Alkaline phosphatase activity and oxygen consumption efficiency in the muglid fish *Planiliza abu* juveniles as biomarkers to a long-term exposure to gas oil

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Received 02/12/2025 Accepted 12/01/2026 Published 17/01/2026

Abstract

The impact of long-term exposure of petroleum hydrocarbons on metabolic rate in *Planiliza abu* juveniles was studied. Alkaline phosphatase activity and oxygen consumption efficiency as biomarkers were tested after exposing to different levels of gas oil in warm and cold conditions. The results showed an elevation in the activity of the ALP enzyme. The activity of the ALP enzyme was higher in warm conditions than in cold conditions. An increase in oxygen consumption was observed after exposure, and it was higher in warm conditions than in cold conditions. The level of hydrocarbons in the blood plasma of fishes was measured using an Oil Content Analyzer, which showed increasing levels in warm conditions compared to cold water. The study concluded that long-term exposure to petroleum hydrocarbons affects metabolic processes as indicated by the excess in enzymatic activity and oxygen consumption rates. The study concluded that increasing in metabolic activity in order to get rid of oil pollutants and avoid their effects on internal systems. The study concluded the exposure to gas oil spill affects the basic biological functions of the fish. On the other hand, ALP enzyme and oxygen consumption rates are suitable as biomarkers for long-term exposure to petroleum hydrocarbons on freshwater.

Keywords: Biomarkers, Hydrocarbons, Iraq, Oil spill, Respiration, *Planiliza abu*.

P-ISSN: 1812-237X, E-ISSN: 2788-5720, https://ijaqua.uobasrah.edu.iq/index.php/jaqua/index

Introduction

Shatt Al-Arab River in Province of Basra, the main estuary of Iraq into the Arabian Gulf. Production and exporting the crude oil and its derivatives were usually associated with oil spill (Chen *et al.*, 2018). These petroleum products such as gasoline, diesel fuel, and light fuel oil are more toxic to aquatic life than crude oil (Ucan-Marin and Dupuis, 2015). Gas oil is a petroleum derivative produced from the refining of crude oil, which involves a fractional distillation process that produces various fractions or cuts of the petroleum hydrocarbon chain. Gasoil fuel contains C20-C12 components (Harayama *et al.*, 1999). Its density ranges from 0.85-0.81 g/cm^3 and its boiling point is from 200-360°C. Gas oil consists of 20% aromatic compounds and 1-3% olefins. The remaining components are saturated compounds (naphthene and paraffins), in addition to 1% sulfur. (Ministry of Oil, 2000)

Although some light fuel oil components evaporate quickly but they are more harmful to fish and aquatic life than heavy fuel oil which is less soluble in water. Dissolution and scattering are important processes causing the toxic effects of petroleum compounds in the water column (Wolfe, 2013), because the dissolved fraction is more accessible to aquatic life than the floating or sinking layer (Reddy *et al.*, 2012). Like other pollutants, petroleum toxicity to fish and aquatic organisms occurs through two main routes: the gills (Wood, 2017) and the digestive tract (Barrowman *et al.*, 1989). Petroleum compounds rapidly enter the fish's body and due to their high affinity for lipids, they concentrate in the fat tissues and cause dysfunction, such as in the nervous system, leading to paralysis and death (Hasan, 2022). Fish species have been observed to differ in their tolerance to the effects of petroleum concentrations (Aldoghachi, and Abdullah, 2021). The sensitivity of fish to petroleum substances varies among individuals according to age, sex, size, physiological condition, and genetic makeup (GESAMP, 1990).

Sensitivity also varies between different life cycle stages. Eggs and larvae are more sensitive than juveniles and adults (Rodríguez *et al.*, 2016). Studies have shown that the toxicity of petroleum compounds in marine waters was greater than in freshwater (Green, and Trett, 1989). Environmental toxicity studies have focused on the long-term effects of sublethal concentrations, or so-called chronic toxicity, which in the case of petroleum pollutants is attributed to polycyclic aromatic compounds (PAAs), which are high-molecular-weight compounds 10-1000 times more toxic than mono- and diaromatic compounds which evaporate rapidly, as well as isoalkanes, both of which are poorly soluble, less volatile, and more stable to biodegradation (Alqassim, 2019; Carls *et al.*, 2001; Rice *et al.*, 2001). Because of the blood's role in transporting oxygen and gas exchange inside the fish body, some pollutants affect this process and causing anemia and tissue hypoxia (Abdel-Tawwab *et al.*, 2019).

Measuring enzyme activity has been used as a biomarker of pollutant exposure. Alkaline phosphatase is a glycoprotein found primarily in vertebrate cell membranes (nuclear membrane, mitochondrial membrane, and cytoplasmic membrane) (Banaee, 2020). It is naturally found in the liver, intestine, bone, and placenta (Lowe *et al.*, 2023),

as well as in the heart muscle, kidney, and liver of fish (McComb, *et al*., 2013). The high levels of alkaline phosphatase in blood plasma are due to its release from cells into the extracellular fluid (Talwar *et al*., 2020). and normally the activity of this enzyme increases with the effects of photoperiod, temperature, and nutrition in fish (Lallès, 2020). Banaee (2020) concluded that alkaline phosphatase can be used as an indicator of disease status in fish. Enzymatic changes in fish have been used as biomarkers of oil pollution (Santana *et al*., 2018). It is known that fish live in environments where the dissolved oxygen content is 8 ppm, and this level is affected by environmental temperature and salinity (Mariu *et al*., 2023). Oxygen consumption has been considered a measure of an organism's metabolic rate (Nelson, 2016).

It is well known that oxygen is required to carry out oxidative phosphorylation and other energy needs in the cell, and respiration is undoubtedly one of the functions affected by pollutants (Wilson, 2017). Aitte, (2020) observed a sharp decrease in blood oxygenation in flatfish exposed to the dissolved fraction of crude oil for 48 hours. Abdel-Tawwab *et al*. (2019) recorded impaired gas exchange and decreased oxygen supply to tissues (tissue hypoxia). Meanwhile, Karem *et al*. (2022) found an increase in oxygen consumption of early-stage fish exposed to crude oil. Given the lack of local studies, whether short or long-term, on the effects of the petroleum derivative gas oil, which is discharged into the aquatic environment from riverboats and petroleum product transport vessels in the Shatt al-Arab River. Ecologically The muglid *Planiliza abu* is an important component of the fish assemblages of inland waters in Iraq and a marketable fish species. The current study aims to determine the effect of exposure to hydrocarbons in gas oil on the activity of the alkaline phosphatase enzyme in blood plasma, to estimate the rate of oxygen consumption. To determine the possibility of using these variables as biomarkers of long-term exposure to oil pollution. And to measure the hydrocarbons concentration in the blood plasma of the exposed fish.

Materials and Methods

Fish sample collecting

Juveniles *Planiliza abu* fish, weighing 6-8 grams, were collected from fish ponds near the Marine Science Center at the University of Basrah campus, at the Garmat Ali site. They were transported to the laboratory in 15-liter plastic containers filled with the water from the fishponds and carefully handled during capture and transport. The fish were distributed among 50-liter circular plastic containers, each containing 40 liters of aged tap water to remove chlorine. The fish were then distributed among the containers, 10 fish per container. The fish were acclimated for 6-7 days under laboratory conditions. Artificial aeration was used to provide dissolved oxygen to the tanks. The fish were given one daily meal of feed (17% protein) at 2% of their body weight. Feeding was discontinued 24 hours before the start of laboratory experiments. A quarter of the tank water was replaced daily using a siphon to remove uneaten food and excreted waste from the acclimated fish.

Preparation of exposure solution

Five liters of gasoil fuel were obtained from the Shuaiba refinery in Basra Governorate. Different volumes of gas oil fuel, namely 40, 32, 16 and 8 ml, were measured and the volumes were completed to one liter, each separately in Volumetric flasks. The solutions were mechanically mixed using a magnetic stirrer for three hours to ensure the mixing of the gas oil with the water. The above concentrations were then added to glass tanks of dimensions 30 x 30 x 60 cm containing 39 liters covered with wooden lids. Thus, we obtained an oil-in-water emulsion of gas oil with initial concentrations of 1, 0.8, 0.4 and 0.2 ml/L.

measurement of oil in the blood plasma

The UNEP (1977) method was followed to extract hydrocarbons from blood plasma. The caudal peduncle of fish was cut after killing it with a blow to the head. A quantity of blood was taken using (1x75) mm heparinized capillary tubes. These capillary tubes were placed in an MSE model HJ-488A microcentrifuge for two minutes, after one end was sealed with artificial clay. Blood plasma was withdrawn from capillary tubes after separation using a 50-microliter syringe, model 705 (Hamilton-Bonaduz Schwetz), diluted with deionized distilled water, and stored in freezer with -20°C temperature until measurement by Oil Content Analyzer (model OCMA-310, HORIBA Ltd., Japan, manufactured in 1995), which uses infrared radiation. The reading was taken directly from the screen in mg/L, and the reading was divided by the concentration factor.

ALP enzyme activity measurement

A colorimetric method was used to measure alkaline phosphatase activity. The Alkaline Phosphatase Kit, manufactured by BioMérieux, is scientifically based on the following equation:

$$Phenyl\ Phosphate \xrightarrow[pH=10]{ALP} > Phenol + Phosphate$$

Phenyl Phosphate ALP/(pH=10)>Phenol+Phosphate

Phenol liberated is measured in the presence of amino-4-antipyrene and potassium ferric cyanide. The presence of sodium arsenate inhibits the enzymatic reaction. Absorbance was measured by spectrophotometry at a wavelength of 510 nm, and alkaline phosphatase activity was estimated according to the following equation:

ALP = X-R×N / Y

X= Absorbance value of the tested sample

R= Absorbance value of the blank sample

N= where n = 20 when the Kind and King U/100 ml.

n = 142 when the Kind and King U/l.

Y= Absorbance value of the standard sample

note: 1 kind-king unit ≈ 0.177 international units (IU/L)

Estimation of oxygen consumption

To measure the oxygen consumption rate in *Planiliza abu* juvenile exposed to different concentrations of gas oil, four fish acclimated to laboratory conditions were taken and acclimated for one day in four artificially ventilated, sealed conical flasks completely covered with an opaque lid. Aeration was then cut off for the four treatments, and three volumes of gas oil were added to the first three after labeling: 0.5, 0.25, and 0.1 ml/L in warm conditions (27°C), and 0.25, 0.1, and 0.05 ml/L in cold conditions (15°C). The fourth treatment was left as a control sample.

Dissolved oxygen concentration was measured using the micro-Winkler method (Sumich *et al.*, 1996) at 0, 30, 60, 120, and 180 minutes, using a Spectrophotometer. Spectrophotometer method: A calibration curve is drawn for fixed oxygen solutions with known concentrations and permeability values measured using a Spectrophotometer. Oxygen concentrations are extracted from the calibration curve after measuring the permeability values of the samples (Sumich *et al.*, 1996). The oxygen consumption rate is calculated in mg per gram of fish weight per hour.

Results

Alkaline phosphatase (ALP) enzyme activity in blood plasma (fig. 1). The results show an increase in the activity of the enzyme under warm conditions throughout the experiment, reaching concentrations of 0.5, 0.25, and 0.1 ml/L on the fourteenth day of 4.15, 3.607, and 2.95 K. KU/100 ml, compared to the control sample on the same day 2.48 K. KU/100 ml.

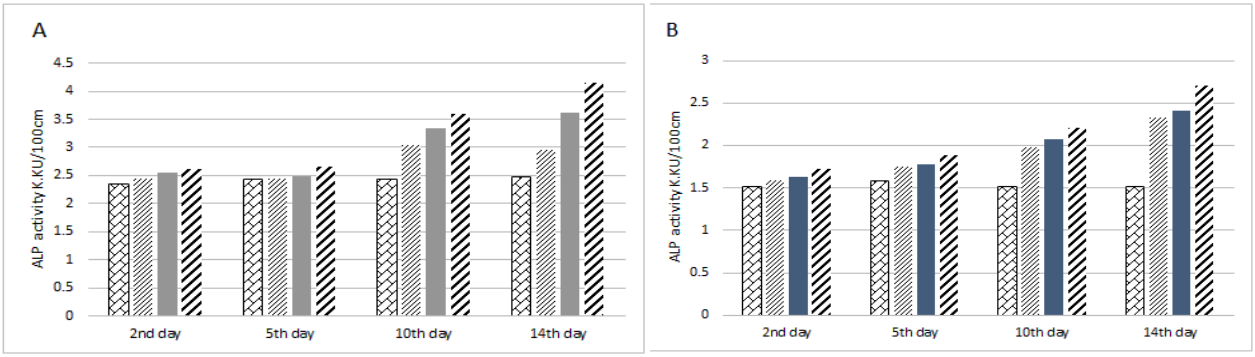
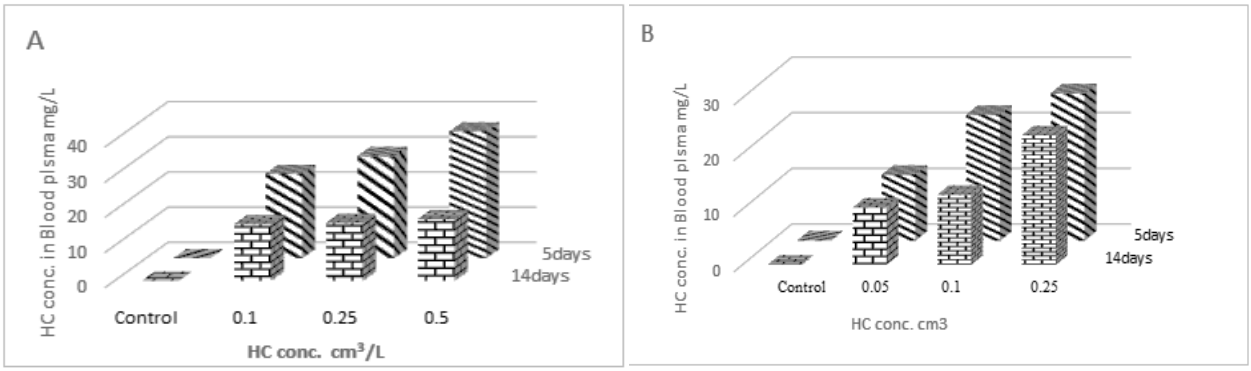


Figure 1. ALP activity (K.K. units/100 ml) in the blood plasma of *Planiliza abu* Juvenile Exposed to Gas Oil. A. At a temperature of 27 ± 2.3°C. B. At a temperature of 15 ± 1.5°C.

Alkaline phosphatase activity in cold conditions showed an increase throughout the experiment at the three concentrations (0.25, 0.1 and 0.05 ml/L), reaching (2.72, 2.405 and 2.33 K. KU/100 ml) in the fourteenth album, compared to the control sample of 1.52 K. KU/100 ml. The results showed that alkaline phosphatase activity in warm conditions is higher than in cold conditions.

Fig. (2) shows the concentration of hydrocarbons in blood plasma. The results of experiments under warm conditions showed a decrease in their concentration in the blood

plasma of fish exposed to initial concentrations of gas oil of 0.5, 0.25, and 0.1 ml on the fourteenth day (17.5, 16.4, and 16) mg/L, respectively. Compared to the fifth day of exposure (36, 29, and 24) mg/L, in the same order. Under cold conditions, hydrocarbon levels in the plasma of fish exposed to concentrations of 0.25, 0.1, and 0.05 ml/L decreased, reaching 23.2, 12.5, and 10.2 mg/L on the fourteenth day, compared to 26.5,



22.6, and 11.8 mg/L on the fifth day. The results showed a greater decrease in the concentration of hydrocarbons in the plasma of juvenile under warm conditions than under cold conditions.

Figure 2. Hydrocarbon concentration in blood plasma of *Planiliza abu* Juvenile Exposed to Gas Oil **A**. At a temperature of 27 ± 2.3°C. **B**. At a temperature of 15 ± 1.5°C.

Table (1) shows the results of estimated oxygen consumption in *Planiliza abu* juvenile exposed to gas oil under warm and cold conditions in the first twenty-four hours. Under warm conditions, the results showed a significant positive correlation (r=0.981) with the concentrations used. The rate of oxygen consumption increased with high concentration. The highest consumption was at the concentration (0.5 ml/L) reaching 0.037 mg O_2/g/h, compared to the control sample of 0.032 mg O_2/g/h.

Table 1. Oxygen Consumption in *Planiliza abu* Juvenile Exposed to Gas Oil.

27° C		**15° C**	
Oil conc.(ml/l)	**Oxygen consumption (mg O_2/gm/h)**	**Oil conc.(ml/l)**	**Oxygen consumption (mg O_2/gm/h)**
control	0.032	control	0.029
0.05	0.032	0.1	0.03
0.1	0.034	0.25	0.03
0.25	0.037	0.5	0.033

Under cold conditions, the results showed a significant positive correlation (r=0.965) with the elevating concentrations used. Oxygen consumption in fish exposed to gas oil increased with high concentration, reaching 0.033 mg O_2/g/h at the concentration (0.25

ml/L) while it was 0.029 mg O_2/g/h in the control sample. Normally the metabolic rate of fish as cold blood animals, in the warm circumstances is higher than in cold conditions.

The results showed that the rate of oxygen consumption in warm conditions was higher than in cold conditions in *Planiliza abu* juvenile exposed to gas oil.

Discussion

The increasing ALP enzyme activity in blood plasma of the fishes on the fourteenth day in all three treatments under high and low temperature conditions. It is worth noting that alkaline phosphatase is an isoenzyme that has no function in blood plasma, and therefore its levels are very low. ALP activity increases with leakage into the blood plasma from tissues that affected by necrosis, especially the liver, intestine, and bone (Sekaran *et al*., 2021). ALP activity also increases with the physiological state, photoperiod, water temperature, and nutritional status of Atlantic salmon (Ma *et al*. 2021). Sandnes *et al*. (1988) reported that the normal alkaline phosphatase activity in the blood plasma of Atlantic salmon (*Salmo salar*) juvenile ranges from 2.5–17 units/100 ml. Johnston *et al*. (2011) reported that its level in males of the same species was 2.9 U/100 ml, and in females, 6 U/100 ml. Alkaline phosphatase in plasma has been used as a biomarker for liver injury (Harper, 1975). Sultan (2001) recorded an increase in alkaline phosphatase activity in the intestines of *Planiliza abu* juvenile under feeding and transfer to a saline concentration of 7 ppt, with ALP enzyme activity reaching 41.32 K. KU/100 ml. Its activity decreased upon starvation and transfer to the same saline concentration (3.92 KK/100 ml) compared to the control sample (4.89 K. KU/100 ml).

It is noted from the results of the current study that ALP enzyme activity levels were higher at high temperatures compared to low temperatures, noting that the *Planiliza abu* juvenile were not fed in both cases. Johnston *et al*. (2011) reported that the ALP enzyme activity level in salmon was 5.1 units/100 ml in December and 5.3 units/100 ml in June.. The results showed that ALP activity increased with increasing gas oil concentration, possibly due to liver damage and diffusion of the enzyme into the bloodstream (Ezenwaji *et al*., 2013).

On the other hand, Gagnon and Holdway (2000) recorded an increase in sorbitol dehydrogenase (SDH) activity [an enzyme with a very low plasma presence] in the plasma of Atlantic salmon and *Platycephalus bassensis* exposed to oil pollution. Wolfe (2013) demonstrated that the dissolved portion of petroleum compounds in the exposure solutions penetrates through the gills. The dissolved hydrocarbons are bound to lipoproteins in the blood, which transport them through the circulatory system to all parts of the fish's body, including muscles (Filatova and Abramochkin, 2023; Omar-Ali *et al*., 2015). The movement of hydrocarbons between water and tissue depends on the concentration ratio between them, diffusion, and the rate of blood flow through the tissue (Ackman *et al*., 1996).

From the current study the concentration of hydrocarbons in blood plasma increases with increasing levels of hydrocarbons in water and decreases with decreasing levels. This

may be explained by the fact that the accumulation of the dissolved fraction continues until equilibrium is reached (Zhou *et al.*, 1997). Hydrocarbons are eliminated through the gills by diffusion, which is one of the factors restricting the accumulation (Ackman *et al.*, 1996). The increasing in the oxygen consumption rate in the fishes that exposed to gas oil with increasing temperature. The consumption rate ranged from 0.032-0.037 mg O_2/g/h at 27°C and from 0.029-0.033 mg O_2/g/h at 15°C. It is well known that energy consumption is often measured indirectly using the oxygen consumption method, which is calculated based on the amount of oxygen consumed in mg per gram of fish weight per hour.

Temperature and pressure are measured because they affect the solubility of oxygen. The oxygen consumption rate can be converted to kilocalories (kcal) according to the following relationship: 1 mg O_2/kg/h is equivalent to 0.00337 kcal/g/h, and 1 kcal/kg/h is equivalent to 297 mg O_2/kg/h (Smith, 1982). Brauner *et al.* (1999) observed a significant increase in air-breathing frequency in gobies exposed to the dissolved fraction of crude oil (WSF). The increasing in breathing could be attributed to a general stress response caused by petroleum compounds. A significant increase in oxygen consumption rate in Australian wolffish (*Maquaria novemaculeata*) exposed to a dispersed oil-in-water re-absorbed fraction at a concentration of 19.2 mg/L (Cohen *et al.*, 2001). After four days, the oxygen consumption rate reached 0.052 mg O_2/g/h compared to the control sample (0.026 mg O_2/g/h). The increased oxygen consumption rate in the current study indicated that excessive need for energy consumption under the stress conditions caused by oil pollution.

The metabolic rate increasing because fish use energy to break down and expel toxic components called polycyclic aromatic hydrocarbons (PAHs) of oil (Filatova and Abramochkin, 2023). The presence of crude oil affects the body physically, increasing the energy required for other tasks, such as elevated heart and respiratory rates. Moreover, Crude oil exposure causes cellular oxidative damage which increases oxygen demand to help stress response and repair process. Fish that were exposed to crude oil need more oxygen to recover after determined activity than unexposed fish.

Conclusions

The study concluded that the long-term exposure to petroleum compounds increases the metabolic rate in *Planiliza abu* juveniles. Whereas the increasing of metabolic rate means to eliminate the hydrocarbon compounds from the fish tissues. While the oxygen consumption rate in *Planiliza abu* juveniles elevate copying with energy consumption. So that using alkaline phosphatase activity in the blood plasma of *Planiliza abu* juveniles as a biomarker of exposure to oil pollution in freshwater environments. As well as using the oxygen consumption rate as another biomarker of exposure to oil pollutants.

Acknowledgement

The authors introduce the gratefulness to the department of Fisheries and Marine Resources in the college of Agriculture in University of Basrah for facilities and chemicals. The research is a part of MSC thesis.

Conflict of interest

No conflict of interest.

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نشاط انزيم الفوسفاتيز القاعدي وكفاءة استهلاك الأوكسجين كمؤشرات حيوية للتعرض طويل الأمد لزيت الغاز في يافعات أسماك الخشني *Planiliza abu*

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تاريخ الإستلام: 2025/12/02 تاريخ القبول: 2026/01/12 تاريخ النشر : 2026/01/17

المستخلص

دُرِسَ تأثير التعرض طويل الأمد للهيدروكربونات النفطية على معدل الأيض لدى يافعات أسماك الخشني *Planiliza abu*. واختُبِر نشاط إنزيم الفوسفاتيز القاعدي وكفاءة استهلاك الأكسجين كمؤشرات حيوية بعد التعرض لمستويات مختلفة من زيت الغاز في ظروف دافئة وباردة. وأظهرت النتائج ارتفاعًا في نشاط إنزيم الفوسفاتيز القاعدي. وكان نشاط الإنزيم أعلى في الظروف الدافئة منه في الظروف الباردة. ولوحظت زيادة في استهلاك الأكسجين بعد التعرض، وكان أعلى في الظروف الدافئة منه في الظروف الباردة. وقِيسَ مستوى الهيدروكربونات في بلازما الأسماك باستخدام جهاز تحليل محتوى الزيت، والذي أظهر مستويات متزايدة في الظروف الدافئة مقارنة بالباردة. وخلصت الدراسة إلى أن التعرض طويل الأمد للهيدروكربونات النفطية يؤثر على العمليات الأيضية كما يتضح من زيادة النشاط الإنزيمي ومعدلات استهلاك الأكسجين. وإن زيادة النشاط الأيضي مؤشر للتخلص من ملوثات النفط وتجنب آثارها على الأنظمة الداخلية للأسماك. كما أن التعرض لكميات من زيت الغاز يؤثر على الوظائف البيولوجية الأساسية. ومن ناحية أخرى، فإن إنزيم الفوسفاتيز القاعدي ومعدلات استهلاك الأكسجين نافعة كمؤشرات حيوية للتعرض الطويل الأمد للهيدروكربونات النفطية على أسماك المياه العذبة.

الكلمات المفتاحية: المؤشرات الحيوية، الهيدروكربونات، العراق، التسرب النفطي، التنفس، *Planiliza abu*.