Assessment of immunomodulator biomarkers (Tnf-α, Il-1β 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, IL1β and TNF-α 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-α (TNF-α), interleukin1β (IL-1β) and interleukin-6 (IL-6) levels were determined in samples of the liver tissue by ELISA kit. The lowest mean IL-1β levels were found at station 6. The mean IL-1β 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-α were found to be significant (p<0.05) among stations. The TNF-α levels significantly decreased in September at stations 1 and 9 (p<0.01). TNF-α, IL-1β 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-α, IL-1β, IL-6, Biomonitoring, Biomarker
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-1β (Zou et al., 1999), TNF-α (Laing et al., 2001), and IL-6 (Kishimoto and Hirano, 1988), are commonly used immune-regulatory genes in fish. Interleukin-1β (IL-1 β) is a key mediator of host response infections and a primary cause of inflammation (Dinarello, 2002), identified in 13 teleost species with a role similar to that in mammals (Mathew et al., 2002).

IL-1β 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-1β in these fish species is analogous to mammalian (Mathew et al., 2002). IL-1β production is stimulated by a variety of agents, including endotoxin, that stimulate molecular pattern receptors.

TNF-α 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., 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 Uzuncayır 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-β, TNF-α 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 Uzuncayır 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.
Biochemical analyses

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

In the lab, the livers of the fish were dissected. The liver tissues were rinsed with 0.9% NaCl and were homogenised in PBS buffer (pH 7.4). The homogenised samples were centrifuged (15,000 g, 10 min, 4°C), and supernatants, if not used immediately, were kept in the deep freeze at −70°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-1β levels**

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

**TNF-α Levels**

TNF-α Levels were assayed by an enzyme-linked immunosorbent assay (ELISA) kit. [CUSABIO BIOTECH CO., LTD. TNF-α 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-α, IL-1β 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>
<thead>
<tr>
<th>Months</th>
<th>Parameters</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>March</td>
<td>Temperature (°C)</td>
<td>9.56</td>
<td>10.0</td>
<td>9.20</td>
<td>9.20</td>
<td>13.9</td>
<td>11.0</td>
<td>13.3</td>
<td>13.5</td>
<td>14.1</td>
<td>13.9</td>
</tr>
<tr>
<td></td>
<td>pH</td>
<td>8.42</td>
<td>8.38</td>
<td>8.33</td>
<td>8.40</td>
<td>8.28</td>
<td>8.38</td>
<td>8.15</td>
<td>8.44</td>
<td>8.32</td>
<td>8.28</td>
</tr>
<tr>
<td></td>
<td>Dissolved Oxygen (mg/L)</td>
<td>15.46</td>
<td>17.77</td>
<td>15.32</td>
<td>16.5</td>
<td>16.44</td>
<td>13.2</td>
<td>14.05</td>
<td>14.07</td>
<td>15.10</td>
<td>13.75</td>
</tr>
<tr>
<td>September</td>
<td>Temperature (°C)</td>
<td>12.6</td>
<td>13.4</td>
<td>12.2</td>
<td>14.1</td>
<td>13.7</td>
<td>20.4</td>
<td>19.4</td>
<td>20.8</td>
<td>23.3</td>
<td>21.1</td>
</tr>
<tr>
<td></td>
<td>pH</td>
<td>7.68</td>
<td>7.44</td>
<td>7.08</td>
<td>0.30</td>
<td>7.78</td>
<td>7.89</td>
<td>8.22</td>
<td>8.10</td>
<td>7.66</td>
<td>8.63</td>
</tr>
<tr>
<td></td>
<td>Dissolved Oxygen (mg/L)</td>
<td>9.44</td>
<td>9.28</td>
<td>10.64</td>
<td>9.23</td>
<td>9.95</td>
<td>9.12</td>
<td>8.28</td>
<td>8.68</td>
<td>7.72</td>
<td>9.55</td>
</tr>
</tbody>
</table>

Some immunomodulatory factors (IL-1β, IL-6 and TNF-α) determined in *Capoecata umbla* collected from ten sampling sites in different seasons, are shown in Table 2.
Table 2: Changes in TNF-α, IL-1β and IL-6 in *Capoeta umbla* captured from different sites in the Uzuncayır Dam Lake in September and March.

<table>
<thead>
<tr>
<th>Station Number / Months /</th>
<th>Mean of IL-1β (pg/ml)</th>
<th>Mean of IL-6 (pg/ml)</th>
<th>Mean of TNF-α (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>March 1151.16±236.22</td>
<td>5.13±0.45</td>
<td>2894.19±117.72</td>
</tr>
<tr>
<td></td>
<td>September 1144.58±14.53</td>
<td>18.10±0.02**</td>
<td>1014.58±70.73**</td>
</tr>
<tr>
<td></td>
<td>Mean 1147.87±105.85&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>11.62±2.91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1954.39±424.76&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>March 1333.58±7.31&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.35±0.53</td>
<td>2689.58±158.60</td>
</tr>
<tr>
<td></td>
<td>September 1121.70±50.20</td>
<td>17.15±0.59**</td>
<td>2366.79±117.57</td>
</tr>
<tr>
<td></td>
<td>Mean 1227.64±52.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.74±2.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2528.19±114.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>March 1058.83±117.94</td>
<td>4.87±0.58</td>
<td>2657.38±223.06</td>
</tr>
<tr>
<td></td>
<td>September 847.12±11.38</td>
<td>4.18±0.23</td>
<td>1665.52±411.83</td>
</tr>
<tr>
<td></td>
<td>Mean 953.00±71.05&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.52±0.32&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>2161.45±305.06&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>March 1314.25±63.71&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.28±0.43</td>
<td>2578.78±5.31</td>
</tr>
<tr>
<td></td>
<td>September 974.53±79.59</td>
<td>2.76±0.98</td>
<td>2279.81±314.55</td>
</tr>
<tr>
<td></td>
<td>Mean 1144.39±88.60&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.52±0.59&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2429.30±155.77&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>March 930.29±24.63</td>
<td>2.70±0.49</td>
<td>2355.50±163.67</td>
</tr>
<tr>
<td></td>
<td>September 1142.55±138.56</td>
<td>3.49±1.61</td>
<td>1845.80±167.78</td>
</tr>
<tr>
<td></td>
<td>Mean 1023.42±82.46&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.08±0.76&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2100.65±154.85&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>March 819.15±115.13</td>
<td>4.12±0.93</td>
<td>1391.30±122.63</td>
</tr>
<tr>
<td></td>
<td>September 949.88±77.31</td>
<td>9.99±4.52</td>
<td>1409.29±106.94</td>
</tr>
<tr>
<td></td>
<td>Mean 884.52±68.56&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.05±2.45&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>1400.30±72.88&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>7</td>
<td>March 1182.55±14.56</td>
<td>3.37±0.56</td>
<td>2500.17±25.59</td>
</tr>
<tr>
<td></td>
<td>September 746.82±256.86</td>
<td>11.72±0.01**</td>
<td>2390.62±562.47</td>
</tr>
<tr>
<td></td>
<td>Mean 964.69±150.77&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.55±1.88&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2445.40±253.00&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>March 1039.48±116.94</td>
<td>6.59±0.22</td>
<td>2721.77±184.43</td>
</tr>
<tr>
<td></td>
<td>September 955.98±44.02</td>
<td>12.24±0.68**</td>
<td>2412.69±133.43</td>
</tr>
<tr>
<td></td>
<td>Mean 997.73±58.92&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>9.42±1.30&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2567.73±122.73&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>9</td>
<td>March 1024.02±127.69</td>
<td>12.68±3.21</td>
<td>3080.11±16.66**</td>
</tr>
<tr>
<td></td>
<td>September 928.90±5.04</td>
<td>6.70±1.33</td>
<td>739.48±4.75</td>
</tr>
<tr>
<td></td>
<td>Mean 976.46±60.94&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>9.69±2.05&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1909.80±523.77&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>March 1128.43±79.39</td>
<td>6.00±0.40</td>
<td>3015.71±362.82*</td>
</tr>
<tr>
<td></td>
<td>September 727.16±128.50</td>
<td>5.80±1.29</td>
<td>1015.41±382.14</td>
</tr>
<tr>
<td></td>
<td>Mean 927.76±275.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.90±0.60&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2015.57±505.57&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

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-1β levels were found at station 6. The mean of IL-1β reached its maximum level at station 2. The IL-1β 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-α at all stations were found to be significant (p<0.05). The TNF-α levels significantly decreased in September at stations 1 and 9 (p<0.01). The lowest mean of TNF-α level was found at station 6. The mean of TNF-α reached its maximum level at station 8. TNF-α level were lower in September than in March at all stations.

Discussion
Domestic wastes of Tunceli are discharged directly to Munzur and Püümür 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 pullution of Uzuncayir Dam Lake by using the changes of IL1β, IL-6 and TNF-α levels in liver of C. umbra (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 IL1β and TNF-α generally were decreased in C. umbra in September. The rise of temperature affects the immune response. The decrease of IL1β and TNF-α 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,
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). IL1β, IL-6 and TNF-α 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-α levels and the lowest dissolved oxygen levels were found in station 9. The TNF-α 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 Swiciki, 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
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-1β the most sensitive cytokine to adrenaline in vitro. On the other hand, adrenocorticotropic hormone (ACTH) rapidly inhibited IL-1β and increased TNF-α, TGF-β1 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-1β and TNF-α 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-1β and TNF-α levels of liver may be due to pollution stress.

To our knowledge, this is the first report that showed the immunonodulatory factors (IL-1β, IL-6 and TNF-α) 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.

Acknowledgment
This study is a part of a comprehensive project that is supported by The Scientific and Technical Research Council of Turkey (TUBITAK), Research Grant Committee of Environment, Earth, Marine, and Atmospheric Sciences (Project no: TUBITAK, CAYDAG 110Y118). This study was carried out under the permission of the Firat University Animal Welfare Committee.

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