

## Assessment of immunomodulator biomarkers (Tnf- $\alpha$ , Il-1 $\beta$ and Il-6) in liver of *Capoeta umbla* for biomonitoring of pollution in Uzuncayir Dam Lake (Tunceli, Turkey)

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### Abstract

This study was aimed to monitor water pollution of Uzuncayir Dam Lake using the changes of IL-6, IL1 $\beta$  and TNF- $\alpha$  levels in *Capoeta umbla* (Heckel, 1843) liver tissue at ten stations in March and September 2011. In this study *C. umbla* (Heckel, 1843) was used as the indicator organism. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin1 $\beta$  (IL-1 $\beta$ ) and interleukin-6 (IL-6) levels were determined in samples of the liver tissue by ELISA kit. The lowest mean IL-1 $\beta$  levels were found at station 6. The mean IL-1 $\beta$  was reached its maximum level at station 2. The difference between the mean levels of IL-6 was found to be significant ( $p < 0.05$ ) among stations. The IL-6 levels were significantly increased in September at stations 1, 2, 7 and 8 ( $p < 0.01$ ) compared to the values in March. The mean levels of TNF- $\alpha$  were found to be significant ( $p < 0.05$ ) among stations. The TNF- $\alpha$  levels significantly decreased in September at stations 1 and 9 ( $p < 0.01$ ). TNF- $\alpha$ , IL-1 $\beta$  and IL-6 levels in *C. umbla* can be used as early diagnostic indicators against adverse environmental events and useful and reliable bioindicators in determining the pollution of the aquatic ecosystem.

**Keywords:** *Capoeta umbla*, TNF- $\alpha$ , IL-1 $\beta$ , IL-6, Biomonitoring, Biomarker

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## Introduction

Vast quantities of pollutants are discharged daily into water bodies, where they affect aquatic life at different stages of development. The studies on these effects on aquatic life can indicate the potential effects of pollutants on humans who are exposed to the toxins through consumption of contaminated food and water. Since these toxicants in water are taken up readily by fishes, they can prove to be a valuable model system to study the consequences of toxicant uptake and bioaccumulation on metabolic activities and immune function (Nayak, 2003; Hook *et al.*, 2006).

Immune function assays have received less attention as a potential bioindicator of exposure and effect of contaminants. However, immunologists have clearly demonstrated in a variety of organisms that several classes of xenobiotics target the immune system resulting in altered immune function (Anderson and Zeeman, 1995).

The immune system and response of fish can be greatly influenced by various external factors such as temperature, light, water quality, salinity and different stress inducers (Magnadottir, 2010). Stresses imposed upon the immune system of fish by environmental pollutants may not be overtly apparent and may act indirectly to exacerbate disease states by lowering resistance and allowing the invasion of environmental pathogens (Zelikoff, 1998).

Releasing cytokine is considered to be very relevant to investigate the toxicity towards the immune system (Carfi *et al.*, 2007).

In particular, pro-inflammatory cytokines, including interleukin-1 $\beta$  (Zou *et al.*, 1999), TNF- $\alpha$  (Laing *et al.*, 2001), and IL-6 (Kishimoto and Hirano, 1988), are commonly used immune-regulatory genes in fish. Interleukin-1 $\beta$  (IL-1  $\beta$ ) is a key mediator of host response infections and a primary cause of inflammation (Dinarelo, 2002), identified in 13 teleost species with a role similar to that in mammals (Mathew *et al.*, 2002).

IL-1 $\beta$  was one of the few cytokines that discovered in fish by homology cloning (Zou *et al.*, 1999). In many species of teleost fish including salmonids (Zou *et al.*, 1999; Ingerslev *et al.*, 2006), cyprinids (Fujiki *et al.*, 2000), gadoids (Corripio-Miyar *et al.*, 2007), perciforms (Pelegrin *et al.* 2001; Covello *et al.*, 2009) and anguilliforms (Tsutsui *et al.*, 2007), it has been cloned, and has also been cloned from cartilaginous fish (Bird *et al.*, 2002). The function of IL-1 $\beta$  in these fish species is analogous to mammalian (Mathew *et al.*, 2002). IL-1 $\beta$  production is stimulated by a variety of agents, including endotoxin, that stimulate molecular pattern receptors.

TNF- $\alpha$  is an inflammatory cytokine in mammals and is produced by macrophages, neutrophils, monocytes, natural killer cells and T cells after stimulation by bacterial lipopolysaccharide. It seems that the same mode were happen in fish species such as rainbow trout (Laing *et al.*, 2001), carp (Saeij *et al.*, 2003), catfish (Zou *et al.*, 2003), red seabream (Cai *et al.*, 2003), Atlantic salmon (Ingerslev *et al.*, 2006), Gilthead seabream (Garcia-Castillo *et al.*,

2002) and the mandarin fish (Xiao *et al.*, 2007).

IL-6 is mainly secreted by T cells and macrophages to stimulate immune response to inflammation (Hirano, 1998) and has been described in Fugu (Bird *et al.*, 2005), rainbow trout (Iliev *et al.*, 2007) and seabream (Castellana *et al.*, 2008).

Increasing attention has been paid to the immun system of fish as a biomarker of xenobiotic stress (Bowser *et al.*, 1994). *C. umbla* is of great commercial importance because it is the most common fresh water fish widely consumed in Tunceli.

The cytokines are important regulators of the immune system in fish (Sahoo and Sakai, 2010), therefore, investigation of cytokine functions may provide data that can be used as a biomarker for monitoring water pollution. The main purpose of this study is to monitor physiological changes in fish that live in the lake water of Uzuncayir Dam Lake that are already collected water about three years. To reveal the effects of the pollution on the living elements in the river and lake, in

fish samples, taken from ten points some cytokines (IL 1- $\beta$ , TNF- $\alpha$  and IL-6) levels were determined.

## Materials and methods

### Sampling

Ten research stations were determined taking into account the pre-dam, Dam Lake and post-dam points at Munzur and Pulumur Rivers and Uzuncayir Dam Lake (Fig1).

Fishes samples were collected during March and September in 2011. Sampling months were selected that could indicate possible seasonal swings in pollution and different biological activities of the fish. The fish were collected by fishing nets.

### Abiotic water parameters

The physico-chemical parameters of the water were measured at each sampling site during each fish-sampling season. The pH, temperature, the dissolved oxygen (DO) content were detected by YSI Professional Plus handheld multiparameter meter.



**Figure 1:** Station 1, Pre-settlement area at Munzur River; station 2, The point just before Munzur River flows into dam lake; station 3, The point just before the discharge point of seepage water into Pulumur River; station 4, The point just before Pulumur River flows into dam lake; station 5, Just after Pulumur River converges with Munzur River in the dam area; station 6, In the middle of the dam lake; station 7, In the middle of the dam lake; station 8, The point in the dam lake near hydroelectric power plant; station 9, Just after hydroelectric power plant; station 10, The point where Munzur River flows into Keban Dam Lake. Map of sampling stations on Uzuncayir Dam Lake, Munzur and Pulumur River systems.

### *Biochemical analyses*

Total 200 male *C. umbla* were captured. At first, fish were anaesthetized to deep sedation with  $0.7 \text{ gL}^{-1}$  benzocaine dissolved in ethyl alcohol (Sardella *et al.*, 2004; Altun and Danabas, 2006) and then were placed in freezer plastic bags and transported to laboratory with ice.

In Lab, The livers of fish were dissected. The liver tissues were rinsed with 0.9% NaCl and were homogenised in PBS buffer (pH 7.4). The homogenated samples were centrifuged (15.000 g, 10 min, 4°C), and supernatants, if not used immediately, were kept in the deep freeze at  $-70^{\circ}\text{C}$  (Yildirim and Yurekli, 2010).

*IL-6 Levels*

IL-6 levels were assayed by an enzyme-linked immunosorbent assay (ELISA) kit [CUSABIO BIOTECH CO., LTD. IL-6 Assay Kit]. Catalog No: CSB-E13258Fh

*IL-1 $\beta$  levels*

IL-1 $\beta$  levels were assayed by an enzyme-linked immunosorbent assay (ELISA) kit. [CUSABIO BIOTECH CO., LTD. IL-1 $\beta$  Assay Kit].Catalog No. CSB-E13259Fh

*TNF- $\alpha$  Levels*

TNF- $\alpha$  Levels were assayed by an enzyme-linked immunosorbent assay (ELISA) kit. [CUSABIO BIOTECH CO., LTD. TNF- $\alpha$  Assay Kit]. Catalog No. CSB-E13254Fh

*Statistical analysis*

One-way ANOVA and the multiple range test of Duncan were used to determine the significance of differences in TNF- $\alpha$ , IL-

1 $\beta$  and IL-6 levels between stations ( $p < 0.05$ ). Independent-samples T test was used for the evaluation of oxidative stress biomarkers between months in the same station.

**Results**

The results of physico-chemical parameters of water sampling sites are shown in Table 1. The lowest dissolved oxygen level among stations was 7.72 mg/L in September at station 9. The pH values ranged from 7.44 to 8.63 throughout the sites; the highest value was determined in September at station 10. The water temperature was higher in September than March.

**Table 1: The physico-chemical parameters of water at sampling time from the sampling stations on Uzuncayir Dam Lake.**

Months	Parameters	Station Number									
		1	2	3	4	5	6	7	8	9	10
March	Temperature (°C)	9.56	10.8	9.20	9.20	10.9	11.8	13.3	13.5	14.1	13.9
	pH	8.42	8.38	8.35	8.40	8.28	8.28	8.55	8.44	8.32	8.28
	Dissolved Oxygen (mg/l)	15.46	13.77	15.32	16.5	16.44	13.2	14.05	14.07	15.18	13.75
September	Temperature (°C)	12.6	13.4	12.2	14.1	18.7	20.4	19.4	20.8	23.3	21.1
	pH	7.68	7.44	7.88	8.30	7.78	7.89	8.22	8.10	7.66	8.63
	Dissolved Oxygen (mg/l)	9.44	9.28	10.64	9.23	9.95	9.12	8.28	8.68	7.72	9.55

Some immunomodulatory factors (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ) determined in *Capoeata umbla* collected from ten sampling sites in different seasons, are shown in Table 2.

**Table 2: Changes in TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in *C. umbla* captured from different sites in the Uzuncayir Dam Lake in September and March.**

Station Number /	Mean of Months /	IL-1 $\beta$ (pg/ml)	IL-6 (pg/ml)	TNF- $\alpha$ (pg/ml)
<b>1</b>	March	1151.16 $\pm$ 236.22	5.13 $\pm$ 0.45	2894.19 $\pm$ 117.72
	September	1144.58 $\pm$ 14.53	18.10 $\pm$ 0.02**	1014.58 $\pm$ 70.73**
	Mean	1147.87 $\pm$ 105.85 <sup>ab</sup>	11.62 $\pm$ 2.91 <sup>a</sup>	1954.39 $\pm$ 424.76 <sup>ab</sup>
<b>2</b>	March	1333.58 $\pm$ 7.31*	4.35 $\pm$ 0.53	2689.58 $\pm$ 158.60
	September	1121.70 $\pm$ 50.20	17.15 $\pm$ 0.59**	2366.79 $\pm$ 117.57
	Mean	1227.64 $\pm$ 52.53 <sup>a</sup>	10.74 $\pm$ 2.88 <sup>a</sup>	2528.19 $\pm$ 114.04 <sup>a</sup>
<b>3</b>	March	1058.83 $\pm$ 117.94	4.87 $\pm$ 0.58	2657.38 $\pm$ 223.06
	September	847.12 $\pm$ 11.38	4.18 $\pm$ 0.23	1665.52 $\pm$ 411.83
	Mean	953.00 $\pm$ 71.05 <sup>ab</sup>	4.52 $\pm$ 0.32 <sup>bc</sup>	2161.45 $\pm$ 305.06 <sup>ab</sup>
<b>4</b>	March	1314.25 $\pm$ 63.71*	4.28 $\pm$ 0.43	2578.78 $\pm$ 5.31
	September	974.53 $\pm$ 79.59	2.76 $\pm$ 0.98	2279.81 $\pm$ 314.55
	Mean	1144.39 $\pm$ 88.60 <sup>ab</sup>	3.52 $\pm$ 0.59 <sup>c</sup>	2429.30 $\pm$ 155.77 <sup>a</sup>
<b>5</b>	March	904.29 $\pm$ 24.63	2.70 $\pm$ 0.49	2355.50 $\pm$ 163.67
	September	1142.55 $\pm$ 138.56	3.49 $\pm$ 1.61	1845.80 $\pm$ 167.78
	Mean	1023.42 $\pm$ 82.46 <sup>ab</sup>	3.08 $\pm$ 0.76 <sup>c</sup>	2100.65 $\pm$ 154.85 <sup>ab</sup>
<b>6</b>	March	819.15 $\pm$ 115.13	4.12 $\pm$ 0.93	1391.30 $\pm$ 122.63
	September	949.88 $\pm$ 77.31	9.99 $\pm$ 4.52	1409.29 $\pm$ 106.94
	Mean	884.52 $\pm$ 68.56 <sup>b</sup>	7.05 $\pm$ 2.45 <sup>abc</sup>	1400.30 $\pm$ 72.88 <sup>b</sup>
<b>7</b>	March	1182.55 $\pm$ 14.56	3.37 $\pm$ 0.56	2500.17 $\pm$ 25.59
	September	746.82 $\pm$ 256.86	11.72 $\pm$ 0.01**	2390.62 $\pm$ 562.47
	Mean	964.69 $\pm$ 150.77 <sup>ab</sup>	7.55 $\pm$ 1.88 <sup>abc</sup>	2445.40 $\pm$ 253.00 <sup>a</sup>
<b>8</b>	March	1039.48 $\pm$ 116.94	6.59 $\pm$ 0.22	2721.77 $\pm$ 184.43
	September	955.98 $\pm$ 44.02	12.24 $\pm$ 0.68**	2412.69 $\pm$ 133.43
	Mean	997.73 $\pm$ 58.92 <sup>ab</sup>	9.42 $\pm$ 1.30 <sup>ab</sup>	2567.73 $\pm$ 122.73 <sup>a</sup>
<b>9</b>	March	1024.02 $\pm$ 127.69	12.68 $\pm$ 3.21	3080.11 $\pm$ 16.66**
	September	928.90 $\pm$ 0.54	6.70 $\pm$ 1.33	739.48 $\pm$ 41.75
	Mean	976.46 $\pm$ 60.94 <sup>ab</sup>	9.69 $\pm$ 2.05 <sup>ab</sup>	1909.80 $\pm$ 523.77 <sup>ab</sup>
<b>10</b>	March	1128.43 $\pm$ 79.39	6.00 $\pm$ 0.40	3015.71 $\pm$ 362.82*
	September	727.16 $\pm$ 128.50	5.80 $\pm$ 1.29	1015.41 $\pm$ 382.14
	Mean	927.76 $\pm$ 275.06 <sup>b</sup>	5.90 $\pm$ 0.60 <sup>abc</sup>	2015.57 $\pm$ 505.57 <sup>ab</sup>

Different letters indicate significant difference, based on Duncan test ( $p < 0.05$ ).

Different letters over the means indicate differences between sites according to multiple range test of Duncan ( $p < 0.05$ ).

Independent-samples t test was used for the evaluation of measured data between months,  $*p < 0.05$  and  $**p < 0.01$  were regarded as statistically significant. The number of sampled male fish from the studied sites in different season was:  $n$ : 10.

The lowest mean of IL-1 $\beta$  levels were found at station 6. The mean of IL-1 $\beta$  reached its maximum level at station 2. The IL-1 $\beta$  levels were lower in September than March at all sampling sites, except stations 5 and 6.

At all stations, the differences among the mean levels of IL-6 were found to be significant ( $p < 0.05$ ). The IL-6 levels were significantly increased in September at stations 1, 2, 7 and 8 ( $p < 0.01$ ) compared to the values in March.

The differences between the mean levels of TNF- $\alpha$  at all stations were found to be significant ( $p < 0.05$ ). The TNF- $\alpha$  levels significantly decreased in September at stations 1 and 9 ( $p < 0.01$ ). The lowest mean of TNF- $\alpha$  level was found at station 6. The mean of TNF- $\alpha$  reached its maximum level at station 8. TNF- $\alpha$  level were lower in September than in March at all stations.

## Discussion

Domestic wastes of Tunceli are discharged directly to Munzur and Pülümür Rivers with no treatment. Therefore, over time, the physico-chemical properties of this water system will change and cause some ecotoxicological impacts on the living elements of this ecosystem.

Biological indicators of exposures to different pollutants and their effects are ancillary approaches to traditional methods in fisheries science to assess the potential impact of environmental contaminants and other anthropogenic sources of stress on fish health (Rice *et al.*, 1995). Fish are one of the important indicators of environmental contaminants of water but there are limited data available on the effects of pollution on fish health and particularly on the immune system (Dunier and Swicki, 1993). The present study was designed to monitor water pollution of Uzuncayir Dam Lake by using the changes of IL1 $\beta$ , IL-6 and TNF- $\alpha$  levels in liver of *C. umbla* (Heckel, 1843) at ten stations in March and September.

Temperature decrease which has an important role on the poikilothermic nature of fish, affects the rate of their physiological functions (Hayward *et al.*, 2009). One recent report indicated that cytokine expression (IL-1, IL-10 and IFNg) was up-regulated in rainbow trout maintained at 25°C (Raida and Buchmann, 2007). Indeed, they found that there was a generalised up-regulation of cytokine expression at both 15°C and 25°C compared to animals at 5°C (Bowden, 2008). In our study, the levels of IL1 $\beta$  and TNF- $\alpha$  generally were decreased in *C. umbla* in September. The rise of temperature affects the immune response. The decrease of IL1 $\beta$  and TNF- $\alpha$  level were monitored as an indicator of degree and duration of fish exposure to pollutants. Artificial environmental stress factors, i.e. pollutants, are generally considered to be immunosuppressive (Dunier and Swicki,

1993). In general, regardless of fish species, elevated water temperatures within the physiological range of fish (i.e. 5-10°C above ambient temperature) frequently enhance immune functions. The mechanisms involved may be attributable to induction of heat shock proteins (HSP) which can function to protect protein folding and trafficking from the adverse effects of elevated temperatures (Dietz *et al.*, 1994). Signalling mechanisms responsible for various stress effects on fish immunity have not been yet well understood, although it is clear that elevated ACTH serum and cortisol levels are involved in some cases. Cortisol can weaken the activity of the immune system (Bly, *et al.*, 1997). The effects of seasonal and in vitro assay temperatures on fish immune function have been well established and there is a little doubt that low temperatures can suppress adaptive (i.e. T and B cell-mediated) immune responses (Carlson *et al.*, 1995). However, there is still controversy over the effects of temperature on innate (non-specific) immune responses. Some studies were reported enhanced activities while the majority of reports were shown immune suppression (Bly *et al.*, 1992).

Changes in environmental pH levels show conflicting results for immune system parameters, such as levels of circulatory lysozyme and IgM (Uribe *et al.*, 2011). In present study, the highest pH values were found in September at station 10 (the point where Munzur River flows into Keban Dam Lake). IL1 $\beta$ , IL-6 and TNF- $\alpha$  levels were found generally low at station 10 compared to other stations. In our study,

station 10 is a dam lake area and in dam lakes, pH levels determine the toxicity of pollutants (Camargo and Alanso, 2006).

Oxygen levels in the environment may modulate the immune response; hypoxia depresses the respiratory burst activity of macrophages and decreases the levels of circulating antibodies, which in turn, are elevated by hyperoxia (Bowden, 2008). In this study, the lowest TNF- $\alpha$  levels and the lowest dissolved oxygen levels were found in station 9. The TNF- $\alpha$  level at station 9 in September confirmed the important contamination effects in station 9 that exhibited lowest dissolved oxygen concentration.

Stress has been defined as a change in the environment (either physical or physiological) that can disrupt normal host homeostasis (Bly *et al.*, 1997). The contribution of the environmental pollutants to stress in fish has been subjected to extensive reviews (Dunier and Swicki, 1993). Several studies showed that the immune function of collected fish from contaminated marine and estuarine environments were severely impaired (Faisal *et al.*, 1991). Stress, at the first phase (alarm phase) affects various aspects of immune function, depending on the nature and duration of the stress (Dhabhar, 2003). For example, stressors can directly affect the cells of the immune system and modulate the secretion of proinflammatory cytokines (Dhabhar and McEwen, 1996; Ruzek *et al.*, 1997).

The mechanism of immunomodulation of pollutants could be a direct toxic effect on immun cells or/and organs, or an indirect effect because the neuroendocrine



system is the first target organ of the pollutants (Dunier and Swicki, 1993). Cytokines play a key role in bidirectional communication between the endocrine and immune systems. They interplay between hormones and cytokines during stress and may influence immune homeostasis in response to environmental challenges (Castillo *et al.*, 2009; Yildirim and Yurekli, 2010) suggested that the cytokine expression in head kidney is highly regulated by stress related hormones, and it is another evidence of the existence of endocrine-immune interactions in the teleost fish. Adrenaline inhibited cytokine expression levels, being IL-1 $\beta$  the most sensitive cytokine to adrenaline *in vitro*. On the other hand, adrenocorticotrophic hormone (ACTH) rapidly inhibited IL-1 $\beta$  and increased TNF- $\alpha$ , TGF- $\beta$ 1 and IL-6 expression. Cortisol inhibited the expression of all cytokines and its immunosuppressive effects is confirmed (Castillo *et al.*, 2009). In our study, the mean IL-1 $\beta$  and TNF- $\alpha$  were at minimum level at station 6 (In the middle of the dam lake) in September. These results indicate that the area is polluted by municipal discharges, and that decrease in IL-1 $\beta$  and TNF- $\alpha$  levels of liver may be due to pollution stress.

To our knowledge, this is the first report that showed the immunomodulatory factors (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ) in fish could be used as bioindicators in fish health and water quality assessment. The results of our experiments suggested that environmental contaminants suppressed the function of immune system. Further studies are needed to understand the exact role of cytokines in response to pollution stress. The study of

biomonitoring of the water pollution is a broad topic, and our group intends to investigate different biomarkers on the suggested fish model.

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