

RESEARCH REPORT

Oxidative stress in oysters (*Crassostrea corteziensis*) exposed to naphthalene**DG Mendoza-López, MI Girón-Pérez, CA Romero-Bañuelos, AE Rojas-García, BS Barrón-Vivanco, IM Medina-Díaz, ML Robledo-Marenco***Universidad Autónoma de Nayarit, Secretaría de Investigación y Posgrado. Boulevard Tepic-Xalisco s/n, Cd. de la Cultura Amado Nervo, C.P. 63190 Tepic, Nayarit, México**Accepted May 5, 2014***Abstract**

Naphthalene is a frequent pollutant in aquatic ecosystems that can affect the physiology of organisms such as molluscs. Oyster *Crassostrea corteziensis* is an endemic species from the tropical West Pacific, with both ecological and economical importance. The objective of this study was to evaluate the oxidative damage in lipids and proteins in the tissue (gills and digestive gland), as well as the membrane stability in hemocytes of oysters *C. corteziensis* exposed to naphthalene (1 or 20 µg/L) sub-acutely (24 and 72 h). The results obtained indicate that under evaluated conditions, this hydrocarbon does not induce oxidation of lipids and proteins. However, the stability of the cell membrane of hemocytes diminished significantly in organisms exposed to 20 µg/L during 72 h. According to the obtained results, it can be suggested that stability of the hemocyte's cell membrane is the most sensitive parameter to naphthalene's effect. It seems that compared to other hydrocarbons, naphthalene has low damage potential on oyster's oxidative parameters.

Key Words: naphthalene; oxidative stress; membrane stability; oyster; *Crassostrea corteziensis***Introduction**

The physiology of molluscs can be altered by polluting substances in aquatic ecosystems (Auffret, 2005; Rodríguez *et al.*, 2005; Baqueiro-Cárdenas *et al.*, 2007; Collin *et al.*, 2010). Bivalve molluscs accumulate a great quantity of water dissolved substances due to their sessile nature and filtration feeding habits (Farrington *et al.*, 1983; Goldberg, 1986). Oysters are one of the most commercially worldwide exploited groups of bivalves. It is calculated that the production of these organisms reaches 4.7 millions of tons per year (FAO, 2012). Pleasure oyster (*Crassostrea corteziensis*), also known as Cortez oyster, is a native species of the Tropical West Pacific, with ecological and economical importance. This mollusc species is distributed along the Gulf of California and all through Panama. Only in Mexico, approximately 1500 tons per year of this species are produced (CONAPESCA, 2011).

A group of pollutants frequently detected in aquatic ecosystems are the polycyclic aromatic hydrocarbons (PAHs). Among these compounds is naphthalene, which has been found in water, suspended organic matter, and sediments, as well as in the tissue of molluscs, particularly, oysters (Meador *et al.*, 1995; Noreña-Barroso *et al.*, 1999; Botello *et al.*, 2002; Guo *et al.*, 2007; Piazza *et al.*, 2008; Maskaoui and Hu, 2009; Ramdine *et al.*, 2012; Girón-Pérez *et al.*, 2013). This hydrocarbon possesses two rings of benzene and is included in the National Priorities List (NPL) of the United States (IARC, 2002). Most of the naphthalene that goes into the environment is released into the air (which mainly results from combustion) from where it enters the soil and water by humid or dry deposition. By possessing only two aromatic rings and low molecular weight, naphthalene, compared to other PAHs, has major water solubility. Therefore, it has major bioavailability for organisms in this environment (IARC, 2002; Iniesta and Blanco 2005).

Several studies that have been made in different type of molluscs indicate that the PAHs can induce the increase in concentration of reactive oxygen species (ROS), which provokes oxidative stress to macromolecules such as lipids, proteins and DNA (Livingstone *et al.*, 1992; Manduzio, 2005; Pichaud *et al.*, 2008). However, up to this moment

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Table 1 Lipid hydroperoxides and protein oxidation in oyster tissue (n = 3) exposed to H₂O₂

	Positive controls oxidation		
	PBS	H ₂ O ₂ (3%)	H ₂ O ₂ (9%)
	Lipid hydroperoxides (μmoles cumene hydroperoxide/gr tissue)		
Gill	3.6 ± 1.0	4.0 ± 0.6	14.1 ± 0.04*
Digestive gland	3.1 ± 0.5	4.5 ± 0.6	14.3 ± 0.2*
	Protein oxidation (μmoles/mg protein)		
Gill	9.4 ± 2.2	6.8 ± 2.5	21.1 ± 5.8
Digestive gland	23.0 ± 6.2	37.6 ± 12.3	33.0 ± 8.9

Values represented as mean ± SE, **p* < 0.05 vs tissue exposed only to PBS

there are no reports of the effect of naphthalene over oxidative stress in the *C. corteziensis* species. Thus the objective of this study was to evaluate oxidative stress in lipids and proteins in tissue (gills and digestive gland), as well as the membrane stability in hemocytes of oysters *C. corteziensis* exposed sub-acutely to naphthalene.

Materials and Methods

Animals and treatment

Crassostrea corteziensis oysters of commercial length and weight (8 ± 2 cm and 70 ± 30 g, respectively, and approximately 7 months old) were acquired at a local market in the State of Nayarit, Mexico and were immediately transported to the laboratory for their depuration during a 30 day period prior to experimentation (Adamo *et al.*, 1997). For this purpose, the oysters were maintained in a 50 l recirculation system with filtered seawater (salinity, 26 ± 2 ‰, pH 8.7 ± 0.2, and temperature, 26 ± 2 °C, 12 h:12 h dark-light cycles) and constant aeration. They were fed daily with dehydrated alga spirulina dissolved in filtered seawater (Castillo-Rodríguez and Garcia-Cubas, 1984). After the depuration period, the oysters were placed in glass fish tanks with 5 l of filtered seawater (without food, under previously mentioned conditions) during a 24 h period for their acclimatization. After this time, the organisms were exposed to sublethal concentrations of naphthalene (1 or 20 μg/L). The naphthalene (Sigma-Aldrich, 99 %) was taken from a stock solution (0.5 g/l), utilizing acetone as solvent at a 1:20 proportion (hydrocarbon:acetone). Three experimental groups were utilized per treatment (n = 20/per group): 1) oysters in seawater; 2) oysters in seawater with acetone, and 3) oysters in seawater with naphthalene. The oysters were exposed to the hydrocarbon during 24 or 72 h, with daily exchanges of water for each fish tank. During the experiment, the oysters were fed daily with alga spirulina.

Concentration of lipid hydroperoxides

For the determination of lipid hydroperoxides the ferrous oxidation-xilenol orange (FOX) method

was used, the tissue (gills and digestive gland) was homogenized with cold methanol (1:9 w/v) and was centrifuged to 1,000xg (3,600 rpm) during 15 min at 4 °C. A mix of reagents was prepared (400 μL FeSO₄ 0.25 mM, 400 μL H₂SO₄ 25 mM, 100 mL xilenol orange 0.1 mM) and it was incubated for 30 min. After that, 100 μL supernatant of the homogenized sample were added. The resulting mix was incubated in room temperature for 18 h in the dark. Once incubating time passed, its absorbance was determined to 550 nm against a standard curve of cumenehydroperoxyde (Monserat, 2003; Zanette *et al.*, 2006).

Oxidation of proteins

To determine the oxidation of proteins, the carbonyl group concentration was evaluated. From every analyzed tissue (gills and digestive gland), 0.4 g of each oyster were obtained and homogenized during 1 min in 1 mL of PBS. After that, it was centrifuged at 9,000 rpm during 20 min. From the centrifuged fraction 100 μL were taken and precipitated with 200 μL of trichloroacetic acid (TCA) at 30 % with slow agitation. The precipitate was added with 500 μL of 2,4-dinitrophenylhydrazine (20 mM in ethanol). The mix was incubated in the dark for 1 h, agitating every 10 min. Once incubation time was completed, it was precipitated with 200 μL of TCA at 30 %. The precipitate was dissolved with 1000 μL of 6 mM urea. The concentration of oxidized proteins was determined at 366 nm and was calculated through molar extinction coefficient (22 mM/cm) (Reznick and Packer, 1994).

Membrane Stability

The hemolymph extraction of the posterior region of the adductor muscle was performed and the membrane stability of the hemocytes was determined through the measurement of neutral red retention (Cantyet *et al.*, 2007; Hannamet *et al.*, 2009). 50 μL of hemolymph in a microwell were incubated during 45 min at 4°C. A rinsing with saline solution was made in order to eliminate the non-added cells. After that, 200 μL of neutral red were added (0.004 %) and incubated during 3 h at room temperature.

Once incubation time passed, it was rinsed with 200 μL of saline solution, and 200 μL of acidified ethanol were added. It was agitated during 10 min and its absorbance was determined at 545 nm.

Determination of protein concentration

Concentration of protein oxidation and membrane stability was adjusted according to the total concentration of proteins in the determined sample, which was measured through the Bradford method (1976).

Positive controls

As positive control of oxidative stress, samples of tissue (gill and digestive gland) ($n = 3$) were exposed to hydrogen peroxide (H_2O_2) (3 and 9%) during 24 h protected from light. After that, the concentration of proteins and lipid hydroperoxides in each sample was determined by following the techniques previously described.

Statistical analysis

For statistical analysis, we employed SigmaStat® (ver. 3.5. statistical software). We determined the normal distribution of the obtained data. For normal data distribution, we used Analysis of Variance (ANOVA) followed by the Bonferroni subtest. For non-parametric data, we used the Kruskal-Wallis test followed by a multiple Tukey-type comparison. To compare statistical difference between two groups, either t-test or Mann-Whitney test were used. The statistical difference was determined with a level of $p < 0.05$.

Results

Previous to the determination of the oxidative damage in lipids and proteins of gills and digestive gland of oysters exposed to naphthalene, oxidation of these biomolecules was induced through the exposure of tissue at H_2O_2 (positive oxidation control). The obtained results showed an increase in the oxidation of lipids and proteins when the tissues were exposed at 9% H_2O_2 . However, significant difference was shown only in the concentration of oxidized lipids (Table 1).

Regarding the effect *in vivo* of naphthalene (1 $\mu\text{g/L}$) on the oxidation of lipids and proteins, no increase in the concentration of such molecules in the gills and digestive gland was detected, after 24 and 72 h exposition (Table 2).

On the other hand, the obtained results indicate that the naphthalene exposition (1 $\mu\text{g/L}$) during 24 and 72 h did not provoke alteration in the membrane stability of the hemocytes. Regarding the oysters exposed to 20 $\mu\text{g/L}$ of naphthalene, a decrease in this parameter was observed. However, this was only significant in the oysters exposed during 72 h ($p < 0.05$) (Fig. 1).

Discussion

In scientific literature, there are a number of studies that evaluate the effect of different PAHs (individualized or mixed) about oxidative stress in different species of bivalve molluscs; however, up to this moment there are no studies published where

Table 2 Lipid hydroperoxides and protein oxidation in oyster tissue exposed to naphthalene

Tissue	Treatment	Naphthalene (1 $\mu\text{g/L}$)		Naphthalene (20 $\mu\text{g/L}$)	
		24 h	72 h	24 h	72 h
Lipid hydroperoxides ($\mu\text{moles cumene hydroperoxide/gr tissue}$)					
Gill	Control	5.3 \pm 1.6	4.0 \pm 1.6	1.1 \pm 0.1	1.6 \pm 0.1
	Naphthalene	2.1 \pm 0.5	10.4 \pm 2.7	0.9 \pm 0.2	1.0 \pm 0.1
		$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$
Digestive gland	Control	8.8 \pm 1.8	13.0 \pm 2.9	2.0 \pm 0.3	1.7 \pm 0.1
	Naphthalene	8.8 \pm 1.9	11.1 \pm 3.0	1.2 \pm 0.2	1.9 \pm 0.2
		$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$
Protein oxidation ($\mu\text{moles/mg protein}$)					
Gill	Control	29.3 \pm 3.8	36.6 \pm 6.2	42.4 \pm 9.7	3.6 \pm 0.1
	Naphthalene	21.5 \pm 3.5	20.3 \pm 2.4	44.7 \pm 8.0	3.5 \pm 0.1
		$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$
Digestive gland	Control	16.2 \pm 3.6	23.3 \pm 4.0	55.0 \pm 12.6	3.5 \pm 0.07
	Naphthalene	28.1 \pm 4.3	20.6 \pm 3.9	43.7 \pm 4.8	3.6 \pm 0.1
		$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$

Values represented as mean \pm SE, $n = 20$ /per treatment

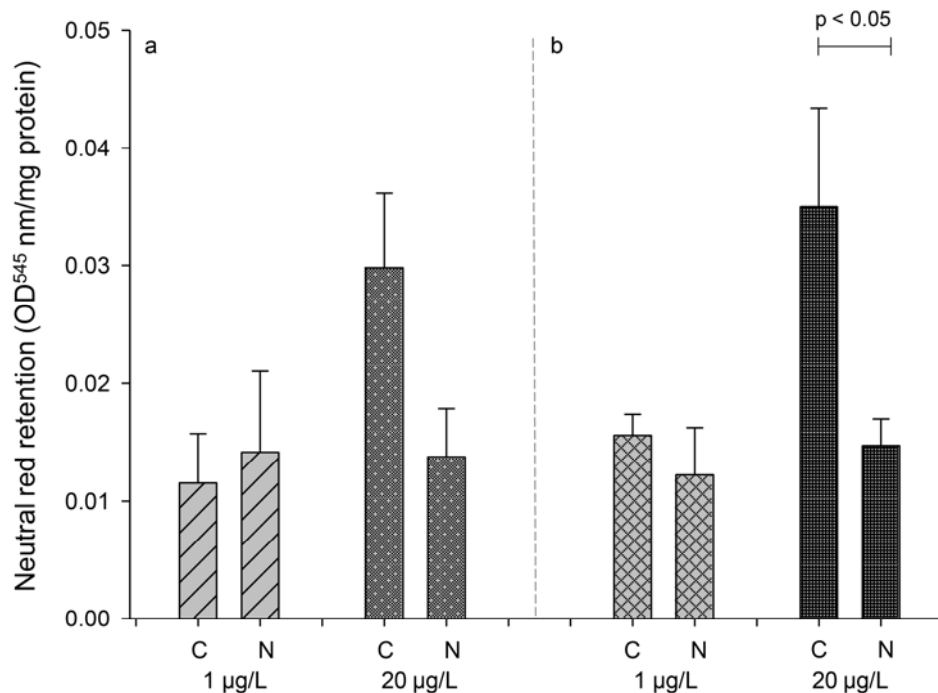


Fig. 1 Membrane stability a) Oysters exposed to naphthalene during 24 h, b) Oysters exposed to naphthalene during 72 h. C = Control, filtered seawater, N = filtered seawater + naphthalene ($\varphi \pm EE$). N = 20/per treatment.

the effect of naphthalene is evaluated on the oxidative stress of the oyster *C. corteziensis*. Therefore, this study represents a first approximation on the effect of this hydrocarbon in such type of oyster with commercial significance in the tropical Pacific.

The obtained results in the bioassays with H₂O₂ (control) suggest major susceptibility of lipids to oxidation for the action of such agent, in comparison with proteins, which can be related with the structure of both types of molecules, since the double bond present in the lipid structures confer to these major susceptibility to oxidation, in contrast with the proteins that are more stable molecules. Besides, there are some mechanisms of protein repair, such as disulfide bonds in cysteines and the formation of sulfoxides in methionines (Hermes-Lima, 2005).

It has been reported that the exposition of *Mytilus galloprovincialis* to benzo [a] pyrene for 7 days, provokes an increase in the lipidic peroxidation in gill (Maria and Bebianno, 2011). While the exposure of the molluscs *Pecten maximus* to sublethal concentrations of phenanthrene (50, 100 and 200 µg/L) causes an increase in the levels of lipid peroxidation in hemolymph (Ansaldo *et al.*, 2005; Hannam *et al.*, 2010), after exposing the molluscs *Nacella concinnato* diesel (0.05 and 0.1 %) during 24, 48 and 168 h, a significant increase was detected in the oxidized lipid concentration in both concentrations after 168 h of exposition.

Contrary to the previously stated, the obtained results in this study, by exposing the *C. corteziensis* to naphthalene, concur with the reported by Lüchmann *et al.* (2011), who by exposing

Crassostrea brasiliana to diesel (2.5, 5, 10 and 20 %) during 96 h, found that there were no significant changes in the oxidation of lipids in the gill and digestive gland. Pichaud *et al.* (2008) reported similar results by exposing bivalve *Mya arenaria* to a mix of PAHs (methylphenanthrene, naphthalene, 2-methylnaphthalene, phenanthrene, anthracene, pyrene, benzo[a]pyrene, and fluoranthene) to a concentration of 47.6 ng/L applied through the consume of phytoplankton during 9 days.

Besides the pro-oxidative potential effect that PAHs can have over molluscs, there are environmental factors that can modulate oxidative damage. In experiments made with *C. gigas* exposed to diesel (0.01, 0.1 and 1.0 mL/L) in different salinity conditions (35, 25, 15 and 9 ups), significant differences were observed in the lipid peroxidation over the different conditions, except for the salinity of 25 ups (Zanette *et al.*, 2011). In this study, the established salinity for the carrying out of the bioassays was of 28 ups (optimal salinity level for *C. corteziensis*), factor that might have had influenced in the oxidative potential of naphthalene, since external factors such as salinity, temperature and low food availability can influence the physiological state of the mollusc, which can provoke additional stress to the one provoked *per se* by the hydrocarbon.

Regarding the protein oxidation levels, in this study there were no significant differences observed in such parameter; contrary to what was reported by Ansaldo *et al.* (2005) when exposing *N. concinnato* diesel 0.05 % during 168 h, who detected a significant increase regarding control.

Membrane stability of hemocytes of *C. corteziensis* was significantly affected after the exposition of naphthalene (20 µg/L) during 72 h. These results agree with the ones obtained by Hannam *et al.* (2010) by exposing scallops *Pecten maximus* to sublethal concentrations of phenanthrene (50, 100 and 200 µg/L), which reported a reduction in the stability of the cell membrane. On the other hand, Anton (2011) also reported destabilization in lysosomal membranes of hemocytes of *Pincta daimbricata*, after the exposition (during 7 days) in soluble fractions of lubricants used in motor vehicles to a 20 % concentration.

One of the possible mechanisms by means of which naphthalene can alter the structure of the lipid bilayer and membrane proteins, is through the induction of ROS: molecules that can alter the biological functions of the membrane and induce cell damage (de la Haba *et al.*, 2013).

The obtained results in this study indicate that under the evaluated conditions, naphthalene does not induce lipid and protein oxidation. However, naphthalene induces destabilization of cell membrane of *C. corteziensis* hemocytes, parameter that has major functional and physiological relevance. On the other hand, evaluated parameters in this study, could have been influenced by a number of factors, among others, the tested concentrations of naphthalene, the established times of bioassays and/or the intrinsic resistance of oyster species.

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