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Effects of xenostrogen treatment (4-Nonylphenol, Bisphenol A) on vitellogenin expression in juvenile *Cyprinus carpio*

Samar Mortazavi[1]*, Ali Reza Riyahi Bakhtiari[2], Abbas Esmaili Sari[2], Fatemeh Rahbarizadeh[3], Nasrin Hassanzadeh[1]

1. Department of Environmental Sciences, Faculty of Natural Resource and Environment, Malayer University, Hamedan, Iran
2. Department of Environmental Sciences, Faculty of Natural Resource and Marine Science, Tarbiat Modares University, Noor, Mazandaran, Iran
3. Department of Medical Biotechnology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

** Corresponding author's E-mail: mortazavi.s@gmail.com*

ABSTRACT

The aim of this study was to examine some potential effects of 4-Nonylphenol (4-NP), Bisphenol A (BPA), and mixture of them on common carp, *Cyprinus carpio* and demonstrate induction of vitellogenin (Vtg) as a biomarker for screening aquatic ecosystems. These compounds with other estrogenic chemicals may be responsible for disrupting effects observed in fish. A total of 100 juvenile carp were treated experimentally with 17β-estradiol (E2), and increasing doses of 4-NP, BPA and also mixture of them. Then the plasma Vtg levels were measured using indirect competitive ELISA. Results showed a significant ($P<0.05$) increase in Vtg levels of exposed carps with E2, maximum 4-NP, BPA and their mixture. The group treated with E2 showed high induction (490%) with respect to controls. The groups treated with maximum dose of 4-NP and BPA showed also induction of Vtg, whereas the group treated with their mixture (3 mg kg^{-1} b.w. 4-NP and 0.01 mg kg^{-1} b.w. BPA) showed the highest induction (2126%). Finally, results showed that mixture of 4-NP and BPA exhibited stronger (synergistic) effects. So that, large scale monitoring of estrogenic effects caused by contamination may be considered as a biomarker in urban and industrial effluents.

Key words: Vitellogenin (Vtg), *Cyprinus carpio*, 17β-Esteradiol (E2), 4-Nonylphenol (4-NP), Bisphenol A (BPA), ELISA.

INTRODUCTION

Environmental problems caused by estrogenic compounds are mostly related to aquatic ecosystem (Matozzo *et al.* 2008). During the last decades, the use of aquatic systems as final receptors of urban and industrial waste waters has increased noticeably, leading to a decrease in the general quality of shores (De los Rios *et al.* 2012), ending in alterations in the composition and structure of communities and finally, damaged ecosystems (Estácio *et al.* 1997; Smith & Shackley 2006; Okus *et al.* 2008; Stein & Cadien 2009; DelPilar Ruso *et al.* 2010).

In such conditions, treated and untreated municipal and industrial sewage, agricultural activities, livestock wastes and system treatment plants (STPs) are considered as the major sources of xenoestrogens entering aquatic ecosystem (Ying *et al.* 2002; Atkinson *et al.* 2003; Tashiro *et al.* 2003; Kuster *et al.* 2004; Matozzo *et al.* 2008). Since sewage and manure are often used as fertilizer, detergents and agricultural effluent are considered as other sources of hormonal pollution in aquatic environments (Orlando *et al.* 2004; Soto *et al.* 2004). Consequently, large amounts of xenoestrogens are discharged in aquatic environments, where they may adsorb to sediments and last for quite a long time (Lai *et al.* 2000).

Among chemicals proven to be endocrine disrupting chemicals (EDCs), alkylphenols (APs) and alkylphenolethoxylates (APEs) are two of the most important classes, not only for the quantity released into the environment, but also for their proved estrogenic activity (Solé *et al.* 2000; Riva *et al.* 2010). Almost all of the

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APEs manufactured worldwide are nonylphenolet- hoxylates (NPEs), a large group of nonionic surfactants used in lubricating oils, emulsifiers, plastics, latex paints, household and industrial detergents, and in the paper and textile industry (Lee, 1999). After usage, NPEs are released into municipal and industrial sewage treatment plants, gradually degrading to nonylphenols (NPs), whose eventual compartment is the aquatic environment (Ahel *et al.* 1994; Taylor & Harrison 1999; Matozzo *et al.* 2008). Among NPs, 4-nonylphenol (4-NP) has been identified as the most important metabolite due to its high resistance to biodegradation, toxicity and intense estrogenic effects (Taylor & Harrison 1999). In addition, 4-NP is a lipophilic compound (log Kow=4.48, (Ahel & Giger 1993) and may therefore be taken up and bio-accumulated by aquatic organisms.

Another important compound existing in the effluent is Bisphenol A (BPA). While primarily applied as an intermediate and additive monomer in plastics and epoxy resins (Hohenblum *et al.* 2004), it is also used as a photo-initiator, or as an additive in insecticides, pharmaceuticals, and adhesives. In 1993 the annual universal production of BPA hit 640,000 tons, out of which an estimated 0.017% (109 tons) were discharged into the environment (Benjonathan & Steinmetz 1998; Staples *et al.* 1998; Matozzo *et al.* 2008).

Due to phenyl ring in their chemical structures, BPA and APs usually possess similar endocrine disrupting activities in general, and estrogenic ones in particular (Gutendorf & Westendorf 2001; Kwack *et al.* 2002; Xu *et al.* 2005). By binding to estrogenic receptors, these compounds can mimic the action of endogenous estrogens.

In fish, one of the best-proven effects of xenoestrogens is the induction of vitellogenins (Vtg), base of the egg-yolk proteins, which provide energy reservoirs for embryo development in oviparous organisms. Vtgs. are high-density glycolipophosphoproteins having Ca and Zn ligands (Montorzi *et al.* 1994; Denslow *et al.* 1999; Matozzo *et al.* 2008; Scognamiglio *et al.* 2016).

Vtg is basically a female particular serum protein. Although the liver synthesizes Vtg in females in reaction to stimulation by 17β-estradiol (E2) (Truscott *et al.* 1992), in males and juveniles Vtg is normally not noticeable. However, exposure to EDCs may cause the induction of Vtg to measurable concentrations even in male and juvenile fish. This has been shown in various laboratory and field studies (Jensen & Ankley 2006; Vega-Lopez *et al.* 2006; Zha *et al.* 2006; Reinen *et al.* 2012).

As pollutants usually occur as mixtures in real aquatic ecosystems, fish are unlikely to be exposed to just one estrogenic chemical. Since we ideally need to know how a fish responds to a mixture of estrogenic chemicals, rather than to an individual chemical, we should try to stimulate the real world, considering the interactions between different chemicals in a mixture, which may result in either a weaker (antagonistic) or a stronger (synergistic) effect.

The main objective of the current study was to examine some potential mechanism(s) of action for 4-NP, BPA, and the mixture of these compounds in sediments of Anzali Wetland, on *Cyprinus carpino*, inhabiting this aquatic ecosystem. The results of the study will relate to the use and design of in vitro bioassays to characterize the endocrine modulating potential of these chemicals and similar compounds.

MATERIALS AND METHODS

4-Nonylphenol (4-NP), Bisphenol A, and 17β-estradiol were purchased from Sigma Aldrich Company (St. Louis, Missouri, USA). Stock solutions of these chemicals were prepared in peanut oil (Sigma) and stored at 20°C in refrigerator in tightly-closed and dark-glass bottles. Juvenile common carps (*Cyprinus carpio*) of 9-10-month old's weighing 80-110 g were obtained from Nasr fish farming, around (airport road) Sari city of Iran, the hatchery of the fish culture. The fish were transferred to the research center of Tarbiat Modares University and were kept in a big fiber glass tank, held in a 12:12 h light-dark photoperiod and not fed during the experiment. The water supply was spring water, quality parameters of which including ammonia, nitrite, dissolved oxygen (DO), pH, hardness, and conductivity were monitored weekly (Table 1). No significant tank-to-tank or week-to-week differences in any of the water quality parameters were indicated (Villeneuve 2000; Casini *et al.* 2002). Water temperature was 17°C and dissolved oxygen was preserved above 60% of the saturation level by continuously aerating the test solutions.

After 5 days, the carps were divided into 9 groups of 9 specimens kept in separate tanks (20 L). The control group was injected intraperitoneally with peanut oil; of the 8 exposed groups, one was injected with 10 mg kg^{-1} b.w. 17β-estradiol in peanut oil and three others were injected with 3, 30 and 60 mg kg^{-1} b.w. 4-nonylphenol in peanut oil respectively. Three of the remaining groups were injected with 0.1, 1 and 10 mg kg^{-1} b.w. Bisphenol A, and

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the last group was injected with 3 and 0.1 mg kg^{-1} b.w. mixtures of 4-Nonylphenol and Bisphenol A. The treatment was repeated after intervals of 7 days for 3 times. 3 days after the last injection, blood samples were obtained from the dorsal vein by puncture for vitellogenin analysis. Blood was collected into heparinized 5ml glass tubes and centrifuged for 4 min at 4000g in a centrifuge. Plasma was pipetted into 1 mL Eppendorf centrifuge tubes, and stored at -80°C until analysis.

Table 1. Average water quality during exposure in weekly monitoring.

Water Quality	Mean ± S.D
Temperature (°C)	17 ± 0.5
pH	7.61 ± 0.02
Dissolved Oxygen (mg L^{-1})	9 ± 0.4
Ammonia (mg L^{-1})	0.57
Nitrite (mg L^{-1})	0.01
Nitrate (mg L^{-1})	0.22
Hardness (mg L^{-1})	120 ± 5
Phosphate (mg L^{-1})	0.36

Vitellogenin Analysis: Plasma levels of Vtg were determined by a commercial enzyme-linked immune sorbent assay developed for *Cyprinus carpio* (ELISA, Biosense Laboratories, Bergen, Norway). This assay had a quantification limit of 0.1 ng mL^{-1}, which did not allow for the accurate determination of the low Vtg concentrations found in the control and some treatments. The procedure of the ELISA was as follows (Christensen et al. 1998; Lindhost *et al.* 2002; Mandich *et al.* 2007): The 96-well polystyrene microtiter plates were supplied and precoated with *C. carpio* Vtg monoclonal antibody. Vtg. standard solutions (range 0.24 to 250 ng mL^{-1} linear two-fold serial dilution) were distributed (50 µL) into dedicated wells while two nonspecific banding (NSB) wells received 100 µL dilution buffer (DB). The plasma samples were diluted 1:10; 1:100 in DB and 100 µL of the diluted samples were dispersed.

Plates were incubated for 1 h at 37 °C and then washed three times with 300 µl washing solution (Phosphate Buffered Saline (PBS), 0.05% Tween-20. In the next step, detecting anti-Vtg antibodies (100 µl) were distributed to each well, incubated for 1 h at 37°C and then washed three times with 300 µL washing solution. Secondary anti-IgG antibodies, horseradish peroxidase conjugate (100 µl), were instantly distributed to each well, incubated for 1 h at 37 °C and then washed five times with 300 µL washing solution for staining. Each well received 100 µL substrate solution (OPD-peroxidase substrate in urea hydrogen peroxide) and the plates were incubated for 30 min at room temperature (20–25 °C), then the reactions were stopped by adding 50 µL 2M H_2SO_4 to all wells. Finally, the absorbance at 492 nm was read with microplate reader.

The working range of the Vtg standard is normally 0.98–125 ng Vtg mL^{-1} and, the intra and inter assay coefficients of variation (% CV) are between 5.6 and 24% within the working range (Table 2).

Table 2. Carp Vtg standard.

Vtg concentration (ng mL^{-1})	absorbance	NSB-corrected absorbance
250	2.062	2.017
62.5	1.738	1.725
15.6	0.816	0.771
1.95	0.190	0.145
0.49	0.0875	0.0425
0.24	0.063	0.018

Statistical analysis

The SPSS version 11.7 was used for statistical analysis. In case of inhomogeneity of variances, the nonparametric Kruskall-Wallis test was performed, followed by the Mann-Whitney U test.

RESULTS AND DISCUSSION

The results obtained from ELISA analysis are shown in (Figs. 1, 2 and, 4). The group treated with 17β-estradiol had high absorbance values (490% variation) (Fig 3). The Mann-Whitney U test results indicated significant difference ($Z= -2.138$, $p < 0.05$) in Vtg levels between groups treated with 17β-estradiol and the control group. On the other hand, groups exposed to different (low to high) concentrations of 4-Nonylphenol showed enhanced

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absorbance values (Fig. 1). In addition, the Mann-Whitney U test results pointed out significant difference (Z= -2.132, $p < 0.05$) between groups treated with maximum 4-Nonylphenol and the control group in them.
Vtg levels. The treatment group injected by maximum concentration of Bisphenol A had high absorbance (Fig. 2). Moreover, Mann-Whitney U the test results showed significant difference (Z = -2.123, $p < 0.05$) between groups treated with maximum Bisphenol A and the control group in Vtg levels. The most important result of the present study belonged to the group exposed to the mixture of Bisphenol and 4-Nonylphenol. This final group had the highest absorbance value (2126%) (Fig. 3). This is while the results of Kruskall-Wallis test (H = 6.4; df = 2; $p < 0.05$) proved significant difference between this latter group and groups treated with 17β-estradiol and control (Fig. 4). All in all, comparison of estrogenic potentials of the four selected chemical compounds in ELISA revealed that relative potency of these chemicals was decreased in the following order: mixture of 4-NP and PBA>17β-Esteradiol>4-NP>BPA (Fig. 4). Statistically significant correlation ($p < 0.01$) was found between dose and response in plasma Vtg levels in treatments injected with increasing dose of 4-NP and BPA (Fig. 5). 100 Juvenile female carps were chosen for this study. Carps are commonly-occurring fish species in Europe and Asia, relatively easy to keep in the laboratory, and large enough to provide tissue and plasma volumes adequate for analyses. In addition, laboratory studies with carp support constant field work involving the use of vitellogenin in wild carp as a biomarker of exposure to xenoestrogens (Folmar *et al.* 1996; Goodbred *et al.* 1997; Snyder 2001; Villeneuve *et al.* 2002; Li *et al.* 2004; Li *et al.* 2010; Gilannejad *et al.* 2016). In this study the sensitivity of carp to xenoestrogens was compared by measuring the effects of natural estrogens and anthropogenic compounds on Vtg induction in primary hepatocytes. The fish tested in this study were exposed to environmentally relevant concentrations of 4-NP and BPA in sediments of Anzali Wetland. 4-NP and BPA concentrations ranging from 0.05 to 29 µg g^{-1} d.w. and 0.01 to 6.97 µg g^{-1}d.w. have been reported respectively (Mortazavi *et al.* 2012).
A representative range of chemicals reported to be estrogenic have been tested to determine whether they are also estrogenic to carp or not, and if so, to assess approximately how potent. Some of the results obtained are shown in Fig. 1. All of the chemicals examined stimulated synthesis of Vtg in a dose-dependent manner. However, some of these chemicals stimulated Vtg synthesis at concentrations reported to be present in the aquatic environment. In natural situations, fish are continuously subjected to any estrogenic chemicals present in the water. The results revealed that exposure to minimum concentrations of 4-NP and BPA in sediments did not cause significant Vtg induction in juvenile female carp (Figs 1 and 2). This is in agreement with other studies demonstrating significant Vtg induction only at greater exposure concentrations. 10 µg L^{-1} NP was reported to be the threshold exposure concentration needed to cause Vtg induction in 2-year-old rainbow trouts (Jobling *et al.* 1996; Villeneuve *et al.* 2002). Another study proved significant induction of Vtg in rainbow trout exposed to concentrations greater than 25 µg L^{-1} NP (Tremblay & Van DerKraak 1998).

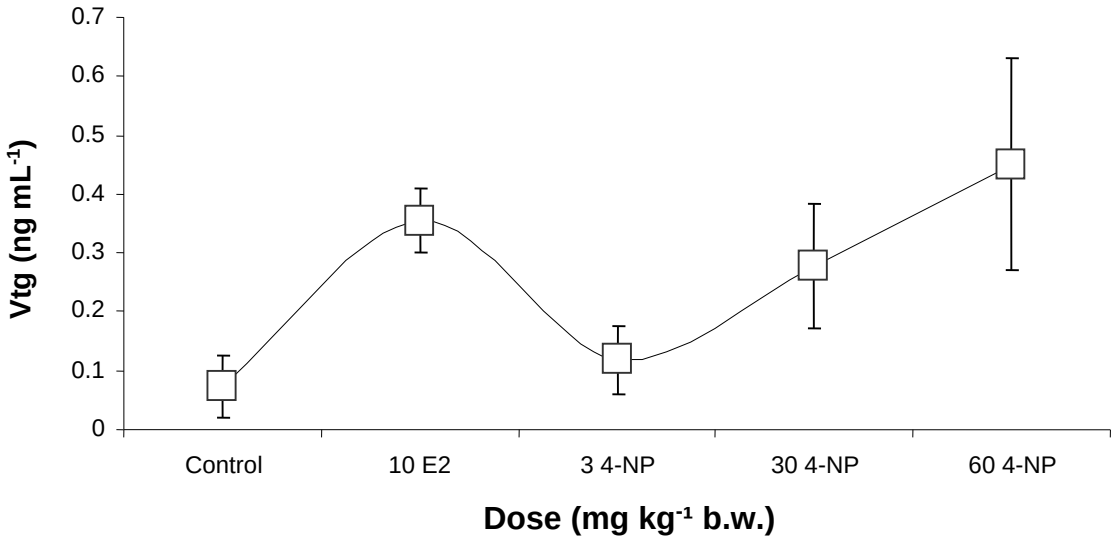

Fig. 1. Plasma Vtg levels (mean ± standard error) in juvenile carps: control, experimentally treated 17β-estradiol (E2) (10 mg kg^{-1} b.w.) and increasing dose of 4-NP (3, 30, 60 mg kg^{-1} b.w.).

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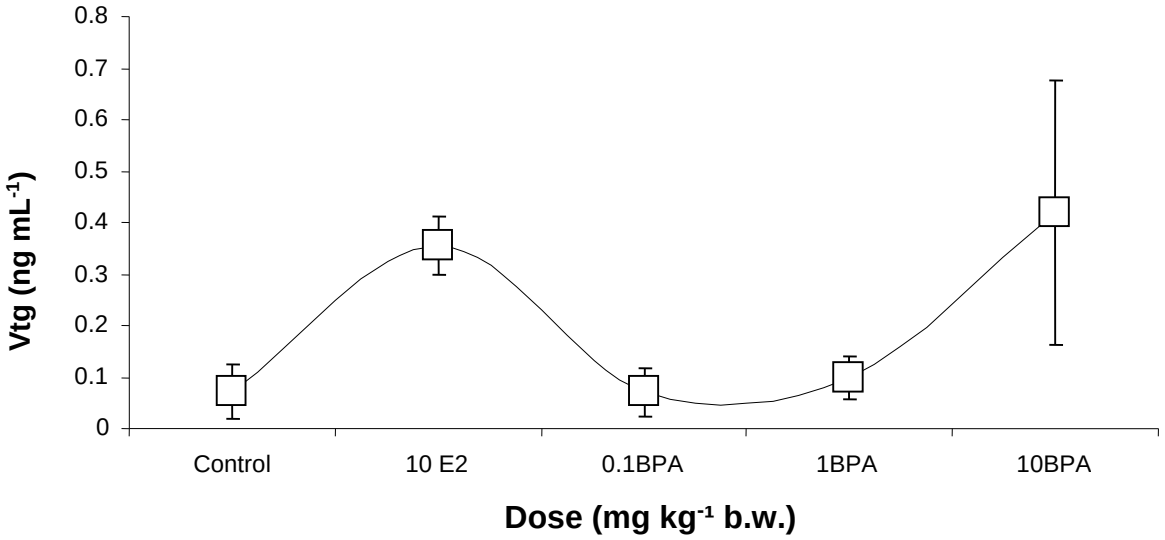

Fig. 2. Plasma Vtg levels (mean ± standard error) in juvenile carps: control, experimentally treated 17β-estradiol (E2) (10 mg kg^{-1} b.w.) and increasing dose of BPA (0.1, 1, 10 mg kg^{-1} b.w.).

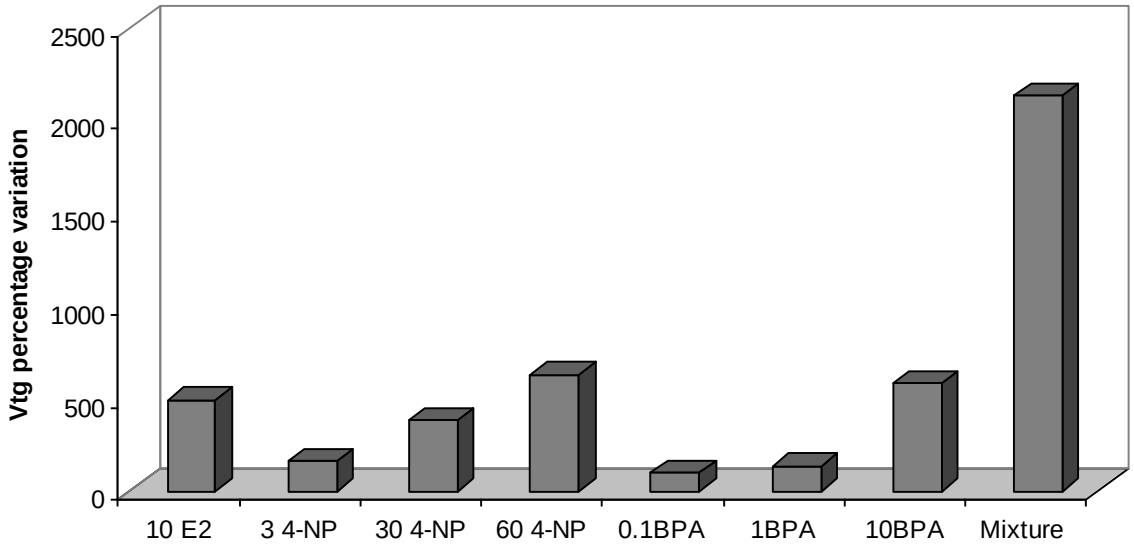

Fig. 3. Percentage variation of plasma Vtg in treated groups with respect to control group.

Another study with fathead minnows exhibited no significant induction of Vtg in males at exposure concentrations up to 2.4 µg L^{-1} NP, but a significant one in females (Giesy *et al.* 2000). Therefore, compared to other reports in the literature, it seems that exposure to high concentrations of the chemicals used for this study were adequate to induce Vtg in juvenile female carp. Other studies also detected marked increases in plasma Vtg concentrations in Atlantic cod, *Gadus morhua*, Atlantic salmon, and *Salmo salar*, after injection of E2 (peaking on day 6) and 4-NP (Hylland & Haux 1997; Ratna *et al.* 2016). Vtg induction has also been observed in various fish species exposed to diluted effluents from wastewater treatment (Jobling *et al.* 2003; Robinson *et al.* 2003; Marin *et al.* 2004). In the present study, mixture of 4-NP and BPA produced significant elevation in plasma Vtg levels. In fact, there was no evidence of synergistic influences of the examined chemicals with respect to their ability to induce plasma Vtg levels. In addition, when NP was injected in combination with BPA, like research of Arukwe *et al.* (2000), Vtg levels were more elevated. Since environmental samples are mixtures of many compounds, including agonists and antagonists, several studies have dealt with this issue. Seifert *et al.* (1999) and Li *et al.* (2004) examined the mixtures of different estrogenic chemicals including antiestrogen tamoxifen with ELRA.

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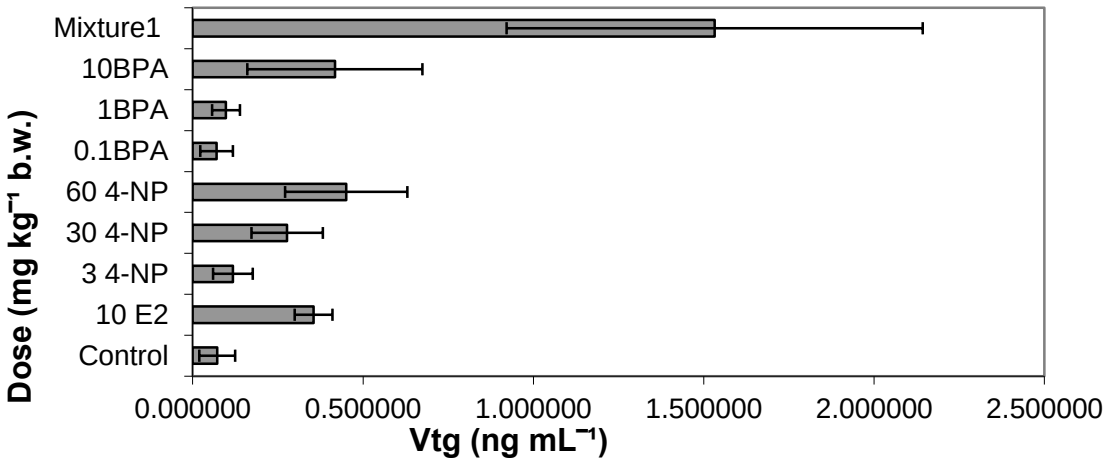

Fig. 4. Plasma Vtg levels (mean ± standard error) in juvenile carps: control, experimentally treated 17β-estradiol (E2) (10 mg kg^{-1} b.w.) and increasing dose of 4-NP, BPA, and mixture of them (3 and 0.1, mg kg^{-1} b.w.) respectively.

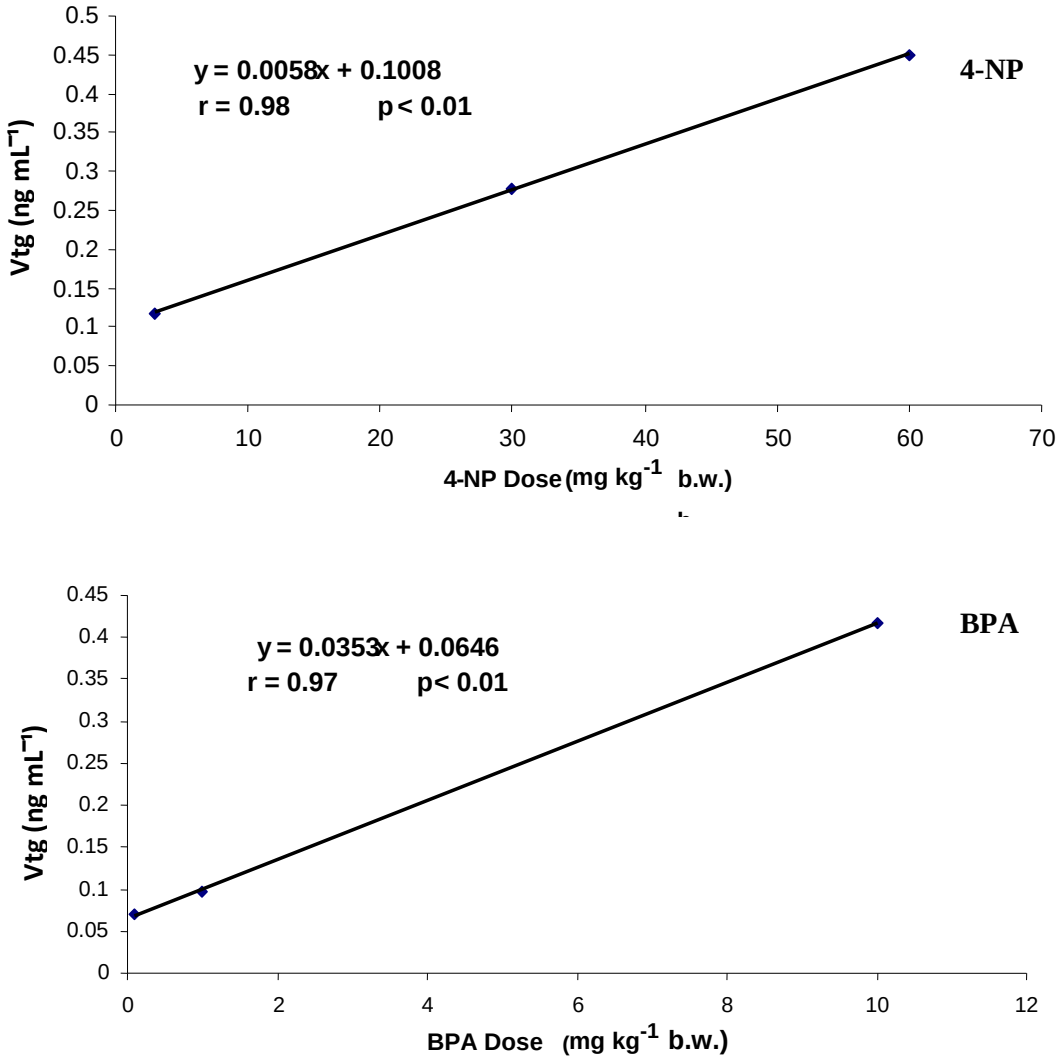

Fig. 5. Significant correlation between dose and response in plasma Vtg levels in Juvenile carps experimentally treated with increasing doses of 4-NP and BPA.

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In all cases, synergism was found. In another study, Silva *et al.* (2002) examined the mixtures of several agonists including Bisphenol-A with YES assay, synergism was also detected. These results validate findings of previous studies on marine fish, especially those by Arukwe *et al.* (1997) in which the treatment of *Salmo salar* with the same two compounds showed induction of Vtg in plasma of the same amount.

Similar to maturely differentiated specimens (Folmar *et al.* 1996; Solè *et al.* 2000; Villeneuve *et al.* 2002), juvenile common carps are also responsive to estrogenic incentives. The fact that this species is also sensitive to relatively low doses of xenoestrogens, such as 4-NP, might have an outcome on natural populations.

With further evidence, this fish might be proposed for large-scale monitoring of estrogenic effects caused by contamination in urban and industrial sewage. Carp is in fact pervasive in most fresh water courses, easily available, and does not migrate (Casini *et al.* 2002) and can therefore represent a good sentinel organism.

CONCLUSION

The findings of laboratory studies show that a rapidly increasing number of chemicals, entering water ways through different sources of effluent, or their degradation products, are being recognized as estrogenic.

As a result, Vtg levels may increase when animals are exposed to a wide group of pollutants having similar feminizing effects. This is while Vtg induction may be considered as a useful tool in evaluating endocrine disruption in aquatic animals, and therefore the potential risk associated with exposure to xenoestrogens. The repercussions of this exposure and the responses to it are unknown for the present time, but they could include opposing effects on physiological processes, particularly reproduction.

In addition, there is a considerable relevance between the ecological and the physiological responses, considering that hormonal changes may cause variations in the sex ratio of a feral population, its reproductive capability, and even the presence of the species in the aquatic ecosystem.

Moreover, using only one (or a few) biomarkers can reduce expensive chemical analyses of individual pollutants and mostly allow detection of their interactions in the aquatic ecosystem.

Further work is required to clarify the effects of other estrogenic chemicals present in the environment on aquatic animals.

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اثرات ترکیبات زنواستروژنی (۴-نونیل فنل، بیسفنل-آ) بر تولید ویتلوژنین در کپورماهیان نابالغ

ثمر مرتضوی[1*]، علی‌رضا ریاحی بختیاری[2]، عباس اسماعیلی ساری[2]، فاطمه رهبری‌زاده[3]، نسرین حسن-زاده[1]

۱- گروه محیط زیست، دانشکده منابع طبیعی و محیط زیست، دانشگاه ملایر، همدان، ایران
۲- گروه محیط زیست، دانشکده منابع طبیعی و علوم دریایی، دانشگاه تربیت مدرس، نور، مازندران، ایران
۳- گروه بیوتکنولوژی پزشکی، دانشکده علوم پزشکی، دانشگاه تربیت مدرس، تهران، ایران

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چکیده

هدف از مطالعه حاضر بررسی برخی از اثرات بالقوه ترکیبات نونیل فنل(4-NP) ، بیسفنل- آ (BPA) و مخلوط آنها بر کپورماهی معمولی در القاء ویتلوژنین (Vtg) به عنوان بیومارکری جهت پایش بوم‌سازگان‌های آبزی است. این ترکیبات و همچنین سایر مواد شیمیایی استروژنی ممکن است مسئول اثرات مخریب مشاهده شده در ماهی باشد. جهت آزمایش ۱۰۰ عدد کپور ماهی نابالغ مورد تزریق با ۱۷بتا استرادیول (E2)، دوزهای افزایشی NP-4، BPA و همچنین مخلوط آنها قرار گرفت. سپس سطح Vtgپلاسما با استفاده از الایزا به روش غیرمستقیم اندازه گیری شد. نتایج نشان داد میزان Vtg افزایش معناداری ($P<0.05$) در برابر میزان E2 ، حداکثر دوز NP-4، BPA و مخلوط آنها دارد. در مقایسه با گروه کنترل، گروه در معرض E2 دارای القاء بالا (۴۹۰٪) بود. گروه های با تزریق حداکثر دوز NP-4 و BPA نیز القاء Vtg را نشان دادند، در حالیکه گروه با تزریق مخلوط آنها ۳ ($mg\ kg^{-1}$ b.w) ۴-نونیل فنل و ۰/۰۱ ($mg\ kg^{-1}$ b.w) بیسفنل آ، بیشترین القاء (۲۱۲۶ ٪) ویتلوژنین را در قیاس با گروه کنترل نشان داد. در نهایت، نتایج نشان داد که مخلوطی از NP-4 و BPA اثرات قوی‌تر (هم افزایی) را نشان می‌دهد. بنابراین نظارت گسترده براثرات استروژنی ناشی از آلودگی ممکن است بعنوان بیوماکردر پساب‌های شهری و صنعتی قابل ملاحظه باشد.

*مؤلف مسئول

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