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The effect of chronic crude oil exposure on some hematological and biochemical parameters of juvenile Beluga (*Huso huso* Linnaeus, 1758)

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Abstract: In the present study, sublethal toxic effects of crude oil on the biochemical and hematological parameters of Beluga (*Huso huso*) were studied. Two hundred juvenile Beluga (120±30g) were supplied by Rajaei fish farm in Mazandaran Province, Iran. Specimens were exposed to the sublethal concentrations of crude oil, including 0, 0.218, 0.327 and 0.436 ppm (equals to control group, 2, 3 and 4 times more than the concentration in Caspian Sea water respectively). Hematological and biochemical parameters were measured once a week for 9 weeks (63 days) of exposure. Results showed the White Blood Cell (WBC), Red Blood Cell (RBC), Hematocrit (Ht) and Hemoglobin (Hb) decreased and Mean Corpuscular Volume (MCV) and Mean Corpuscular Hemoglobin (MCH) were mainly significantly higher in fish exposed to crude oil concentrations compared to control group. A biphasic response was observed in their value throughout the study period. Results of the leukocyte numbers showed that, eosinophil numbers increased, while lymphocyte numbers decrease in comparison to the control group and during the experiments. Monocyte numbers showed no significant differences (except 49th and 63th day) while the neutrophil numbers were higher in treatment groups than the control. Biochemical parameters showed an increase in serum glucose and other parameters including total protein, Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP) and Lactate Dehydrogenase (LDH) activities were decreased in treatment groups and mainly during the experiments. The alterations of these parameters can be used as suitable biomarkers in monitoring of crude oil pollution in the aquatic environment and to protect aquatic life.

Keywords: Biomarker, Blood, Great sturgeon, Immunology, Oil Pollution

Introduction

Among different types of pollutants, petroleum products are one of the most relevant to aquatic ecotoxicology (Pacheco and Santos, 2001a). Crude oil is a complex mixture of organic compounds, 75% of which consist of short and long chain hydrocarbons (Neff, 1979). Polycyclic Aromatic Hydrocarbons (PAHs) can enter the body via many routes including breathing, eating, or drinking. Exposure to PAHs can also occur by skin contact. Once PAHs enter the body of a living organism, each are metabolized to form highly reactive molecules such as diol epoxides that are PAH intermediate metabolites (Trabelsi and Driss, 2005; Arias *et al.,* 2009; Zhang *et al.,* 2011).

Exposure to crude oil and its derivatives can induce a variety of toxic symptoms in experimental aquatic animals. Petroleum hydrocarbons can act as a mediator in free radical generation in fish that causing various side effects in their tissues (Engelhardt *et al.,* 1981; Davison *et al.,* 1992; Alkindi *et al.,* 1996; Khan, 1998; Pacheco and Santos, 2001a, 2001b; Achuba and Osakwe, 2003; Khan, 2003; Zang *et al.,* 2003, 2004). An increase in antioxidant defenses in animals after exposure to different concentrations of the water-soluble fraction of diesel oil (WSD) has shown in goldfish *Carassius auratus* for various experimental times (Zang *et al.*, 2003, 2004). Other studies have also indicated that the exposure of fish to the petroleum derivatives causes different effects in cortisol plasma concentrations (Alkindi *et al.*, 1996; Pacheco and Santos, 2001a, b), suggesting that these contaminants might interfere in the fish stress response. It was also reported that Crude oil hydro-carbons and surfactants promote structural damage in gills specially in branchial respiratory epithelium (Engelhardt *et al.*, 1981; Rosety Rodriguez *et al.*, 2002) affecting gas exchange, processes and limiting oxygen transfer in aquatic organism (Alkindi *et al.*, 1996; Val and Almeida-Val, 1999). Histopathological findings in liver also showed many cellular damages after exposing to the water soluble fraction of crude oil

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(Khabakhsh *et al*., 2014).

During the recent years, production of fossil fuel in Caspian Sea increased. Although Iran currently has no oil or natural gas production in the Caspian regions, it potentially has significant reserves and plays as a transit center for oil and natural gas exports from other Caspian Sea countries (Tolosa *et al*., 2004). In addition, sea currents probably transport and circulate the entrapped pollutions along the Iranian coast of Caspian Sea (Mohammadi Zadeh *et al.,* 2010). Therefore, as a land-lock system, pollutant discharges into the Caspian Sea, remain trapped within the basin (Karpinsky, 1992). Previous studies reported a detectable contamination of PAHs in different parts of Iranian coast of Caspian Sea (Pak and Farajzadeh, 2007; Habibi *et al.,* 2008). Such conditions seriously affect the life of the Caspian Sea aquatic animals including sturgeon species, the most valuable species of Caspian Sea. The accumulation of toxic agents in sediments which disturb the migration and reproduction of the sturgeon species (Billard and Lecointre, 2001; Kajiwara *et al.,* 2003) has significant effects on their population as the high levels of tumors, morphofunctional abnormalities in gonad development and gametogenesis, and morphogenesis of various organs have been noticed in Caspian sturgeons (Kajiwara *et al.,* 2003; Pourkazemi, 2006). Whereas they are already imperiled, because of a poorly regulated fishery, illegal catch, poaching, over harvesting, spawning habitat loss, and water quality (Billard and Lecointre, 2001; Kajiwara *et al.,* 2003). Also, some sturgeon characters such as high lipid content in body, long living period, long juvenile stage and benthivorous diet behavior make them to be quite potent target for exposing to PAHs (Jaric *et al*., 2011).Therefore, these valuable fish species may be at a high potential risk via the accumulating of PAHs in their organs. In previous study by Khoshbavar Rostami *et al*. (2012), mean concentrations of 16 PAHs were reported in water, sediment and tissues samples from five sturgeon species collected from the southern the Caspian Sea coasts and reported mean levels of PAHs have increased and were higher that described by Nasrollahzadeh and Malekshomali (2002). The acute toxicity (LC_{50}) of crude oil in this species was carried out by Jahanbakhshi *et al*. (2012) and short term exposure indicating adverse effect on some biochemical and hematological parameters (Hedayati and Jahanbakhshi, 2012; 2013; Jahanbakhshi and Hedayati, 2013). Infusion of crude oil indicating histopathological damages including many gill lesions (epithelial lifting, erythrocyte infiltration, lamellar aneurism, hyperplasia, and lamellar fusion), and several abnormalities in the gill structure (Jahanbakhshi and Hedayati, 2012; Khabakhsh *et al*., 2014). The objective of this study was to determine the effect of long term (63 days) exposure of sublethal concentration of crude oil on some hematological indices and biochemical parameters of Beluga (*Huso huso*). This was done because the greatest risks due to long term exposure to small doses of such chemical pollutant can be resulted in a decline in the population, retardation of reproduction in second generation, damage to life cycle and enhancing of fish susceptibility to infectious diseases.

Material and methods

Chronic exposure of crude oil

Juvenile specimens of Beluga (n=200), average weight 120±30 g, were supplied by Rajaei fish farm in Mazandaran Province, Iran and safety brought to the laboratory of Gorgan Research Center of Inland Aquatics Stocks. Specimens were kept in eight fiberglass tanks (2000-L). Prior to the toxicity tests, fish were acclimated to laboratory conditions for a minimum of two weeks. Water temperature, dissolved oxygen, pH and total hardness were measured continuously during the experiments period (Hedayati *et al.,* 2010) which were 22±1°C, 8.2±0.8mg/L, 7.5±0.1 and 145±5mg/L respectively. Other water quality parameters were measured by spectrophotometry as follow, ammonia <0.02mg/L, nitrite <0.1mg/L, nitrate<0.503mg/L and phosphate <0.285mg/L. Also fish were maintained under natural photoperiod (14L:10D). Fish also were fed with commercial pellet (protein 36%, lipid 14%, ash 11%, fiber 3.5%, phosphorus 1%, moisture 11%,

carbohydrate 22.5%) and fish meal 50% twice a day. Prior to the commencement of experiment, healthy Juveniles were collected randomly and transferred into twenty tanks (300 L capacity) which were continuously aerated.

Fish were subjected to different concentrations of crude oil at 0 (control group), 0.218 (group B), 0.327 (group C) and 0.436 (group D) ppm, performed in tank 300 L, each consisting ten fishes. The concentrations were chosen based on a preliminary examination (Khoshbavar Rostami *et al.,* 2012) and were 2, 3 and 4 folds more than those detected in Caspian Sea water. During the experiment, Water renewal was performed once a day corresponding to whole of the water tank with dechlorinated tap water and the stocks of crude oil were used to achieve above mentioned concentrations in test tanks daily. Water was monitored continuously monitored for temperature, dissolved oxygen, pH, and total hardness during the experiment. No mortality was observed during the exposure period.

Blood samples were collected after 24 hours and thereafter every week until 9 weeks (63 days) of exposure to the toxicant. Five fish per treatment were sampled at each sampling time.

Hematological and biochemical analyses

Immediately after removing the fish from the tank, they were anesthetized with clove powder (200 ppm, 20min; Hedayati and Safahieh, 2011) and blood samples were taken from the caudal vein by plastic syringes and transferred to heparinized and non-heparinized tubes (Hedayati and Safahieh, 2011).

Determinations of the blood indices were performed immediately on heparinized blood. The White Blood Cell (WBC, number per cubic millimeter) and Red Blood Cell (RBC, number per cubic millimeter) were determined by diluting heparinized blood with Giemsa stain at 1:30 dilution and cells were counted using a hemocytometer Neubauer Chamber Counting using light microscope (Stevens, 1997). The leukocyte differential count was made in peripheral blood smears stained by Merck Giemsa (Beutler *et al.,* 2001). Blood smears were prepared, and leukocytes were categorized into lymphocytes, monocytes, neutrophils and eosinophils (Banaee *et al.,* 2008).

Hematocrit values (Ht, percent) were immediately determined after sampling by placing blood in glass capillary tubes and centrifuged for 5 min at 3,000 rpm (1006 GV) in a microhematocrit centrifuge (Hettich, Germany) then measuring the packed cell volume (Goldenfarb *et al.,* 1971); hematocrit determined by use of a microhematocrit reader. Hemoglobin levels (Hb, milligrams per deciliter) were determined colorimetrically by measuring the formation of cyanomethemoglobin according to Lee *et al*. (1998). Erythrocytes indices including Mean Corpuscular Volume (MCV, femtoliter), Mean Corpuscular Hemoglobin Concentration (MCHC, percent), and Mean Corpuscular Hemoglobin (MCH, picogram) were calculated according to Lee *et al*. (1998).

The non-heparinized blood samples were centrifuged for 15 minutes at 400 g and separated sera were used to determine biochemical parameters. Ultrapure water was used for all serum dilutions and standard preparations and duplicate readings were recorded for standards and serum samples. The quantitative determination of serum glucose (gram per deciliter), serum total protein (gram per deciliter), Aspartate Aminotransferase (AST, IU per liter), Alanine Aminotransferase (ALT, IU per liter), Alkaline Phosphatase (ALP, IU per liter) and Lactate Dehydrogenase (LDH, IU per liter) activities were carried out with Pars-Azmoon Diagnostics Infinity kits by a Technicon RA1000 auto-analyzer.

Statistical analysis

For each index, the data were tested for normality and homogeneity of variances. An One-way analysis of variance with Duncan's post hoc were used to determine statistically differences (α=0.05) to evaluate the effect of chronic concentrations exposure of crude oil on fish blood parameters. The differences between means were analyzed at the 5% probability level. Data are reported as means ± standard deviation. The software SPSS, version 17 (SPSS, Chicago, Illinois state) was used as described by Dytham (1999).

Results

The fish exposed to constant sublethal concentrations

of crude oil mainly showed significantly difference in the hematological and biochemical levels in comparison to the control group. RBC reported significantly a lower level during whole exposure period in all treatment compared to control group(P≤0.05). Moreover the RBC showed a decline trend during the experiments (63 days), it was significant in the fishes exposed to group C (P≤0.05, Fig.1a).

During the nine weeks of the experiments, WBC was higher in control group(P≤0.05, Fig. 1d), but it showed some variations in its level in other treatment groups. In group B, WBC decreased after 7 days. Then it increased slowly after 21 days. In group C and D an increasing trend was observed during the experiment. However there was no significant difference in the WBC of all the treatment groups during the nine weeks of exposure (P>0.05, Fig. 1d).

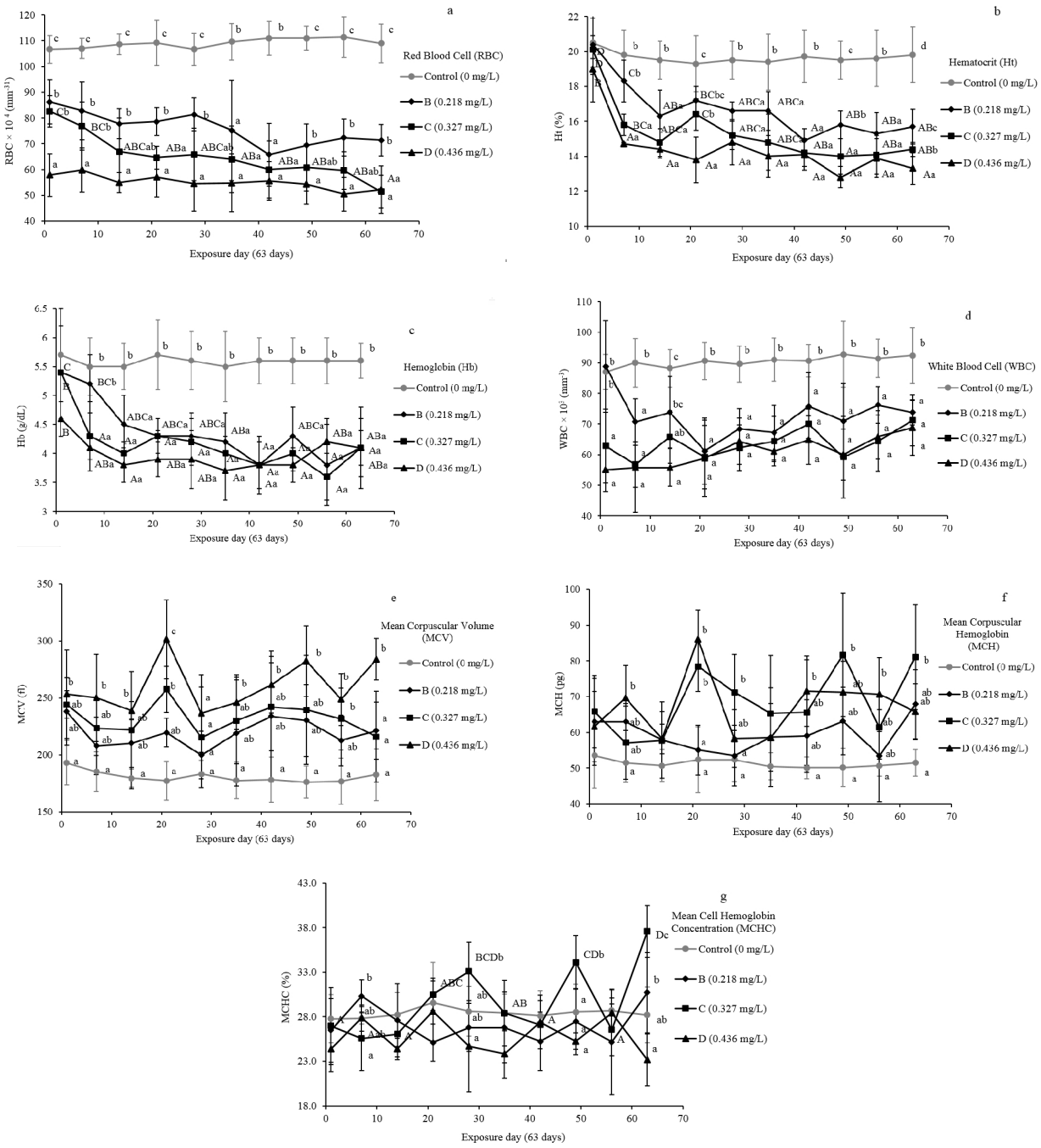

Fig. 1: Hematological parameters of Beluga after long exposure to crude oil. Different small and capital letters show significant differences between treatment groups and during of the exposure respectively (α=0.05).

Also Ht was significantly lower during whole exposure period in all treatment compared to control group except in the first day after treatment (P≤0.05, Fig.1b). It showed a biphasic trend in different treatment groups during the experiment as the Ht decreased after 14 days in group B and C, after that it increased in the third week (21 days) of the experiments and then it showed a decline trend. similar results were in group D except that an increase were observed in Ht level in fourth week (28 days) of the experiments (P≤0.05, Fig.1b).

The Hb values were significantly decreased in treated fish compared to control one except first week of exposing (P≤0.05, Fig. 1c). Hb values of the fishes in different treatment groups decreased up to seventh week (49 days) except in group C (P≤0.05, Fig. 1c). In group C, the Hb decreased up to the second week (14 days). Then it had a trend like a sinuous one from 14 days to the 63 days of the trial (P≤0.05, Fig. 1c).

Erythrocyte index of MCV was significantly lower in control group in comparison to the treatment groups during the trails except of its value in fourth week (P≤0.05, Fig. 1e). The index decreased from first day to day of 14 in the other groups. After that it increased after 21 days. Then it showed an increasing trend up to seventh week (49 days). Although there was no significant differences (P>0.05, Fig. 1e).

Similar results were observed in MCH level as it was significantly lower in control group except of first, 14 and 35 days of the exposure (P≤0.05; Fig. 1f). However other groups showed no specific trend during the experiment period (P>0.05). Maximum values of the index were calculated in the last, seventh, and third weeks of the experiments for group B, C, and D respectively(P>0.05; Fig. 1f).

Also, there was significantly differences between MCHC values of different treatments with the control group in 1, 28, 35, and 63 days of exposure (P≤0.05, Fig. 1g). In group B and C, the maximum MCHC values were calculated after 63 days while it was maximum in after 21 days of the experiment for group D. The differences between MCHC values during the experiment was significant in group C (P>0.05, Fig. 1g).

Lymphocyte count was significantly decreased when the fish exposed to more toxicant concentrations as the minimum frequently were in group D (P≤0.05, Tab. 1). In each treatment group, the maximum lymphocyte were counted in the first day of the exposure while the minimum were in 63, 21, and 7 days of the exposure in B, C, and D group respectively (P≤0.05, Tab. 1). Although a decline trend was observed in the lymphocyte frequently in all the groups, it was significant in group B and C (P≤0.05, Tab. 1). There was no significant differences in monocyte population in treated groups compared to control except in the 49th and 63th day (P>0.050.05, Tab. 2). In the day of 49, Monocyte frequency increased in croup B and then it decreased in group C and D (P≤0.05, Tab. 2). Similar results were observed in the 63th day, except that the monocyte increased in group D to the maximum frequency (P≤0.05, Tab. 2).

Tab. 1: Lymphocyte changes of Beluga after long exposure (63 days) to crude oil

Day/Treatment	Control (0 mg/L)	B (0.218 mg/L)	C (0.327 mg/L)	D (0.436 mg/L)
1	59.2 ± 2.8^{Ac}	48.8 ± 4.0^{Db}	40.8 ± 6.7^{Aab}	39.2 ± 2.8^{Ca}
7	59 ± 1.9^{Ac}	45.8 ± 3.4^{BCDb}	40.8 ± 3.4^{Ab}	29.8 ± 1.6^{Aa}
14	57.8 ± 1.9^{Ab}	39.6 ± 1.8^{ABCa}	36 ± 5.1^{Aa}	36.6 ± 2.9^{BCa}
21	57.8 ± 1.9^{Ac}	40.6 ± 3.4^{ABCDb}	33.8 ± 3.1^{Aa}	31.6 ± 2.9^{ABa}
28	58.8 ± 2.3^{Ad}	48.4 ± 5.1^{CDc}	41.2 ± 1.1^{Ab}	31.6 ± 3.2^{ABa}
35	60.8 ± 4.2^{Ab}	41.4 ± 8.9^{ABCDa}	37 ± 3.5^{Aa}	36 ± 3.4^{BCa}
42	58.2 ± 1.9^{Ac}	43.2 ± 3.7^{BCDb}	37 ± 3^{Aa}	33.2 ± 3.3^{ABa}
49	59.6 ± 1.7^{Ac}	47.6 ± 4.2^{CDb}	36.6 ± 2.2^{Aa}	36.4 ± 1.4^{BCa}
56	58.6 ± 2.6^{Ab}	38 ± 4.9^{ABa}	35.4 ± 2.9^{Aa}	35 ± 2.2^{BCa}
63	59 ± 2.5^{Ab}	34 ± 2.7^{Aa}	34.2 ± 3.7^{Aa}	35.2 ± 2.9^{BCa}

Data presented as mean ± standard deviation. Different small and capital letters show significant differences between treatment groups and during of the exposure respectively (α=0.05).

Tab. 2: Monocyte changes of Beluga after long exposure (63 days) to crude oil

Monocyte	Control (0 mg/L)	B (0.218 mg/L)	C (0.327 mg/L)	D (0.436 mg/L)
1	1.8 ± 0.8^{Aa}	1.7 ± 1.2^{Aa}	1.3 ± 0.6^{ABCa}	1.5 ± 0.7^{ABa}
7	2 ± 0.8^{Aa}	2 ± 1.4^{Aa}	2 ± 1.2^{BCa}	2 ± 0^{Ba}
14	2 ± 1^{Aa}	2.5 ± 0.7^{Aa}	1.8 ± 1.1^{BCa}	2 ± 0^{Ba}
21	4.4 ± 3.2^{Aa}	2 ± 0.8^{Aa}	2 ± 0^{BCa}	2 ± 0.8^{Ba}
28	2 ± 0.7^{Aa}	1.8 ± 0.5^{Aa}	1.3 ± 0.6^{ABCa}	3 ± 1.4^{Ba}
35	1.8 ± 1^{Aa}	2.2 ± 1.1^{Aa}	1 ± 0^{ABa}	1.5 ± 0.6^{ABa}
42	2.2 ± 0.4^{Ab}	3.3 ± 1^{Ac}	0^{Aa}	0^{Aa}
49	2.8 ± 1^{Aa}	1.7 ± 0.6^{Aa}	2.7 ± 1.2^{Ca}	2 ± 1.7^{Ba}
56	1.8 ± 0.8^{Aa}	1.5 ± 0.7^{Aa}	1^{ABa}	0^{Aa}
63	1 ± 0^{Ab}	1.3 ± 0.6^{Ac}	0^{Aa}	2^{Bd}

Data presented as mean ± standard deviation. Different small and capital letters show significant differences between treatment groups and during of the exposure respectively (α=0.05).

The eosinophil count was significantly increased when fishes were exposed to the more toxicant concentration except in the first day after treatment (P≤0.05, Tab. 3). In group B and C, there was a significant increase in eosinophil count during the experiment. the eosinophil count in the day 21th was the exceptional though (P≤0.05, Tab. 3). Similar results were observed in group D except that the eosinophil frequency in week 8 was lower that it was expected (P≤0.05, Tab. 3).

Also, the neutrophil count was in treated fish was higher than control group during the experiment (P≤0.05, Tab. 4). In Group B, minimum and maximum number were count in the week 7 and 2 respectively (P≤0.05, Tab. 4). There was no significant differences in neutrophil during the trail in group C (P>0.05, Tab. 4) but there was the decline trend in it in group D (P≤0.05, Tab. 4).

Tab. 3: Eosinophil changes of Beluga after long exposure (63 days) to crude oil.

Eosinophil	Control (0 mg/L)	B (0.218 mg/L)	C (0.327 mg/L)	D (0.436 mg/L)
1	13.4 ± 3.2^{Aa}	15.4 ± 2.7^{Aa}	17.2 ± 3.6^{ABa}	14.6 ± 2.1^{Aa}
7	10 ± 2.9^{Aa}	14.2 ± 2.6^{Aab}	15 ± 4.6^{Aab}	20.4 ± 2.7^{ABb}
14	11 ± 1.9^{Aa}	15.4 ± 3^{Aab}	21.4 ± 2.3^{BCb}	21.8 ± 6.1^{BCb}
21	9.4 ± 2.6^{Aa}	24.2 ± 2.3^{Cb}	29.4 ± 3.7^{Ebc}	32.8 ± 4.4^{Dc}
28	11.6 ± 2.7^{Aa}	19 ± 2^{ABb}	22.8 ± 2.2^{BCDb}	$28.2\pm3D^{c}$
35	10.2 ± 2^{Aa}	19 ± 3.6^{ABb}	23.6 ± 3.2^{CDEb}	30.6 ± 3^{Dc}
42	13 ± 2^{Aa}	20.4 ± 1.7^{BCb}	28.2 ± 3.3^{DEc}	30 ± 3.2^{Dc}
49	12 ± 1.2^{Aa}	23.2 ± 2.4^{BCb}	26.8 ± 2.8^{CDEbc}	31 ± 2.9^{Dc}
56	13 ± 0.7^{Aa}	20.4 ± 2.9^{BCb}	28 ± 2.8^{DEc}	26.8 ± 3.4^{CDc}
63	13.4 ± 2.3^{Aa}	23.8 ± 2.4^{BCb}	28.2 ± 3^{DEbc}	32.8 ± 2.8^{Dc}

Data presented as mean ± standard deviation. Different small and capital letters show significant differences between treatment groups and during of the exposure respectively (α=0.05).

Tab. 4: Neutrophil changes of Beluga after long exposure (63 days) to crude oil.

Neutrophil	Control (0 mg/L)	B (0.218 mg/L)	C (0.327 mg/L)	D (0.436 mg/L)
1	21.2 ± 2.3^{Aa}	29.8 ± 3.7^{ABb}	35.8 ± 4.4^{Ab}	43.2 ± 3.6^{Cc}
7	23.2 ± 4^{Aa}	32.4 ± 2.7^{ABCb}	36.6 ± 4^{Abc}	42.2 ± 1.3^{Cc}
14	22.2 ± 2.6^{Aa}	40.2 ± 1.3^{Db}	37.4 ± 2.5^{Ab}	40.4 ± 3.2^{Cb}
21	21.8 ± 1.1^{Aa}	33.6 ± 1.3^{ABCb}	36.4 ± 3.6^{Ab}	34 ± 2.7^{ABb}
28	21.8 ± 2.3^{Aa}	31.2 ± 4.4^{ABCb}	35.2 ± 2.3^{Ab}	34.6 ± 3.8^{ABb}
35	20.6 ± 2.2^{Aa}	30.2 ± 2^{ABCb}	37 ± 4.5^{Ab}	35 ± 1.4^{ABb}
42	20.6 ± 1.7^{Aa}	33.8 ± 3.8^{ABCb}	34.8 ± 2.9^{Ab}	33.6 ± 3^{ABb}
49	20.2 ± 2.6^{Aa}	28.2 ± 3.3^{Ab}	34.8 ± 1.3^{Ac}	32.8 ± 1.8^{ABc}
56	20.2 ± 2.2^{Aa}	36.4 ± 5.4^{CDb}	36.2 ± 1.5^{Ab}	37.8 ± 1.5^{BCb}
63	21.2 ± 2.3^{Aa}	35 ± 1.9^{BCDb}	36.2 ± 2.9^{Ab}	30.2 ± 4.8^{Ab}

Data presented as mean ± standard deviation. Different small and capital letters show significant differences between treatment groups and during of the exposure respectively (α=0.05).

The total protein was mainly significantly lower in treated fish compared to control fish during 63 days of exposing (P≤0.05, Fig. 2a). In all treatment groups, the total protein decreased after 7 days of exposing significantly (P≤0.05, Fig. 2a). It did not varied significantly after 7 days up to the last week (63 days) though. (P>0.05, Fig. 2a).

The glucose level was also higher in treated fish during the experiment but it was significant in 14, 42, and 49 days of the experiment (P≤0.05, Fig. 2b). No significant difference was detected in glucose level in treatment groups during the time of the toxicant exposure. The glucose level reached to the maximum after 14 days of exposing in group B and D, while it happened after 42 days in group C(P>0.05, Fig. 2b).

ALT enzyme activity also was lower than control fish from second to the last week (P≤0.05, Fig. 2c). In all the treatment groups, ALT enzyme activity showed a decline trend as the maximum and minimum enzyme activities were in the first day and 56 days of the exposing respectively (P≤0.05, Fig. 2c).

Also, levels of AST enzymes activity did not change significantly in first 21 days (P>0.05, Fig. 2d) but it decreased in treated fish than control group (P≤0.05, Fig. 2d). Like the ALT, AST activities mainly decreased significantly during the exposing period the treatment groups (P≤0.05, Fig. 2d). The minimum AST activity was measured after 56 days of exposure in all the groups (P≤0.05, Fig. 2d).

ALP activity was lower in treated fish than control group, but the differences were not significant except at day 49 (P>0.05, Fig. 2e). Similarly, no significant level was measured in ALP activity during the experiment in all the groups (P>0.05, Fig. 2e). The minimum activities were measure in the day 49, 49, and 35 in group B, C, and D respectively.

Results of LDH activity showed a decrease in treated fish and its level was significantly difference from control fish after 21–63 days (P≤0.05, Fig. 2f). It also decreased during the trial in all groups (P≤0.05, Fig. 2f).

Discussion

Measurement of biochemical and physiological parameters is commonly use as a diagnostic tool in aquatic toxicology and biomonitoring (McDonald and Milligan, 1992; Folmar, 1993; Soimasuo *et al.,* 1995; Kang *et al.,* 1999, 2003). In addition, hematological factors can be sensitive indicators of changes in ecophysiological condition (Vinodhini and Narayanan, 2009). Our results declared an increase of MCV, MCH and decrease of RBC, WBC, Hb, and Ht during chronic exposure to crude oil (P≤0.05).

Generally, the Ht value depends on the oxygen carrying capacity of the blood (Larsson *et al*., 1985). In present study the observed decrease in Ht value may be due to the less oxygen content of in the blood of fish. Moreover, lower Ht values also indicate shrinkage of cell due to toxicant stress on erythropoirtic tissue (Saravanan *et al*., 2011).

A progressive change in fish hematological parameters occurred, due to physiological stress in fish, probably. The crude oil can affect RBC, causing a hemolysis by a disruptive effect on the erythropoietic tissues of spleen and kidney. The decrease in hemoglobin concentration may be due to either an increase in the rate at which hemoglobin is destroyed or a decrease in the rate of hemoglobin synthesis. The erythrocyte indices obtained in present study confirms that chronic exposure to crude oil can stimulate erythropoiesis in Beluga.

Hedayati and Jahanbakhshi (2013) measured a decreased of RBC, Hb, Ht and MCV in *Huso huso* exposed to crude oil. The exposure of *Huso huso* water soluble fraction of diesel oil for 48 h and 7 days caused a decrease in hematocrit and hemoglobin due to hemolysis (Hedayati and Jahanbakhshi, 2013). Prolonged exposure (96h -15 days) to hydrocarbons present in water soluble fraction of diesel oil (WSD) inducted hemolysis in *Prochilodus lineatus* which caused a reduction in Ht and Hb accompanied by an increase in plasma concentrations of potassium (Simonato *et al*., 2008). Ramesh *et al*. (2014) reported that some hematological parameters of Indian major carp *Labeo rohita* induced by sublethal concentration of water borne selenite such as Ht, Hb, and RBC decreased significantly during the long time exposure (35 days). The authors pointed out that the significant

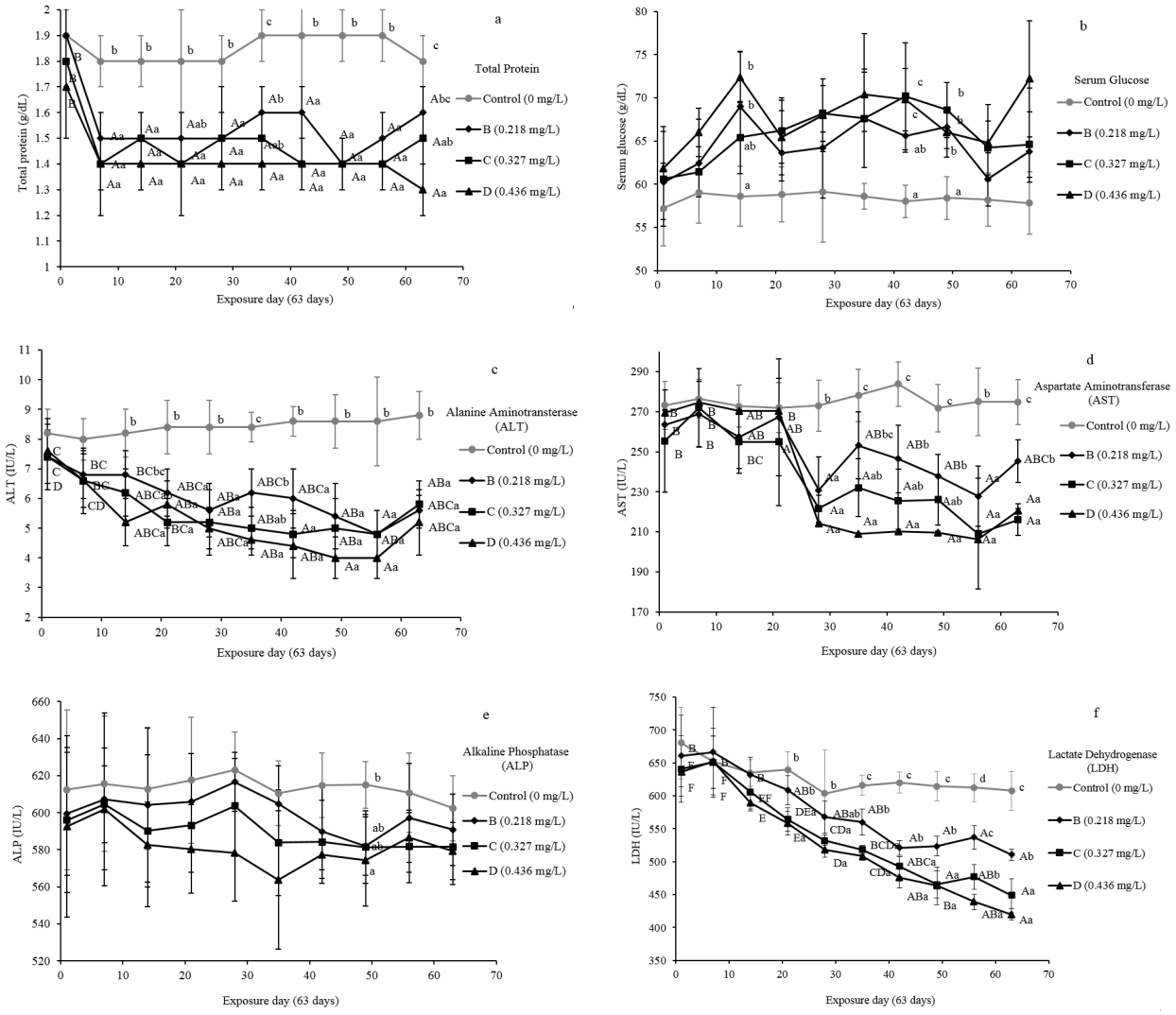

Fig. 2: Serum biochemical parameters of Beluga after long exposure to crude oil. Different small and capital letters show significant differences between treatment groups and during of the exposure respectively (α=0.05).

decrease in hematological parameters usually indicate the anemic condition of fish which might have resulted from the hemolysis caused by the toxicant. Moreover, accumulation of toxicants in the gill region may damage the structure of gill and induced impaired osmoregulation (Saravanan *et al*., 2011). Therefore, failure of erythrocyte production, internal hemorrhage or impaired osmoregulation during stress condition may leads to a reduction in RBC count (Joshi *et al*., 2002; Adedeji *et al*., 2009; Kavitha *et al*., 2010). Similar hematological responses detected in RBC, Ht, MCHC and Hb of olive flounder (*Paralichthys olivaceus*) exposed to single PAH, phenanthrene (Jee *et al.,* 2004). Also, a decrease in RBC level was seen in *Siganus rivulatus* at acute exposure to high concentration of crude oil (Eisler, 1975).

The chemical derived alternation in MCV, MCH, and MCHC have been attributed to direct or feedback responses resulting in hemolysis and impairment in hemoglobin synthesis, stress related release of RBCs from the spleen and hypoxia (Matsubara *et al*., 1985; Wiersma *et al*., 1998). The increase in MCV and MCH value indicate the anemia was of a macrocytic type or increasing RBC volume as suggested by Talas and Gulhan (2009). An increase in MCV and MCH values along with a reduction in MCHC value were observed in Indian major carp exposed to Ibuporfen for 35 days (Saravanan *et al*., 2012). Brucka Jastrzebska and Protasowicki (2005) and Saravanan *et al*. (2011) observed an increase in MCV and MCH value during sublethal treatment of cadmium , nickel, clofibric acid didofenac in common carp (*Cyprinus carpio*) due to

macrocytic anemia. Moreover, high concentration of smaller immature erythrocytes in the circulation due to hyperplasia in the erythropoietic (erythrocyte forming) sites also leads to higher value of MCV (Ferrando and Andreu Moliner, 1991). The lower value of MCHC in group B and C indicates a decrease in Hb synthesis. Our results were accordance with Lemly (1993), Sforcin (2007), and Ramesh *et al*. (2014). Whereas the observed increase of MCHC value during sublethal treatment (like in group D) may be due to congenital sphaerocytosis as suggested by sobecka (2001). In this study, we conclude that the alternations of these hematological parameters may provide the general health condition of fish under crude oil intoxication in fish.

Leucocytes are involved in the control of immunological function and the decrease of WBC counts after exposure to various toxicant may indicate a decrease in nonspecific immunity of the fish (Saravanan *et al*., 2011). Our results were accordance with a significant reduction in WBC count with increase in crude oil concentration in juvenile *Clarias gariepinus* and *C. anguillaris* (Awoyinka *et al*., 2011).

A significant decrease in lymphocytes was observed while neutrophil and eosinophil levels were increased mainly in treatment groups in comparison to the control group and during the exposure indicating a significant stressor effect by crude oil in fish immune-physiology. This phenomenon is because of lymphocytes role in responsibility for both hormone and cell mediated immunity; and so a decrease in its level will damaged such immune responses to the both pathogenic and non-pathogenic agents.

Also, an increase in eosinophil and neutrophil level shows that, the crude oil exposed fish, tried to actively respond to the toxin via enhancing the phagocytic activity. Under such long-term exposure condition, fish phagocytosis will abolish by damaging of hematopoetic tissues. Therefore, this result showed the chronic exposure of Beluga to crude oil can cause leucoctyotoxic in the fish via induction of leucocyte toxicity in the fish.

A significant decrease of leucocyte count (WBC) and the occurrence of lymphopenia and neutrophilia characterize the leucocyte profile of *Huso huso* after the acute exposure to diazinon at 22°C (Khoshbavar Rostami *et al*., 2004). Similar results were observed by Svododa *et al*. (2001) after exposing common carp to the acute effect of diazinon at 19-21°C. The changes in differential leucocyte count observed here indicate a decreased level of non- specific immunity in fish after chronic exposure to the toxicant.

Generally, the presence of pollutants in aquatic environment exerts its effect at cellular or molecular level which results a significant changes in biochemical responses and for monitoring of aquatic environment analysis of biochemical methods offer as important biomarkers (Vutukuru, 2003). Among the biochemical profiles, plasma glucose has been extensively used as a sensitive indicator of environmental stress in fish (Nemcsok and Boross, 1982). In the present study, the animals also showed a hyperglycemic response during exposure to crude oil, indicating the provision of energy reserves for immediate utilization (Oliveira *et al*., 2011). Similarly, Alkindi *et al*. (1996) observed a significantly elevated plasma glucose concentration in flounder fish after 3-h exposing of fish to the crude oil water-soluble fraction, indicating occurrence of stress condition in fish. This is due to early impact of the stressors on increase in glucose level. The hyperglycemic condition observed in many teleost fish under stress condition is mainly mediated by effect catecholamines on glucose release from liver (main carbohydrate store in fish) (Min and Kang, 2008). Similar results were reported by Talas and Gulhan (2009), Saravanan *et al*. (2011), Saravanan *et al*. (2012), and Ramesh *et al*. (2014).

Likewise, protein serves as an immediate source of energy during stress condition in many organisms. A significant decrease in protein serum level occurred, may be due to their possible utilization for metabolic purposes. Accumulation of toxicants in organs such as liver and kidney may leads to impaired protein synthesis (Lemly, 1993; Elia *et al*., 2011). Therefore, liver and kidney disorder due to toxicant stress may also lead to decrease in protein levels (Layanya *et al*., 2011). Also, a decrease in total protein in fish exposed to toxicants could be attributed to either a state of

hydration and change in water equilibrium as similar findings were reported by Jee *et al*. (2006), Simonato *et al*. (2008) and Jahanbakhshi and Hedayati (2013). Level of total protein were decreased in fish exposed to diazinon (28 days) due to chronic liver diseases (Banaee *et al*., 2011). Other authors also found that the level of total protein was decreased in fish exposed to different pollutants and pesticides (Vijayan *et al*., 1997; Velisek *et al*., 2008).

Enzyme activities are considered as sensitive biochemical indicators and widely used to assess the health of the organism in aquatic toxicology (Gul *et al*., 2004). Several soluble enzymes of blood serum have been considered as indicators of the hepatic dysfunction and damage. Among the array of enzymes used the AST, ALT, ALP and LDH are widely used to detect the cellular damage caused by the toxicants (Jung *et al*., 2003; Roy and Bhattacharya, 2005; Datta *et al*., 2007; Gad, 2007).

In our study, activity of these enzymes inhibited by crude oil as demonstrated by Gabriel *et al*. (2012) who confirmed metabolic enzymes activities of AST, ALT, ALP and LDH in plasma of *Clarias gariepinus* exposed to cypermethrin. Therefore, chronic/long-term exposure of Beluga to crude oil could induce a hepatotoxicity in fish via an inactive transamination and oxidative deamination functions. Kavitha *et al*. (2010) suggested that the significant decrease of AST and Alt activity during acute and sub-lethal treatment may be due to damaged hepatocytes are no longer capable of synthesizing AST protein. Further the significant decrease in ALT activity might have been resulted from the renal failure. Likewise decrease in serum ALT activity during arsenic exposure in fish indicated a congested condition in liver (Datta *et al*., 2007).

The LDH is an enzyme found in almost all body tissues, such as heart, kidneys, liver, skeletal muscle, brain, erythrocyte and gills (Hasnain, 2005). LDH measurement is used to detect tissue disorders and as an aid in the diagnosis of tissue damage (Hasnain, 2005; Rao, 2006). Layanya *et al*. (2011) pointed out that the reduction of enzyme activity can be attributed of toxicant accumulation in liver which in turn leads to death of liver cell. The author added inhabitation of LDH activity during sub-lethal exposure to inorganic arsenic t may be due to impaired carbohydrate metabolism. Similar results were observed by Banaee *et al*. (2011) in 7 days of exposure to diazinon in rainbow trout.

Conclusion

In the present study, it is concluded that, crude oil has a profound influence on the hematological, biochemical, and enzymological profiles of fish. Our results confirmed, crude oil had a disruptive action on erythropoietic cells and inhibits all enzymatic activities. These parameters could be effectively used as potential biomarkers of crude oil toxicity to the freshwater fish in the field of environmental biomonitoring.

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