Ecotoxicology and Environmental Safety 158 (2018) 69–77

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Biochemical and molecular alterations in freshwater mollusks as biomarkers for petroleum product, domestic heating oil

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ARTICLE INFO

Keywords:
Domestic heating oil
Comet assay
AChE
MDA
SOD
CAT. Mollusks

ABSTRACT

To investigate the effect one of the oil products, domestic heating oil (DHO), on freshwater mollusks, *Unio tigridis* and *Viviparous bengalensis* were exposed to three DHO concentrations for each species (5.8, 8.7, and 17.4 ml L^{-1} for mussels; 6.5, 9.7, and 19.5 mlL^{-1} for snails, respectively). Antioxidant enzymes (superoxide dismutase, catalase), malondialdehyde, acetylcholinesterase and DNA damage in both species tissues were monitored over 21 days. The results showed that both antioxidant enzymes concentration (SOD and CAT) increased in the lowest DHO concentrations (5.8, and 8.7 ml L^{-1}), and then decreased in the highest concentration (17.4 ml L^{-1}) as the same pattern for *Unio tigridis*, but this not occurred for *Viviparous bengalensis*. MDA values recorded significantly increased compared to control. No reduction was observed in AChE concentrations in soft tissues of both mollusks may due to that DHO was a non-neurotoxicant to *Unio tigridis* and *Viviparous bengalensis*. The results of DNA damage parameters were showed significant differences ($p \leq 0.05$) between control and DHO concentrations except lowest concentration for each parameter measured in digestive gland of *Unio tigridis*. As well as, these significant differences were recorded between control and three concentrations of DHO exposure for comet length, and tail length parameters, and between control and highest oil concentration for tail moment in *Viviparous bengalensis*. DHO has the ability to prevent the reproduction of *Viviparous bengalensis* snail relation to control, that is what we considered strong evidence of the toxicity properties of DHO on the reproductive status of this species of snails. SOD, CAT, and MDA were useful biomarkers for evaluating the toxicity of DHO in mussel and snails, and comet assay was a good tool to assess the potential genotoxicity of DHO.

1. Introduction and literatures review

All over the populated parts of the earth, the quality of natural freshwater is being disturbed by human activities (Falkenmark and Allard, 1999). Oil spills do occur in freshwaters as a consequence of the many oil-related activities in this environment. Hence, it is important to study impacts of petroleum spills in freshwater habitats. Oils have been found in sediments at low energy sites as much as 5 years after the occurrence of spills, and they may be released into the water column long after the initial spill. Thus, water-column organisms as well as species inhabiting the sediment may be affected by oil spilled in the environment (Green and Trett, 1989). As well as, if oil is dispersed into small droplets, filter-feeding organisms can be exposed to whole oil through ingestion of droplets (Laetz et al., 2015). Therefore, the measurement of the biological effects of these pollutants have become of major importance for the assessment of the environment quality (Gray, 1992). In water, oil film floating on the water surface may be prevents natural aeration and leads to death of fresh water life (Ukoli, 2005).

There are many forms of liquid petroleum products which used as a fuel oil for furnaces or boilers in buildings. Domestic heating oil (DHO) is one of these products descripted by low viscosity, and consists of a mixture of petroleum-derived hydrocarbons, and was used mainly for home heating. Many researchers were determined petroleum hydrocarbons in water bodies (Olufemi et al., 2011; Obiakor et al., 2014; Freije, 2015), and others were studied the effects of oil spills and other petroleum hydrocarbon products on a different types of aquatic fauna included phytoplankton (Adekunle et al., 2010), bacteria (Jurelevicius et al., 2013), crustacean and midge (Bhattacharyya et al., 2003; Klerks et al., 2004), in addition to the various mollusks species (da Silva et al., 2005; Halldórsson et al., 2008; Fenghua et al., 2015; and Bhagat et al., 2017).

According to McCarthy and Shugart (1990), biomarkers are measurements at the molecular, biochemical, or cellular level in either wild populations from contaminated habitats or in organisms experimentally

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https://doi.org/10.1016/j.ecoenv.2018.04.006
Received 10 March 2018; Received in revised form 2 April 2018; Accepted 4 April 2018

exposed to pollutants that indicate that the organism has been exposed to toxic chemicals, and the magnitude of the organism's response to the contaminant. Biomarker studies have been applied in different animal species, but mollusks in the aquatic environment and among aquatic organisms more suitable for biological monitoring (Zhoua et al., 2008).

Antioxidant enzymes such as SOD and CAT are considered the most common biomarkers used in environmental quality monitoring (Regoli et al., 1998). MDA has been one of the first compounds to be used as an index of lipid peroxidation, and it consider one of the better known used as a biomarker of oxidative damage (Gagné, 2014a). As well as, AChE is a commonly used biomarker not only in aquatic ecotoxicology studies, but also ecotoxicology (Alloh et al., 2018; El-Nahhal and Lubbad, 2018) while DNA damage (measured by comet test) is used as a biomarker of genotoxicity under acute or chronic conditions (Gagné et al., 2014b).

Many studies used antioxidant enzymes, lipid peroxidation, AChE, DNA damage in mollusks tissues as biomarkers for oil spill (Bocquene´et al., 2004; Moreira et al., 2004), and many liquid petroleum products pollutants (Siu et al., 2004; Zheng et al., 2013; Bhagat et al., 2015), but no occurred any study used *Unio tigridis* and *Viviparous bengalensis* as biomarkers for domestic heating oil at world level. This reason, in addition to, the biomarker importance in aquatic environments as tool or early warning for disturbance diagnosis, we chose our study idea.

2. Materials and methods

2.1. Animals

Freshwater mollusks, *Unio tigridis* (7 ± 0.5 cm length, 3 ± 0.5 width, and 25 ± 2 g weight), and *Viviparous bengalensis* (2.8 ± 0.1 cm length, 2 ± 0.1 width, and 4 ± 0.5 g weight) were sampled from Hilla River (A branch of the Euphrates River in central Iraq). Only mollusks of the similar size were collected to be used in exposure experiments, then cleaned, transported to the laboratory where the experiments were carried out, and identified according to the standard keys (Ahmed, 1975; Plaziat and Younis, 2005).

2.2. Acute toxicity assay of DHO and calculate mortality percent

Experiments were conducted in 20 L plastic containers, each one containing 18 L of pre-aerated dechlorinated tap water, benthic sediments, and 6 adult mollusks. The specimens were not fed a day prior to and during toxicity tests to reduce fecal and excess food contaminating the test solution (Khalil, 2015). A geometric dilution series of eight concentrations was used in each toxicity test, with two replicate containers per concentration (i.e. 12 mollusks per concentration) and one blank control container. The oil was bought at a commercial gas station in the center of Hilla city, and was directly added to 10 L of water and mixed throughout for 5 min, than the volume was completed to 18 l with chlorine-free tap water.

DHO was tested at 50, 60, 70, 80, 90, 100, 110, and 120 ml L^{-1} according to da Silva et al. (2005). The six adult mollusk for each species were placed in the containers under 25 ± 0.75 °C, and 12 h:12 h dark/light regime, and the live/dead mollusks were counted after 24, 48, 72, and 96 h by the individual movement monitoring. Mussels were counted as dead when become unable to enter its soft tissues and closing their shells, while floating on the water surface of the aquarium was mark death in the snails. The probit analysis was carried out using by Finney's (1971) method, and then the LC_{50} was derived from the best-fit line obtained. Mortality rate of *Unio tigridis* and *Viviparous bengalensis* were calculated by the ratio between the total numbers of mollusks at the start to dead number of mollusks at the end of experiment.

2.3. Subchronic toxicity test for DHO

One control and three treated groups of mollusks (mussels and snails) were used in experiments. Treated groups were exposed to different concentrations of DHO (5.8 ml L^{-1}, 8.7 ml L^{-1} and 17.4 ml L^{-1} for mussels; 6.5 ml L^{-1}, 9.7 ml L^{-1}, and 19.5 ml L^{-1} for snails respectively), and during 21 days, these three concentrations for each species of mollusks were calculated by 1/15th, 1/10th and 1/5th of the 96 LC_{50} value (Al-Sawafi and Yan, 2013). The same experimental conditions of acute toxicity assay were considered to assess the effective concentration of DHO. Sufficient number of tested and control animals were removed at 21 days. For the biochemical analysis, mollusks were dissected and the soft tissues of specimens were weighted and used as source of measurements, while the digestive glands of two mollusks species were used for molecular analysis. All steps of the living tissue separation were carried out in the ice box to maintain the measured qualities from change and damage.

2.4. Biomarker measurements

The soft tissues of mollusks were homogenized by Pestle Motor Mixer provided by Argos Technologies (U.S) Cat. No.A0001 in phosphate buffer solution (PBS; pH 7.4) as a ratio 1:3, centrifuged at 15,000 × *g* for 15 min at 4 °C for biochemical assays. Superoxide dismutase (SOD: E-EL-H1113), and Acetylcholinesterase (AChE: E-EL-R0355) concentrations were measured by ELISA method using Elisa Kits (Elabscience Biotechnology Co., Ltd., China). Catalase (CAT: SEC. 418Hu), and Malondialdehyde (MDA: CEA597Ge) concentrations were measured by ELISA method using Elisa Kits (Cloud Clone Crop, USA). DNA damage was measured by take 100 mg of digestive gland of mollusks, then added to Ca^{2+}/Mg^{2+}-free PBS as ratio 1:5 (w: v), and used methods were described by (Conners, 2004;Gagne *et al.,* 2014) for DNA single-strand breaks measuring with the Comet assay. DNA damage was quantified by measuring comet length, tail length, and tail moment using computerized image analysis system (TriTek CometScore™ Freeware v1.5). Fifty cells were scored per slide, and geometric means were used to describe the damage.

2.5. Statistical analysis

Analysis of variance (ANOVA), F-test, t-test and least significant differences (LSD) were used to explain the differences between means at ($p \leq 0.05$), express that as mean ± SE. Principle Component Analysis was used for correlation between biochemical and molecular biomarkers (Gerry and Michael, 2002).

3. Results and discussion

3.1. Acute toxicity assay

The most common toxicity test is the 96 LC_{50}, which determines the concentration of toxicant that results in a 50% lethal response over a period of 96 h exposure (Gosling, 2003). This test was used in our study, and the results showed that 96 LC_{50} for *Unio tigridis* and *Viviparous bengalensis* were exposed to eight concentration of DHO were 87.17 ml L^{-1}, and 97.5 ml L^{-1}, respectively (Table 1; Fig. 1).

In a study to investigate the acute toxicological effects of the water-soluble fractions (WSF) of oils in the bivalve *Donax faba*, the results showed that 96 LC_{50} of diesel oil, kerosene, and the mix of diesel oil and kerosene were 3.916%, 13.461%, and 4.631% respectively (Devi and Ravichandran, 2016). Not occurred any previous study to known the effects of DHO of our selected mollusk species in all previous studies.

3.2. Sub lethal toxicity of domestic heating oil

Most research on the fate and effects of oil entering the aquatic

Table 1
LC_{50} of domestic heating oil (DHO) after 96 h for Left: *Unio tigridis*, and Right: *Viviparous bengalensis*.

Con. (mL^{-1})	Log of Con.	Mortality %	Probits	96 LC_{50} (mL^{-1})	Con. (mL^{-1})	Log of Con.	Mortality %	Probits	96 LC_{50} (mL^{-1})
50	1.69	0	0	**87.17**	**50**	1.69	16	4.01	**97.5**
60	1.78	16	4.01		**60**	1.78	16	4.01	
70	1.85	16	4.01		**70**	1.85	16	4.01	
80	1.90	48	4.95		**80**	1.90	32	4.53	
90	1.95	48	4.95		**90**	1.95	32	4.53	
100	2.00	66	5.41		**100**	2.00	65	5.39	
110	2.04	82	5.92		**110**	2.04	65	5.39	
120	2.08	81	5.88		**120**	2.08	65	5.39	

environment has focused on marine systems; therefore, parallel concern for the freshwater environment has lagged behind (Bhattacharyya et al., 2003). The results of the study are shown below in sequence.

3.2.1. Antioxidant enzymes changes

The relationships between exposure to pollutants and alterations in several enzymes have increased their use as environmental biomarkers in aquatic ecosystems (van de Oost et al., 2003). SOD and CAT activities play important roles in the antioxidant protection of invertebrates (Livingstone, 2003).

In the soft tissues of *Unio tigridis* mussel, the mean of SOD concentrations were 4608.5 pg/ml in control, while were 5121.5, 7515.4, and 5376.5 pg/ml in 0, 5.8, 8.7, and 17.4 ml L^{-1} of DHO concentrations for 21 day, respectively (Fig. 2A). Either CAT concentrations were 221 pg/ml in control as mean, while were 309, 459.33, and 372 pg/ml in 0, 5.8, 8.7, and 17.4 ml L^{-1} of DHO concentrations, respectively (Fig. 2B). By other hand, both enzymes increased in the lowest DHO concentrations (5.8and 8.7 ml L^{-1}), and then decreased in the highest DHO concentration (17.4 ml L^{-1}) as the same pattern. This may be in agreement with the study which noted that a decrease in SOD activity could be followed by a parallel decrease in CAT activity because the two enzymes are linked functionally and occur sequentially (Asagba et al., 2008).

As for the snail *Viviparous bengalensis*, the results showed that SOD concentrations were 57.59 pg/ml as mean in control, while were 63.92, 88.76, and 98.75 pg/ml in 0, 6.5, 9.7, and 19.5 ml L^{-1} in the soft tissues of snail exposed to DHO concentrations for 21 day, respectively (Fig. 2C). In addition to, CAT values were 291.67, 196, 207, and 228 pg/ml in control, lowest, median, and highest concentration of DHO (ml L^{-1}) which tested (Fig. 2D).

The results of present study were showed significant differences ($p \leq 0.05$) between control and treatments of SOD concentrations in soft tissue of the both *Unio tigridis* and *Viviparous bengalensis* exposed to DHO. The similar results of significant differences ($p \leq 0.05$) between control and treatments of CAT concentrations were recorded in the both mollusk species (Table 2A and B). Also, a significant positive correlation was recorded between SOD and CAT biomarkers in the soft tissues of *Unio tigridis* (r = 0.88; $p \leq 0.05$; Fig. 3).

As well as, Fig. 3 showed that SOD and comet length, tail length, MDA, tail moment, and AChE biomarkers in *Viviparous bengalensis* tissues were significantly correlated (r = 0.89, 0.86, 0.82, 0.78, and 0.69; $p \leq 0.05$, respectively).

The elevated activity of antioxidant enzymes in different mollusk species exposed to oil-originated chemicals has been reported by several authors, CAT activity increased in the digestive gland of *Mytilus edulis* has been observed after exposure to the water-accommodated fraction of crude oil (Cajaraville et al., 1992). The presence of diesel hydrocarbons in the digestive gland of the limpet *Nacella concinna* was associated with increased activities of SOD, and CAT in a dose-dependent and temporal manner (Ansaldo et al., 2005). CAT Activity in the digestive glands of *Crassostrea rhizophorae* was not different compare to control when exposed to the various diesel oil concentrations (0.01, 0.1, and 1 ml L^{-1}; da Silva et al., 2005), this result is not consistent with our results may be due to the lack of tested concentrations; but, compatible with other studies such as a study Jifa et al. (2006) which reported unaltered changes in *Lateolabrax japonicus* CAT activity after (B[a]P) exposure. Also found that CAT activity in *Eisenia fetida* was unaltered suggesting that PAH exposure does not induce increased CAT activity (Wu et al., 2011).

As well as, after a 21-day exposure, SOD activity in freshwater snail *Bellamya aeruginosa* under all treatments was significantly greater than control, and showed a response with increased concentration of ethylbenzene (Zheng et al., 2013), this compatible with our SOD results in *Viviparous bengalensis* tissues exposed to DHO. But, CAT values recorded a significant increase in lowest concentration then decrease in highest concentration of water accommodated fractions (WAFs) of diesel (Verlecar et al., 2012), anyway, this clearly appeared in our CAT related results in *Unio tigridis* tissues exposed to DHO.

3.2.2. Lipid peroxidation changes

MDA has been used to assess effects of various pollutants (Viarengo and Canesi, 1991; Pellerin-Massicotte, 1997). The mean of MDA

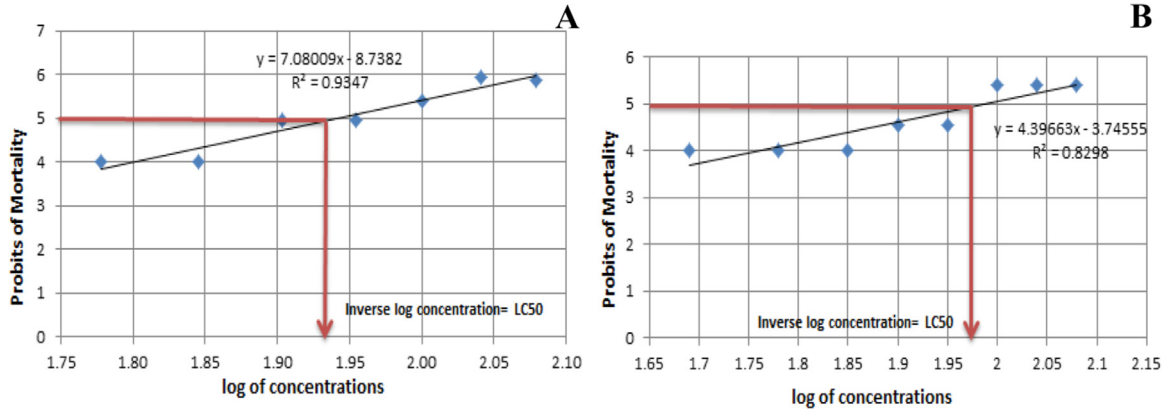

Fig. 1. Toxicity curve of DHO after 96 h. of exposure for A: *Unio tigridis*, and B: *Viviparous bengalensis*.

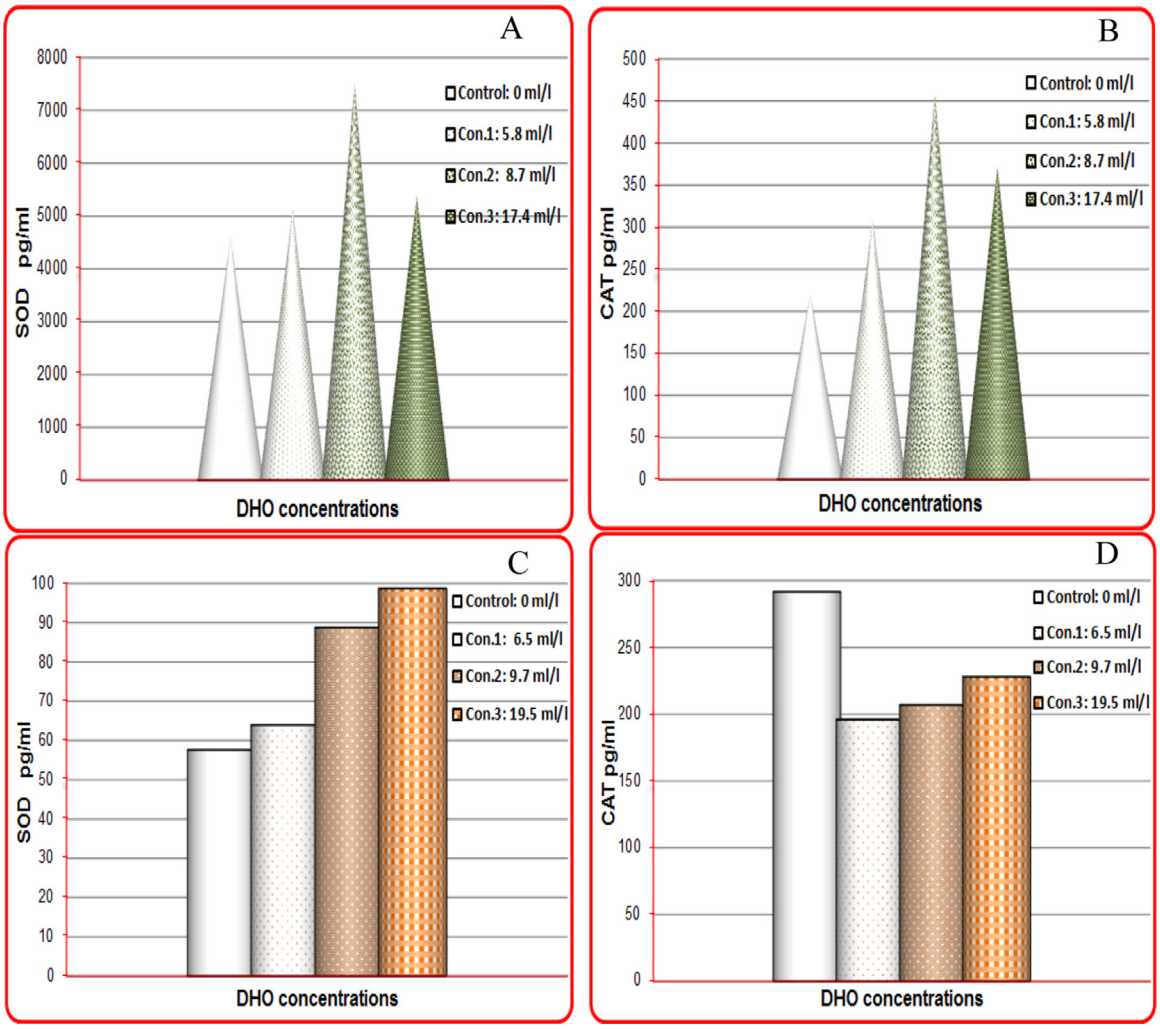

Fig. 2. Means of SOD (left), and CAT (right) concentration in *Unio tigridis* (A, B), *and Viviparous bengalensis* (C, D) exposed to DHO sublethal concentrations with control sample.

Table 2
Mean ± SE of biochemical and molecular biomarkers parameters in *Unio tigridis* (**A**), and *Viviparous bengalensis* (**B**) exposed to DHO sublethal concen-trations.

A

Parameters Concentrations	SOD (pg/ml)	CAT (pg/ml)	MDA (ng/ml)	AChE (pg/ml)	Comet Length (μm)	Tail Length (μm)	Tail Moment (μm)
Control: 0 ml/l	4608.5 ^{c} ± 35.95	221 ^{d} ± 10.02	1126 ^{b} ± 60.09	244.33 ^{b} ± 3.48	53.69 ^{c} ± 0.43	2.09 ^{c} ± 0.12	0.56 ^{c} ± 0.01
Con.1: 5.8 ml/l	5121.5 ^{b} ± 53.65	309 ^{c} ± 5.20	1791.4 ^{a} ± 51.58	258.67 ^{a} ± 2.33	55.50 ^{c} ± 0.31	3.56 ^{c} ± 0.36	1.46 ^{c} ± 0.30
Con.2: 8.7 ml/l	7515.4 ^{b} ± 222.49	459.33 ^{a} ± 13.53	1907.4 ^{a} ± 48.46	245 ^{b} ± 4.04	58.96 ^{b} ± 0.68	7.3 ^{b} ± 0.19	3.03 ^{a} ± 0.48
Con.3: 17.4 ml/l	5376.5 ^{a} ± 99.01	372 ^{b} ± 7.21	1953.7 ^{a} ± 151.61	265.67 ^{a} ± 3.48	64.41^{a} ± 0.95	10.19 ^{a} ± 1.26	4.10 ^{a} ± 0.60
LSD P ≤ 0.05	**410.82**	**31.04**	**289.89**	**11.06**	**2.09**	**2.17**	**1.34**

B

Parameters Concentrations	SOD (pg/ml)	CAT (pg/ml)	MDA (ng/ml)	AChE (pg/ml)	Comet Length (μm)	Tail Length (μm)	Tail Moment (μm)
Control: 0 ml/l	57.59 ^{d} ± 1.29	291.67 ^{a} ± 9.39	25 ^{c} ± 0.36	426 ^{b} ± 12.42	42.96 ^{c} ± 0.80	4.98 ^{d} ± 0.53	1.65 ^{b} ± 0.07
Con.1: 6.5 ml/l	63.92 ^{c} ± 0.61	196 ^{b} ± 3.21	33.91 ^{b} ± 0.31	441 ^{b} ± 18.15	48.38 ^{b} ± 0.77	6.24 ^{c} ± 0.10	1.82 ^{b} ± 0.05
Con.2: 9.7 ml/l	88.76 ^{b} ± 2.07	207 ^{b} ± 7.94	37.53 ^{ab} ± 1.10	448 ^{ab} ± 4.04	50.75 ^{b} ± 1.57	7.51 ^{b} ± 0.29	2.06 ^{b} ± 0.09
Con.3: 19.5 ml/l	98.75 ^{a} ± 1.23	228 ^{b} ± 16.56	38.33 ^{a} ± 2.10	485.67 ^{a} ± 9.24	56.54 ^{a} ± 1.30	14.13 ^{a} ± 0.46	5.66 ^{a} ± 0.36
LSD P ≤ 0.05	4.56	34.04	3.95	39.45	3.78	1.26	0.62

Note: Small letters indicate to comparison in column, similar letters are non-significantly differences between means at ($p \leq 0.05$), using (LSD test).
Note: Small letters indicate to comparison in column, similar letters are non-significantly differences between means at ($p \leq 0.05$), using (LSD test).

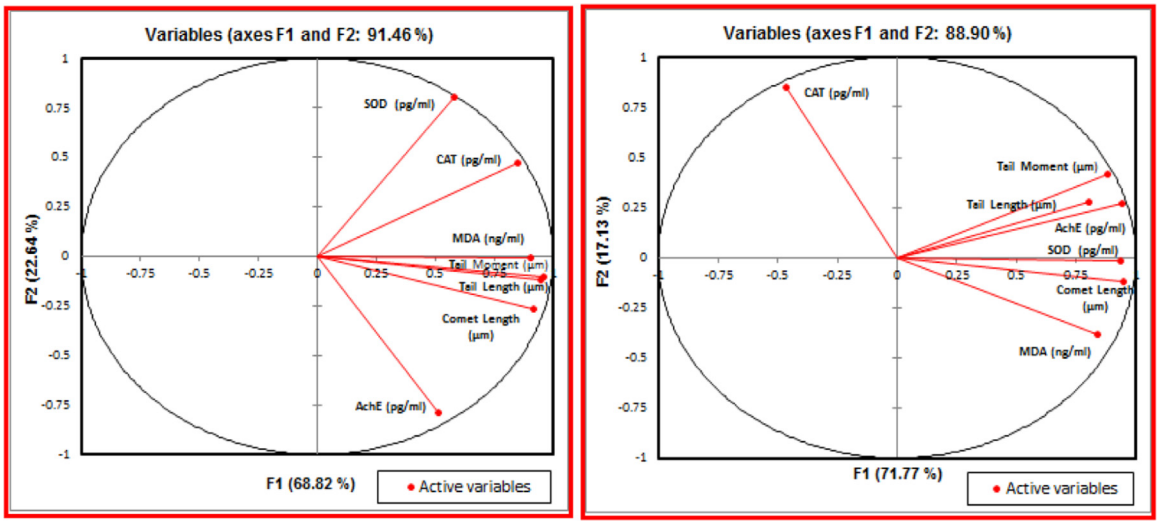

Fig. 3. PCA for correlation between biochemical and molecular biomarkers in left: *Unio tigridis*, and right: *Viviparous bengalensis* exposed to DHO sublethal concentrations.

concentrations in control of the present study was 1126 ng/ml in the soft tissues of *Unio tigridis* mussel, MDA concentration increased in lowest, median, and highest DHO concentration compared to control sequentially (1791.4, 1907.4, and 1953.7 ng/ml, respectively; Table 2A, and Fig. 4A). As for to the snail *Viviparous bengalensis*, MDA concentration was 25 ng/ml in control, and 33.91, 37.53, and 38.33 ng/ml in 6.5, 9.7, and 19.5 ml L^{-1} of DHO (Table 2B, and Fig. 4B). Also, the results were showed significant differences ($P \leq 0.05$) between control, and first, second, and third concentration of MDA in soft tissue of *Unio tigridis*, and *Viviparous bengalensis* (Table 2A and B).

Strongly significant positive correlation were recorded between MDA concentrations and tail moment, tail length, CAT, and comet length ($r = 0.83, 0.79, 0.78$, and 0.74; $p \leq 0.05$, respectively), in the soft tissues of *Unio tigridis* mussel (Fig. 3). As well as, it has appeared positive correlation between MDA concentrations and SOD, comet length, tail length, AChE, and tail moment ($r = 0.82, 0.76, 0.66, 0.61$, and 0.54; $p \leq 0.05$, respectively), and a significant negative correlation between MDA concentrations and CAT ($r = -0.64$; $p \leq 0.05$) in the tissues of *Viviparous bengalensis* snail (Fig. 3).

MDA concentrations under all treatments (in mussels and snails) were generally greater than control, this indicating without a doubt that the antioxidant system, including SOD, and CAT, ineffectively prevented lipid peroxidation. These results may be in agreement with several studies which recoded increased MDA in mollusk tissues after exposed to oil and their products e.g. the study Porte et al. (2000) which noted that levels of lipid peroxidation in mussel digestive gland tissue increased after accidental oil spills.

As well as, in the tissues of *Mytilus edulis*, MDA activity was high during the six months after the "Erika" oil spill incident (Bocquene´ et al., 2004). A significant increase in MDA content was seen on day 15 in *Perna viridis* exposed to water accommodated fractions (WAFs) of diesel at 0.5% and 5% concentrations in the digestive gland tissue relation to the control, as for petrol, these increased values did not differ significantly compare to control (Verlecar et al., 2012).

3.2.3. Acetylcholinesterase changes

Little information is available regarding the activity of AChE in mollusk exposed to petroleum hydrocarbons compare with pesticides (Olivares-Rubio et al., 2017). Our results showed that AChE values were 244.33, 258.67, 245, and 265.67 pg/ml as means in 0, 5.8, 8.7, and 17.4 ml L^{-1} of DHO concentrations for *Unio tigridis* mussel, respectively (Table 2A; Fig. 4C). As for to the snail *Viviparous bengalensis*, the means were 426, 441, 448, and 485.67 pg/ml in 0, 6.5, 9.7, and 19.5 ml L^{-1} of DHO concentrations, respectively (Table 2B; Fig. 4D). Also, the results showed significant differences ($p \leq 0.05$) between control, and first and third concentrations of DHO in soft tissues of *Unio tigridis* (Table 2A), and between control and highest concentrations of DHO in soft tissues of *Viviparous bengalensis* (Table 2B). Moreover, were observed significant positive correlation between AChE concentrations and all biomarkers tested of two mollusk species except SOD in *Unio tigridis*, and CAT in *Viviparous bengalensis* which negative correlated with their ($r = -0.30, -0.64$; $p \leq 0.05$, respectively; Fig. 3). DHO can be dissolved in the body of the tested organism and reached the nervous system and stimulate the release of AChE, consequently increased level was observed. This explanation is in accordance with El-Nahhal (2017) who found increased level of AChE due to treatments with Toxogonin.

The results from used AChE enzyme as biomarkers showed that it was inhibited by many environmental contaminants, such as undetermined components of fuel oils and complex mixtures of pollutants at concentrations that may be ecologically relevant (Moreira and Guilhermino, 2005; Binelli et al., 2006; Tim-Tim et al., 2009). AChE activity in *Mytilus edulis* was significantly lower during the first year of the survey after the "Erika" oil spill, suggesting a general stress syndrome in the mussels (Bocquene´ et al., 2004). Other study showed that activities of AChE in the digestive glands of *Crassostrea rhizophorae* exposed to the various diesel oil concentrations (0.01, 0.1, and 1 ml L^{-1}) were not different compare to control (da Silva et al., 2005), these results do not correspond to our observation may due to that DHO was a non-neurotoxicant to *Unio tigridis*, and *Viviparous bengalensis*, and we not in agreement with proposed for consideration that AChE inhibition as a generalist biomarker (Leinio and Lehtonen, 2005). AChE activities may be differentially modulated depending on the pollutant tested (Ozretic and Krajnovic-Ozretic, 1992).

3.2.4. DNA damage changes

DNA damage (comet assay) was correlated with pollution loads (Fernández-Tajes et al., 2011), and the size of the comet tail reflecting the extent of DNA damage (Rigonato et al., 2005). In the digestive gland of *Unio tigridis* mussel, the results of comet length were 53.69, 55.50, 58.96, and 64.41 µm in 0, 5.8, 8.7, 17.4 ml L^{-1} of DHO concentrations, and for tail length were 2.09, 3.56, 7.31, and 10.19 µm, either for tail moment, its results were 0.56, 1.46, 3.03, and 4.10 µm in control, lowest, median, and highest concentrations of DHO, respectively (Table 2A; Figs. 4E, and 5A and C). The results of DNA damage parameters were showed significant differences ($p \leq 0.05$) between control and the treatments of DHO concentrations except lowest

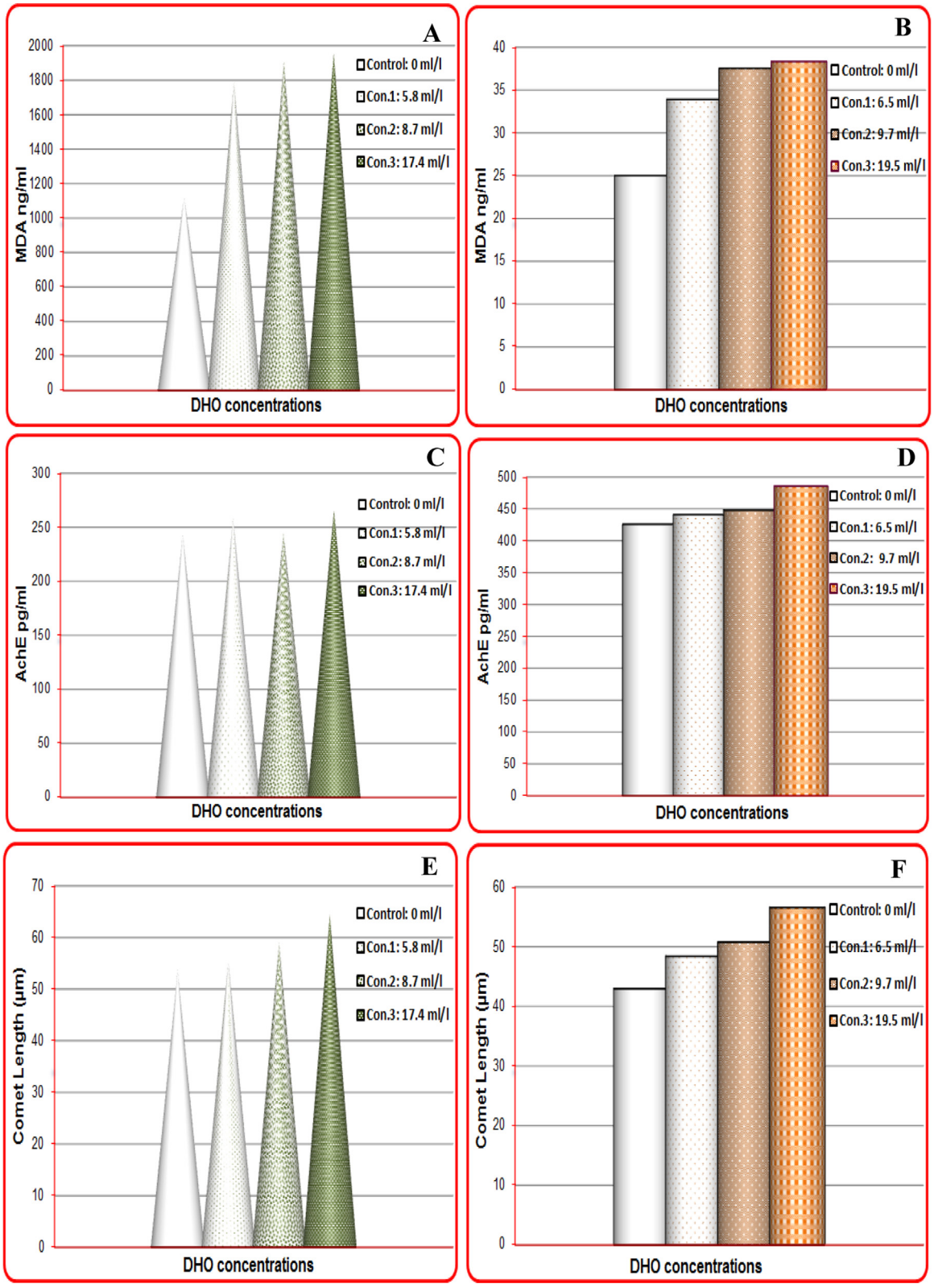

Fig. 4. Means of MDA concentration (A, B); AChE (C, D); Comet length (E, F) in *Unio tigridis* (left)and *Viviparous bengalensis* (right) exposed to DHO sublethal concentrations with control sample.

concentration for each parameter measured in digestive gland of *Unio tigridis* (Table 2A), also, the results showed a significant positive correlated between comet length and tail length ($r = 0.96$; $p \leq 0.05$), and tail moment ($r = 0.95$; $p \leq 0.05$), and between tail length and tail moment ($r = 0.95$; $p \leq 0.05$, Fig. 3).

As for to the snail *Viviparous bengalensis*, the results of comet length, tail length, tail moment in the digestive gland were (42.96, 48.38, 50.75, and 56.54 µm), (4.98, 6.24, 7.51, and 14.13 µm), and (1.65, 1.82, 2.06, and 5.66 µm) in 0, 6.5, 9.7, and 19.5 ml L^{-1} of DHO concentrations, respectively (Table 2B; Figs. 4F, and 5B and D).

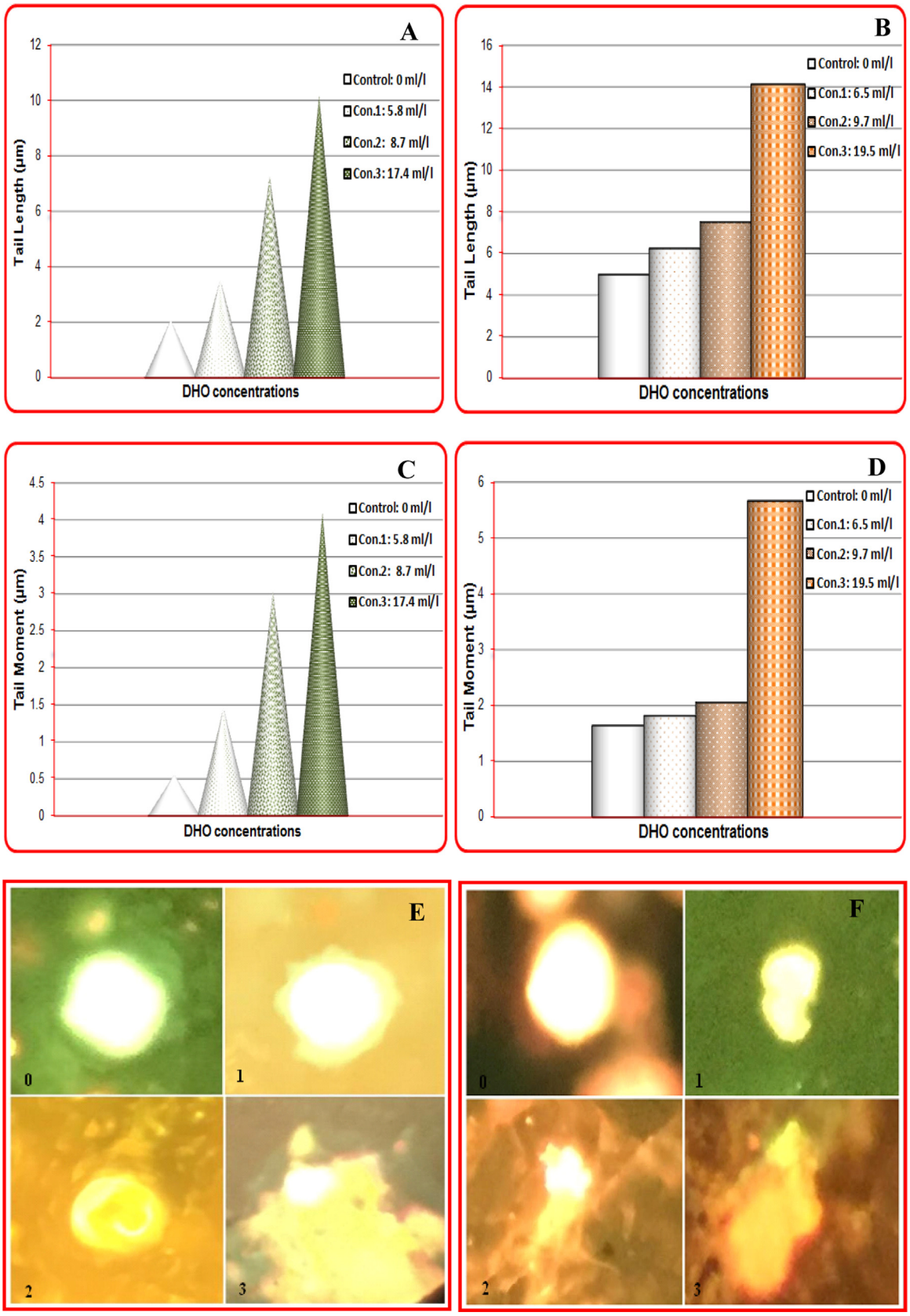

Fig. 5. Means of Tail length (A, B); Tail moment (C, D); Comet assay (E, F; class 0: un-damaged cells; classes 1, 2 and 3: from the less to more damaged cells) in *Unio tigridis* (left)and *Viviparous bengalensis* (right) exposed to DHO sublethal concentra-tions with control sample.

Statistically, significant differences ($p \leq 0.05$) were recorded between control and three concentrations of DHO exposure for comet length, and tail length parameters, and between control and highest concentration of DHO exposure for tail moment were measured in digestive gland of *Viviparous bengalensis* (Table 2B). Also, the results showed a significant positive correlated between comet length, and tail length ($r = 0.90$; $p \leq 0.05$), tail moment ($r = 0.82$; $p \leq 0.05$), and between tail length and tail moment ($r = 0.98$; $p \leq 0.05$, Fig. 3). DNA damage (Fig. 5E and F) which happened in mussels and snails measured over 21 days of exposure to DHO indicated to the toxic nature of these petroleum products to *Unio tigridis* and *Viviparous bengalensis*, which linked with inefficient antioxidant enzymes. This may be agreed with several studies which noted genotoxic effects of petroleum products on other mollusk species.

Adverse effects, including genotoxicity, of water soluble petroleum products on aquatic biota, *Perna perna* mussel inclusively, are well documented (Groner et al., 2001; Francioni et al., 2007). Sarkar et al. (2008) were reported a decrease in DNA integrity in the gastropod, *Cronia contracta*, and were reported to be attributed to exposure to polycyclic aromatic hydrocarbons.

The results of pervious study from used *Perna viridis* mussel as an experimental animal to detect DNA damage induced by water-borne benzo[a]pyrene in serial concentrations, suggested that an increase in the proportion of DNA damage was occurred dose-dependently (Siu et al., 2004). Moreover, by using the same concentration and type of pollutants demonstrated that tissues of *Perna viridis* showed an increase of DNA damage after prolongation of the animals exposure until 24 days (Ching et al., 2001). This was clearly reflected in our study. But, a significant decrease of DNA damage was observed after 12 days exposure to benzo[a]pyrene may indicated that recovery or DNA repair on cells of green mussel was occurred, and these not agreed with our results of present study, may due to that genotoxins can directly or indirectly inhibit DNA repair enzymes (Vasseur et al., 2013).

It is important to mention that DHO has prevented the reproduction of *Viviparous bengalensis* snail in the present study relation to control, and that is what we considered strong evidence of the toxicity properties of DHO on the reproductive status of this species of snails. A wide variety of environmental pollutants, directly or indirectly affecting DNA, have a significant toxicological relevance since they are implicated in many pathological processes (Lewis and Galloway, 2009; Devaux et al., 2011), and exposure to these pollutants can lead to abnormal physiological responses and cause adverse effects on the development, growth, behavior, and reproduction (Lee and Peart, 2000; Eganhouse and Sherblom, 2001; Ginebreda et al., 2014).

4. Conclusion

The results of the present study showed that 96 LC_{50} of DHO were 87.17 ml L^{-1} for mussels, and 97.5 ml L^{-1} for snails, and these not detected in previous studies. In summary, SOD, CAT, and MDA were effective biomarkers for evaluating the toxicity of DHO in mussel and snails. The results were indicating without a doubt that the antioxidant system, including SOD and CAT, ineffectively prevented lipid peroxidation. DHO was non-neurotoxicant to *Unio tigridis*, and *Viviparous bengalensis* after exposure, therefore, we consider that AChE specialist and not generalist biomarker for all pollutants, and DHO can be dissolved in the body of the tested organism and reached the nervous system and stimulate the release of AChE, consequently increased level was observed. As well as, comet assay was a good tool to assess the potential genotoxicity of DHO.

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