ig+cells at short exposition times. However, after 28 days, respiratory burst and phagocytic activity returned to control levels while the count of B cells remained decreased (Karrow *et al.*, 2001). Moreover the treatment of rainbow trout with 10-70% sewage plant effluents (containing PAHs among other contaminants), also reduced the number of circulating lymphocytes but increased their in vitro proliferation capacity. Strikingly this effluent failed to alter any other immune functions such as respiratory burst phagocytosis, lysozyme activity, leucocyte populations other than lymphocytes and *Aeromonas salmonicida* specific IgM production (Hoeger *et al.*, 2005). By contrast intra-peritoneal (ip) injection of diesel oil based drilling mud extracts produced no effect on the IgM levels and complement activity, suppression of the serum lysozyme, and elevated head–kidney lymphocyte proliferation in response to phytohemaglutinin (Tahir and Secombes, 1995). The modulatory effects of estradiol on complement activity, serum peroxidase activity and IgM in gilthead sea bream by Cuesta *et al*. (2007) had also demonstrated that intra-peritoneal (ip) injection of E2 treatment enhanced the complement activity 1 day post-injection and peroxidase after 3 and 7 days. Concomitantly, E2 treatment suppressed complement activity and production of IgM at the latest experimental time points (Cuesta *et al.*, 2008). These results coincide with the suppression of IgM synthesis and inhibition of IgM producing cells in rainbow trout (Hou *et al.*,1999). 17-β- estradiol (E2) has been shown to induce lymphocyte proliferation and IgM production in some studies (Cook, 1994; Suzuki *et al*., 1997 ;Thilagam *et al.*, 2009), but other studies led to opposite conclusions (Wang and Belosevic, 1994) (Suzuki *et al*, 1996; Hou *et al.*, 1999; Hou and

Han, 2001; Cuesta, 2007). Moreover androgens are negatively correlated with plasma IgM during the reproductive cycle in rainbow trout (Suzuki *et al.*, 1997). In accordance with these physiological impacts, the AR agonist TBT causes a decrease in lymphocyte numbers and inhibits lymphocyte proliferation. The relationship between sex steroids and the number of leukocytes underscores the difficulty of maintaining immune homeostasis in maturing fish (Misumi *et al*., 2004; Harford *et al.*, 2005). Taken together, these studies indicate that estrogen-like EDCs depress the immune proteins.

According to the above mentioned results, the activity of lysozyme in the plasma of fish injected with the median dose (50 $\mu g g^{-1}$ bw) of 4-NP treatment had meaningful differences with the control groups (p<0/05), whereas lysozyme activity were found to be decreased in 3 and E2 treatment in *C. carpio carpio* in 21 days after the start of the experiment (p<0/05). The humoral immune response is a compilation of proteins and glycoproteins with defense functions found in the fish plasma and other body fluids such as mucus or sexual products. An important bacteriolytic enzyme is the lysozyme, mainly found in eggs, mucus, plasma and leucocytes (Magnadottir, 2006). In agreement with this study several of innate immune proteins have been demonstrated to be targets for estrogenic compounds. Some of these compounds are known to have effects on plasma lysozyme activity. EE2, NP and BPA also indicate that innate immune proteins may be affected by estrogenic compounds (Moens *et al.*, 2006). In fish, lysozyme disrupts the cell walls of gram+bacteria by exposure to PCB via the diet caused a decrease in lysozyme enzymatic activity in the mucus of Arctic charr (*Salvelinus alpinus*) (Maule *et al*., 2005). Nakayama *et al*. (2008) had also evaluated the effects of heavy oil contamination (3.8 g L^{-1} for 3 days) in Japanes flounder (*Paralichthys olivaceus*) using cDNA microarrays. They have found an alteration of expression in immune related genes including down-regulation of immunoglobulin light chain, CD45, major histocompatibility complex class II antigens and macrophage colony stimulating factor precursor, and up-regulation of interleukin-8 and lysozyme. Morevere, in vitro incubation with oil, pure and single PAHs, of European sea bass plasma produced significant changes in lysozyme and alternative complement activities (Nakayama *et al.*, 2008). Similarly PAHs mixture spiked- sediments (10 mg kg^{-1} dry wt) failed to change the serum lysozyme but reduced the ROS activity of kidney leucocytes of Dab *(Limanda limanda)* (Hutchinson *et al.*, 2003). Moreover, Dunier and Siwicki (1994) have also demonstrated, in rainbow trout, intra-peritoneal injection of lindane (10-100 mg kg^{-1} bw) greatly depressed the number of antibody-secreting cells, serum lysozyme levels, respiratory burst activity and

myeloperoxidase (contributes together with ROS and RNI to pathogen killing) proliferating capacity of cells, but not of T cells, and its percentage in the head-kidney but at the same time increased the plasmatic ceruloplasmin, an acute phase protein (Dunier and Siwicki, 1994). PCBs mixture (Aroclor 1242, 1254 and 1260) failed to modify lysozyme and ROS activity in L.limanda (Vazquez-Duhalt *et al.*, 2005). Siwicki *et al*. (1990) indicated that, trichlorfon exposure decreased the serum lysozyme, lymphocyte proliferation respiratory burst and phagocytosis of common carp leucocytes (Siwicki *et al*., 1990), but unchanged the production of specific antibodies (Cossarini-Dunier *et al*., 1990). Among them phenol, pyrocpyrocatechol and hydroquinone decreased the cell-mediated cytotoxic activity of spleen lymphocytes in common carp (Taysse *et al.,* 1995). Additionally, pentachloro –phenol reduced macrophage production of cytokines in goldfish (Chen *et al*., 2005), but activated phagocytosis and unaltered other immune functions and disease resistance in rainbow trout (Shelley *et al.,* 2009). On the other hand, the stimulation of specific immunity has ever been reported to be associated with changes in sex-steroid levels. For instance, serum E2 levels were dramatically reduced in mildly infected seabream, *(Sparus sarba)*, and remained at a low level during the spread of the infection. Also changes in acquired immunity are sometimes observed in fish treated with estrogeno mimetic.

This effect is first detected by measuring the level of antibodies that are specific against various Bacteria Aroclor1254, TBT, and NP suppressed the level of specific antibodies when given alone or in mixture (Rice and Xiang, 2000; Iwanowicz *et al.,* 2009). Recent work by Jin *et al*. (2009) has also demonstrated zebrafish embryos exposed for 3 days to 17α-ethynyestradiol, permethrin atrazine and nonylphenol (0.1-12.5 μg L^{-1}) altered the expression of immune- relevant genes (TNFα, IFN, IL-1β, IL-8, CXCL-Clc, CC-chemokines, iNOS, etc.) and indicating their single and combined effects upon fish immune response (Jin *et al*., 2009). Also in channel catfish, the injection of PCB 126 (ER antagonist) at 0.01 mg kg^{-1} increased the number of specific antibody secreting cells (SASC) against *Edwardsiella ictaluri* (Regala *et al*., 2001). In contrast, PCB 12 exposure caused a decrease in the number of plasma antibodies against Vibrio anguillarum but did not influence the acquired response to *Listonella anguillarum* in Chinook salmon (*Oncorhynchus tshawytscha)* (Powell *et al*., 2003), when administered at 1 mg kg^{-1}. Thus, although the impact of EDCs on specific antibody production appears real, the effect (stimulatory versus inhibitory) is inconsistent. Overall, changes in leukocyte functioning, immune-related proteins or antibody production by xenoestrogens gens, renders the animal more sensitive to

pathogens. This study demonstrated that 4-nonyl-phenol caused immunological impairment in fish which weakened its immune system and ultimately led to death of the fish.

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