Caspian Journal of Environmental Sciences
Online ISSN: 1735-3866
Print ISSN: 1735-3033

# Effects of diazinon on olfactory epithelium and genes related to olfactory signal transduction in Caspian roach, *Rutilus caspicus*

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## ABSTRACT

Olfaction in fishes is biologically-essential chemoreceptors. It provokes important behaviors for the survival of fish such as feeding, reproduction, avoiding predator and intraspecific communication. Pesticides can affect the olfactory epithelium and genes related to olfactory transduction and impair olfactory function. The present study aims to determine how toxicity of diazinon impacts olfaction of Caspian roach, *Rutilus caspicus* in histological and gene levels. We exposed fish (0.7 ± 0.05 g in weight) to 0, 1, 2 and 3 mg $L^{-1}$ diazinon (60 fish in each 3-replicate 60-L glass tank) for 7 days in freshwater. Diazinon concentrations were chosen based on reports on its concentration in surface waters. Mortality rate, histological events of olfactory epithelium, and genes expression at the 7$^{th}$ day after diazinon exposure included a significant decrease in the number of olfactory receptor cells (ORCs), while increased goblet cells (GCs). In addition, G-protein αi (Gpαi) was significantly upregulated, whereas calmodulin-dependent kinase II α (CaMKIIα) was significantly downregulated after 7 days as compared to the control group. These results indicate that diazinon can impair olfactory function through effect on the olfactory epithelium and olfactory signal transduction pathways in the Caspian roach fingerlings.

**Keywords**: Diazinon; Olfactory epithelium; Signal transduction; Caspian roach.

## INTRODUCTION

Caspian roach, *Rutilus caspicus* is one of the freshwater species and native to the Caspian Sea (Coad 1980; Soleimani *et al*. 2012). In addition to the high nutritional and economic value for the people (Kiabi *et al*. 1999), it has an important ecological role in the commercially-important fish food chain, such as sturgeons, perch and pike-fish (Keyvanshokoh & Kalbasi 2009). Their population has recently declined in the Caspian Sea because of overfishing, degraded habitat and pollution (Kiabi *et al*. 1999). Consequently, artificial production of the Caspian roach fingerlings (about 1 g in weight) has been taken place in order to rehabilitate stock population at the sea through releasing fingerlings in its rivers. Reports indicated that high levels of contaminants such as diazinon (especially in the summer) were found in the southeast rivers of the sea such as Gharesou (3.7 mg $L^{-1}$, Khosravi Katuli *et al*. 2013) and Torkrood (0.1-2.1 mg $L^{-1}$) due to the high application of diazinon in paddy fields, northern Iran, which is coincidentally with the release of the Caspian roach to these rivers (Shayeghi *et al*. 2001). The contaminants in water are taken up by fishes, influencing on olfactory organ (Pourgholam *et al*. 2013), olfactory sensory neurons (Tierney *et al*. 2010), endocrine system ( Khosravi Katuli *et al*. 2014; Hajirezaee *et al*. 2016; Mojazi Amiri *et al*. 2017), and genes related to olfactory signal transduction (Sandahl *et al*. 2005; Tilton *et al*. 2011; Maryoung *et al*. 2014, 2015), all with potential to abolish or cause aberrant olfactory-driven behaviors (Tilton *et al*. 2011; Maryoung *et al*. 2014, 2015). Olfaction in fishes is a biologically-essential chemoreception and very important in fish survival due to its role in feeding, reproduction, avoiding predator and intraspecific communication (Hara 1975).

Caspian J. Environ. Sci. Vol. 19 No. 2 pp. 211~218
DOI:
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Received: June 07. 2020 Accepted: Nov. 10. 2020
Article type: Research

The surface of the lamellae is covered by olfactory epithelium. Different types of cells are included into the epithelium, mostly olfactory receptor cells (ORCs), supporting cells (SCs), goblet cells (GCs), and basal cells (BCs), of those, ORCs are the primary sensitive nervous cells. New cells of the olfactory epithelium, receptor, supporting, and goblet ones, having limited longevity, develop and differentiate from BCs (Kasumyan 2004). Odorants bind to ORCs, activating the G-protein coupled receptor (Gp) and adenylyl cyclase (AC) to transmit olfactory signals (Pifferi *et al.* 2010). This process is regulated by calmodulin-dependent kinase (CaM) (Choi *et al*. 1992; Cali *et al*. 1994). Chemical neuro-behaviorals such as pesticides and metals have ability to impair the olfactory processes in vertebrates (Sandahl *et al*. 2005; Tierney *et al.* 2010). Few olfactory toxicological studies have endeavored to describe effects across organizational levels. Hence these studies should be continued to link toxicity mechanisms in fish behavior (Tierney *et al*. 2010). Histological examination of the olfactory epithelium revealed that exposure to high level of cadmium caused a decrease in ORNs and SCs (William and Gallagher, 2013). However, there is little study about effects of pesticides on olfactory sensory neurons in fish (Tierney *et al*. 2010). Maryuang *et al*. (2015) reported that exposure to hyper-salinity and chlorpyrifos upregulated four genes (chloride intracellular channel 4, Gprotein zgc: 101761, calcium calmodulin dependent protein kinase II delta, and adrenergic alpha 2C receptor) in juvenile rainbow trout, *Oncorhynchus mykiss* that inhibit olfactory signal transduction (Sandahl *et al*. 2005; Tierney *et al*. 2010). By increased number of contaminants followed by unpredictable changes in environmental condition (such as temperature), and by invasive species, the challenge is to determine how olfaction will function to ensure the success of fishes in a modern ecology (Tierney *et al*. 2010). Therefore, in this study we tried to find that how diazinon can affect olfactory system in Caspian roach, *R. caspicus* by examining olfactory epithelium and genes related to olfactory signal transduction.

## MATERIALS AND METHODS

### Experimental fish

The Caspian roach fingerlings (1± 0.3 cm in length and 0.7 ± 0.05 g in weight) were supplied from the Sijowal Caspian Sea Teleost Fish Propagation & Cultivation Centre (Golestan Province, the Southern Caspian Sea, Iran), and were transferred to the laboratory of Fisheries Research Center in Gharehsou, Iran. Fish were maintained in 14-h light:10-h dark cycle at 26 ˚C for 7 days in a 1000-L tank to acclimatize. Water was continuously aerated and 50% of the water was changed daily. During acclimation and experimental period, fish were fed with commercial fish diet (Commercial concentrates, JRS Co, Germany) three times a day (Imanpoor & Roohi 2015). All procedures were carried out in accordance with the Animal Care and Use Committee guidelines at the Faculty of Sciences of the University of Tehran (357, November 8, 2000).

### Fish exposure

After the acclimation period, fish were randomly transferred into 12 × 60-L tanks (60 fish per tank) and maintained at the above-mentioned light cycle and temperature. Then, they were exposed to diazinon for 7 days to investigate its long-term effects on olfactory system. Fish were exposed to 0 (control), 1, 2 and 3 mg $L^{-1}$ diazinon in freshwater (FW) for 7 days in three replicates per treatments. Thereafter, 50% of water was changed with freshwater daily and then diazinon was calculated and added to the water based on the volume of added water. Survival rate was monitored every 24 h during the exposure period. Diazinon concentrations were chosen based on reports on its concentration in the river surface waters discharging to the Caspian Sea, i.e. 3.7 mg $L^{-1}$ (Khosravi Katuli *et al*. 2013), 2.1 and 1mg $L^{-1}$ (Shayeghi *et al*. 2001). Hedayati *et al*. (2016) also reported that the median lethal concentration ($LC_{50}$ - 96h) of diazinon on the Caspian roach fingerlings exposed is 1.71 mg $L^{-1}$.

### Sampling

At the end of diazinon exposure period, three fish per replicate (9 fish per treatment) were euthanized in clove oil (150 ppm) for 40-50s (Holloway *et al*. 2004), and the olfactory organ was removed and fixed in Bouin's fluid for 48 h for histological examination (Roberts 1989). Four fish per replicate were euthanized and their olfactory tissue was pooled and placed in Cryotube and stored at -80 °C until total RNA isolation (Tilton *et al.* 2011).

### Histology

After fixation in Bouin's fluid for 48 h, the samples were washed in water and stored in 70% ethanol. The olfactory organs were embedded in paraffin and serial transverse cross-sections (3 µm) were made using a microtome. Dewaxed and rehydrated sections were stained with hematoxylin and eosin (Liu *et al*. 2015). The histological alterations in olfactory organ including olfactory receptor cells (ORCs), supporting cell (SCs) and goblet cells

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DOI:
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Received: June 07. 2020 Accepted: Nov. 10. 2020
Article type: Research

(GCs) from each fish, were determined using a light Microscope equipped with an ISCapture camera. Numbers of ORCs, SCs and GCs per 100 $\mu m^2$ of olfactory epithelium were counted using light microscope equipped with micrometer.

**Molecular studies**

Total RNA was isolated from olfactory tissues using TRIzol according to the manufacture's protocol (Ueda *et al.* 2016). The quantity (ng $\mu L^{-1}$) of RNA was determined by NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and the RNA purity was assessed measuring OD260/280 and OD260/230 ratios. Total RNA was then converted to cDNA using Promega Reverse Transcription System kits (Thermo Fisher Scientific, Waltham, MA). Primers were designed using Primer3 software for Gpαi and CaMKIIα. Primer sequences are listed in Table 1. The qPCR was run for each gene with β-Actin as the housekeeping gene using iScript three-step RT-PCR kit with SYBR Green from Bio-Rad. Thermocycling parameters were as follows: 15 min at 95 °C; 40 cycles of 15 s at 95 °C, 20 s at 58 °C, and 20 s at 72 °C; 15 s at 95 °C, 1 min at 65 °C, and 15 s at 95.1 °C.

**Table 1.** Primer sequences (5'-3').

| Target | Primer Sequence |
|---|---|
| Gpαi | TGAACAGAATAAGGCCAACGC |
| | GTGGAGTCTGACAGCTGGTAC |
| CaMKIIα | CAT(CT)CAGCAGATTTTGGAGGC |
| | TCTGCCAACTTCACAGCTGC |
| β-actin (housekeeping gene) | GATGAGGCTCAGAGCAAGAGAGGT |
| | AACACGCAGCTCCATTGTAGAAGG |

**Statistical processing and data analysis**

Data analyses were performed using SPSS 19.0 software (SPSS, Chicago, IL, USA). All data were tested for normality (Kolmogorov-Smirnov test) prior to conducting statistical analyses. One-way analysis of variance (ANOVA) followed by a post-hoc Tukey's test were used to assess the significant effects of diazinon concentrations on mortality rate, the number of ORNs as well as SCs and genes expression. A value of $p < 0.05$ was considered statistically significant for all tests. All the data were expressed as the mean ± standard deviation (SD).

## RESULTS AND DISCUSSION

**Mortality rate**

The Caspian roach fingerlings showed a dose-dependent mortality following exposure to diazinon. Mortality rate in freshwater ranged from 1% at 1mg $L^{-1}$ to 10% at 3 mg $L^{-1}$ (Fig. 1). The highest mortality was happened at the highest concentration of diazinon. Mojazi Amiri *et al*. (2017) reported that rainbow trout showed a dose-dependent mortality rate with chlorpyrifos (organophosphate pesticide) and most mortality occurred at high concentration of this pesticide, consistent with result of the present study. Their results revealed that organophosphate pesticides have negative effects on survival rate in fish especially in higher concentrations of 2 to 3 mg $L^{-1}$. It is well known that there is a positive relationship between the toxic component levels in water and mortality rate. Absorption of toxic substances in the body rises with increased concentrations of toxic chemicals in water (Pesce *et a*l. 2008; Saravanan *et al*. 2011).

**Histopathological changes**

According to table (2) and Fig. (2), histopathological alterations in the olfactory epithelium of the Caspian roach fingerlings exposed to different levels of diazinon in FW revealed that significant differences were observed in the numbers of ORCs and SCs by increased diazinon concentration ($p < 0.05$). The numbers of ORCs and SCs significantly decreased at high levels of diazinon (3 mg $L^{-1}$, $p < 0.05$). However, the number of GCs significantly increased in fish exposed to 2 and 3 mg $L^{-1}$ ($p < 0.05$). Also condensed nuclei (CN) were observed in fish exposed to high concentration of diazinon compared to control group (Fig. 2 & Table 3). In the present study, we indicated that exposure to diazinon as an organophosphate pesticide can negatively affect olfactory epithelium in fish. Histological results showed that high concentration of diazinon caused an increase in the GCs number, while a decline in ORCs and SCs. Some authors reported that the effects of chemicals were partially decreased by the


Received: June 07. 2020 Accepted: Nov. 10. 2020
DOI:
Article type: Research
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increase in GCs associated with elevated mucus production (Bettini *et al*. 2006). Therefore, raised number of GC, may be a defensive response of olfactory to diazinon in the environment. Nevertheless, increase in mucus secretion can negatively influence the diffusion of odorants, olfactory sensitivity, and olfactory signal transduction (Elinder & Arhem 2003; DeMaria & Nagi 2010; Tierney *et al*. 2010). Contaminants in the water could interact with the olfactory neurons as readily as odorants, which is problematic and may impair neuron functionality, and even loss of olfaction in fish (Tierney *et al*. 2010). Therefore, diffusion of odorants in fish exposed to diazinon in this study can decline due to the elevated number of GCs, while the drop in ORCs, hence consequent reducing in olfactory sensitivity to odorants and chemical information on the environment.

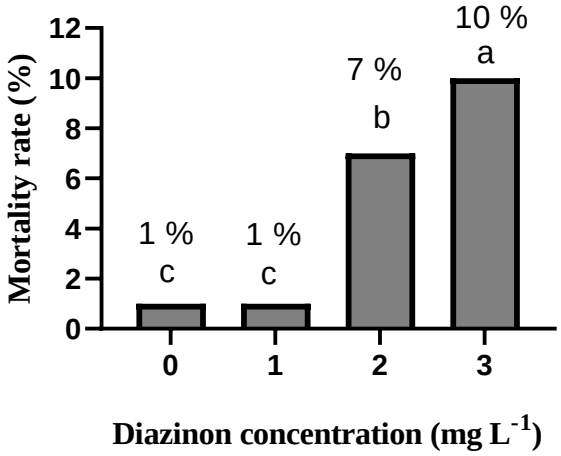


**Fig. 1.** Mortality rate after seven days exposure to diazinon.

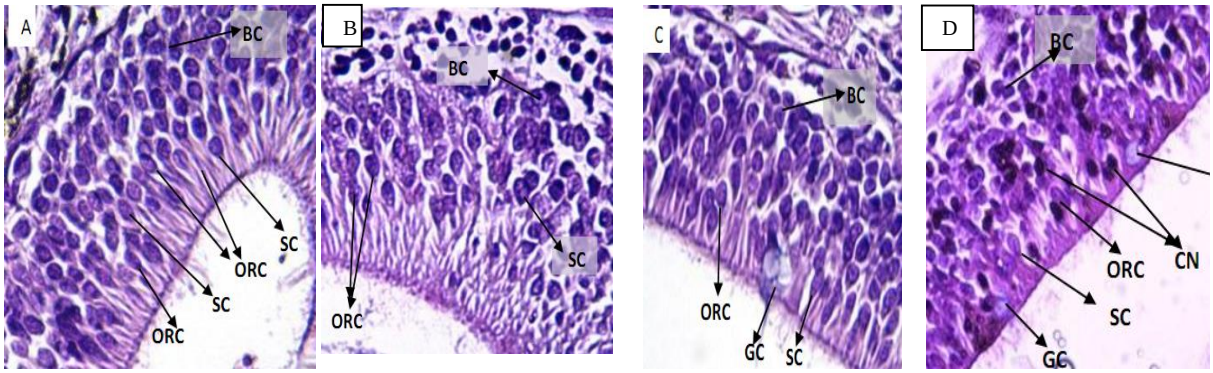


**Fig. 2.** histopathological alterations in the olfactory epithelium of Caspian roach fingerlings exposed to different levels of diazinon (A: Control, B: 1 mg $L^{-1}$, C:2 mg $L^{-1}$, D:3 mg $L^{-1}$). light macrophotograph (40 ×). BC: Basal cell, CN: condensed nuclei, GC: goblet cell, ORC: olfacrory receptor cell, SC: Supporting cell.

**Table 3**. Summary of histopathological alterations in olfactory epithelium of the Caspian roach fingerlings exposed to diazinon.

| Diazinon concentration | Increase in the GCs number | Decrease in the ORCs number close to the epithelial surface | Decrease in the SC number | Presence of CN |
|---|---|---|---|---|
| 0 mg $L^{-1}$ | + | - | - | - |
| 1 mg $L^{-1}$ | + | - | - | - |
| 2 mg $L^{-1}$ | +++ | ++ | + | - |
| 3 mg $L^{-1}$ | +++ | +++ | ++ | +++ |

None (-), mild (+), moderate (++), and severe (+++)

## Molecular analyzing

Gpαi was significantly upregulated, while CaMKIIα was significantly downregulated after 7 days exposure to diazinon in FW as compared to the control group ($P < 0.05$). Different diazinon concentrations significantly altered

Caspian J. Environ. Sci. Vol. 19 No. 2 pp. 211~218
DOI:
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Received: June 07. 2020 Accepted: Nov. 10. 2020
Article type: Research

gene expression in fish. We observed a dose-dependent alteration in Gpαi and CaMKIIα expression. Gpαi exhibited an upregulation, while CaMIIα was downregulated in fish exposed to diazinon, such that the highest alterations were found at its highest concentration. Gpαi is an inhibiting olfactory signal transduction (Maryoung *et al*. 2015) which decreases intracellular AMP levels via inhibiting AC (Schulz & Schoneberg 2003). Some subunits of AC and cAMP synthesis are stimulated by CaM and CaM-binding proteins (Choi *et al*. 1992; Cali *et al*. 1994). In addition, the calmodulin-stimulated state of AC is inhibited by Gpαi family members (Taussig *et al*. 1994). Therefore, in the present study, upregulation of Gpαi may be via inhibition in CaMIIα regulation effect on cAMP pathway and decreased olfactory signal transduction in fish, hence consequent impaired olfactory function. Tilton *et al*. (2011) reported that exposure to CPF disrupts olfactory function via inhibition of Gp signaling, leading to an olfactory system that is less responsive to odorants. If the olfactory receptors are affected by chemicals in the environment, behavior related to olfactory sense might be affected negatively, followed by depleting energy reserves, less growth and decreased fitness (Olsen 2011). Studies concerning to pesticide effects on the olfactory sense demonstrated that even its low concentrations can influence the olfactory detection of chemical cues (Moore & Waring 1996, 1998; Waring & Moore 1997). In addition, exposure of crayfish, *Orconectes rusticus* to non-lethal concentration of the herbicide metalochlor led to a decreased ability in responding to chemical stimuli (Wolf & Moore 2002). Moore & Waring (1996) reported that diazinon can reduce the electro-olfactogram (EOG) response to L-serine and PGF2a even at low level (1 mg $L^{-1}$) in fish.

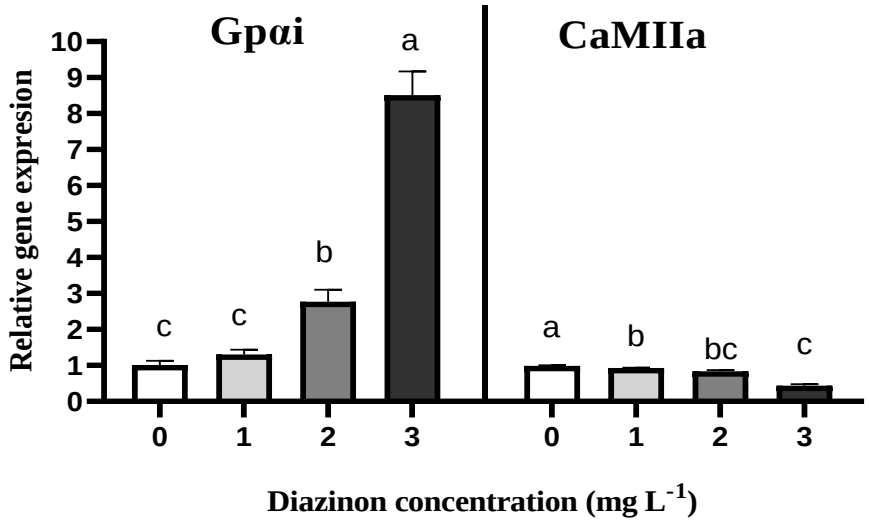


**Fig. 3.** Relative gene expression of Gpαi and CaMIIa after 7 days exposure to diazonon. Different lowercase letter indicates significant differences among diazinon concentrations ($p < 0.05$, One-Way ANOVA, Tukey's post hoc test).

## CONCLUSION

In general, diazinon can decrease the number of ORCs in olfactory epithelium where the odor molecules bind and transform the odorant molecules as neural signals to the brain (Rospars *et al*. 2010), while the increased GC number leads to a reduced transmission of information. In addition, exposure to diazinon can results in upregulation of Gpαi in fish that can inhibit CaM regulation and transmission pathway. Therefore, detection and transmission of odorant information may be disturbed in fish and as a consequence, olfactory-driven behavior may be impaired or lost in fish as a result of exposure to diazinon in FW. In this study we demonstrated that diazinon can be an olfactory toxicant in Caspian roach due to its various effects on olfactory signal transduction pathways or to its influences on olfactory epithelium.

## ACKNOWLEDGEMENT

This work was supported by the University of Tehran and Iran National Science Foundation under the project number 96009514, 29 April 2018. The authors are grateful to Sijowal Caspian Sea Teleost Fish Propagation & Cultivation Centre, Golestan Province, Iran, and also Fisheries Research Center of Gharesou, Iran for providing fish and equipment in our study.


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Received: June 07. 2020 Accepted: Nov. 10. 2020
Article type: Research

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Caspian J. Environ. Sci. Vol. 19 No. 2 pp. 211~218
DOI:
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Received: June 07. 2020 Accepted: Nov. 10. 2020
Article type: Research

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Caspian J. Environ. Sci. Vol. 19 No. 2 pp. 211~218
DOI:
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Received: June 07. 2020 Accepted: Nov. 10. 2020
Article type: Research

# تأثیر دیازینون بر بافت پوششی بویایی و ژن‌های مربوط به انتقال اطلاعات بویایی در ماهی کولمه (*Rutilus caspicus*)

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(تاریخ دریافت: ۹۹/۰۳/۱۸ تاریخ پذیرش: ۹۹/۰۸/۲۰)

## چکیده

بویایی در ماهی‌ها یکی از گیرنده‌های شیمیایی اساسی است. بویایی عامل مؤثر در ایجاد رفتارهایی از جمله تغذیه، تولید مثل، فرار از شکارچی و ارتباطات درون‌گروهی است که برای بقای ماهی ضروری هستند. آفت‌کش‌ها می‌توانند بر بافت پوششی بویایی و ژن‌های مربوط به انتقال اطلاعات بویایی تأثیر بگذارند و عملکرد بویایی را مختل کنند. هدف از مطالعه حاضر تعیین چگونگی اثرات سم دیازینون بر بویایی بچه‌ماهیان کولمه (*Rutilus caspicus*) در سطح بافت و بیان ژنی بوده است. بچه‌ماهیان (۰/۰۵ ± ۰/۷ گرم) به مدت ۷ روز در معرض ۰ ، ۱ ، ۲ و ۳ میلی‌گرم در لیتر دیازینون (۶۰ ماهی در هر ۳ مخزن با گنجایش ۶۰ لیتر) در آب شیرین قرار گرفتند. غلظت دیازینون بر اساس گزارش‌های مربوط به غلظت این سم در آبهای سطحی انتخاب شد. پس از هفت روز از قرار گرفتن در معرض سم دیازینون، میزان مرگ و میر، اثرات بر بافت پوششی بویایی و میزان بیان ژن‌ها سنجش شد. نتایج نشان داد که قرار گرفتن در معرض سم دیازینون باعث کاهش قابل توجهی در تعداد سلول‌های گیرنده بویایی (ORC) شد، در حالی که تعداد سلول‌های ترشح کننده موکوس (GC) افزایش یافت. ۷ روز پس از قرار گرفتن در معرض سم دیازینون، جی-پروتئین آلفا آی (Gpαi) به طور معنی‌داری افزایش و کلسیم کالمودولین ۲ آلفا (CaMKIIα) به طور قابل توجهی نسبت به گروه شاهد کاهش یافت. این نتایج نشان می‌دهد که سم دیازینون می‌تواند عملکرد بویایی را از طریق تأثیر بر بافت پوششی بویایی و مسیرهای انتقال اطلاعات بویایی در بچه‌ماهیان کلمه مختل کند.

*مولف مسئول

***Bibliographic information of this paper for citing:***

Hosseinzade, M, Mojazi Amiri, B, Iri, Y, Poorbagher, H 2021, The effects of diazinon on olfactory epithelium and gene related to olfactory signal transduction in Caspian roach (*Rutilus caspicus*)**.** Caspian Journal of Environmental Sciences, 19: 211-218

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Caspian J. Environ. Sci. Vol. 19 No. 2 pp. 211~218
DOI:
Received: June 07. 2020 Accepted: Nov. 10. 2020
Article type: Research
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