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Oil, Phthalates and Biotoxin Analyses of Mussels (*Mytilus galloprovincialis*) Collected from Dardanelles

Çanakkale Boğazı Midyelerinde (*Mytilus galloprovincialis*) Petrol, Ftalat ve Biotoksin Analizi

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Abstract

Oil, phtalates and biotoxin pollution on mussels were investigated at the entrance and exit of Dardanelles between June 2001 and May 2002. The max. oil pollution was found in May 2002 in the Çanakkale and Gelibolu samples as 28.26 µg/g and 17.26 µg/g (wet weight), respectively. The detected of oil compounds were aliphatic group as C10-C22 and aromatic group as phenol, naphthalene, anthracene, phenanthrene, indol, thiophene, dibenzothiophene derivatives. Phthalates derivatives, dibutyl, diisobutyl phthalate, DEHP (bisethyl hexyl phthalate) were identified by GC/MS analyses. Biotoxine analysis was made by bioassay; PSP and DSP mouse tests were found positive. This is first record in Turkey.

Keyword: Oil, phthalates and biotoxin, mussels, Dardanelles.

Introduction

Dardanelles strait connects the Marmara Sea to the Aegean Sea. It is 60 km long, 1.2 km wide at the narrowest and 6.5 km at the widest points and 60-100 m deep. Water exchange between the Marmara Sea (the Black Sea water, upper layer) and the Aegean Sea (the Mediterranean water, lower layer) produces characteristic layeud water structure within the strait (Memorandum, 1941). The upper layer flows into the Marmara Sea (Southward) at the rate of 0.8 m/s (1.6 knots) and lower layer of 0.2 m/s (0.4 knot) in reverse direction at the bottom.

The oil pollution in the Dardanelles orginates from diverse sources such as tanker and ship traffic and sewage of the cities. Increasing vessels traffic and especially tankers have caused oil pollution in the area. It has a transit traffic of 42.000 ships of which are 5.500 tankers per annum on the average.

Oil pollution in entrance and exite of sea water of Dardanelles was investigated by Güven et al., (2002).

The mussel watch was proposed for the survey of pollutants such as oil, minerals etc. Mussels are filter feeder organisms and rapidly take up pollutants from sea water. They do not metabolize of oil components and discharge %90 of it to fresh sea water within 3 days.

Petroleum contamination on mussel was investigated by various authors. Oil pollution in mussels was investigated after the Nassia accident occured in the Bosphorus in March 1994 (Güven et al.,1995; 1998). Bioaccumulation of PAHs was studied in Slovenian coastline of the Northern Adriatic Sea (Notar and Lekovsek, 2000) and found 643-685 ng/g wet weight, in Baltic Sea sample PAH ranges from 90 to 3900 n/g (Baumard et al., 1999).

The mouse bioassay in PSP was first used by Sommer and Meyer (1937). This method was also used by regulation of Ministry of Agriculture of Turkey. They were used 1ml acidic extracts of mussels injected i.p. into three mice, followed by death in < 15 min by respiratory arrent. Mouse unit is defined as the amount of PSP toxin required to kill a 20 g mouse within 15 min. This assay is only quantitative when the mouse death occurs between 5 and 7

min. Many countries accept 80 μg STX eq μg STX eq per 100 g of soft tissue. The pH values in extraction influence on this assay. AOAC method establishes pH ranging between 2 and 4. Epimerisation occurs with this hot acid treatment resulting of β-to α- epimers, but it effect on the assay in low (minor). The amount of salts in extract decreases PSP toxicity on the bioassay.

Mouse bioassay in DSP. The acceptable criteria varies from two or three mice deaths in less than five hours two or three mice deaths in less than 24 hrs. Free fatty acids may be toxic to mice by i.p. injection yielding fats positive reaction for DSP toxicity (Takagi et al., 1984). Hexane washing was used for removing free fatty acids.

Mussels can directly absorb lower weight PAHs through interstitial filtered water. Phenantrene is estimated to be absorbed at a ratio of 88% through the gills, and this value decreases to 74% for Pyrene. PAH levels found are 0.036-0.362 μg/g in native mussels and 142-207 μg/g (dry weight) in caged mussel sampled in the Spring and Autumn (Piccardo et al., 2001).

Phthalates are derivatives of benzene dicarboxylic acid. They were used as alkyd resins, polymeric polyesters, drying/ non-drying oils, plasticizers for dyes and fibers etc. They were identified in sea water of the Bosphorus and Dardanelles (Güven et al., 1997) and also in marine algae (Güven et al., 1990).

Biotoxins examined are PSP (Paralythic shell fish poison) and DSP (Diarhaetic shell fish poison). The detection of biotoxins were made by immunoassay, mouse bioassay and HPLC methods. The most sensitive method is immunoassay; pg quantities of by toxins are detectable.

Caustive organisms of PSP are Alexandrium catenella, A. minatau, A. tamarense, Gymnodinium catenatum, Pyrodinium bahamense. It causes the symptoms middle case: within 30 min tingling sensation, numbness around lips, gradually spreading to face and neck, prickly sensation in finger tips and toes, headache, dizzines, nausea, vomiting, diarrhoea and extreme case: muscular paralysis, pronounced respiratory difficulty, choking sensation, death through respiratory paralysis may occur within 2-24 hrs (Hallegraeff, 1995). Shell fish containing more than 1.8 μg dinophysis toxin-1 per gram

of hepatopancreas are considered unfit for human consumption (Lee et al., 1987).

Caustive organism of DSP are: Dinophysis acuminata, D. acuta, D. fortii (Igarashi and Fujita, 1978), D. norvegica, Prorocentrum lima. DSP toxins are okadaic acid, dinophysis toxin, pectenutoxin and sulfated yessotoxin. Its symptoms are; mild case: after 30 min to a few hrs diarrhoea, nausea, vomiting, abdominal pain; and extreme case: tumor formation in the digestive system promoted due to chronic exposure.

In this study, the oil, phthalate and biotoxin pollutions were investigated in the mussels collected from the Dardanelles during June 2001- May 2002.

Material and Methods

Mussel (*Mytilus galloprovincialis*) samples were taken from the Dardanelles: in entrance Gelibolu, Lapseki and in exit Çanakkale, Kilitbahir during the period of June 2001 and May 2002 (Fig.1).

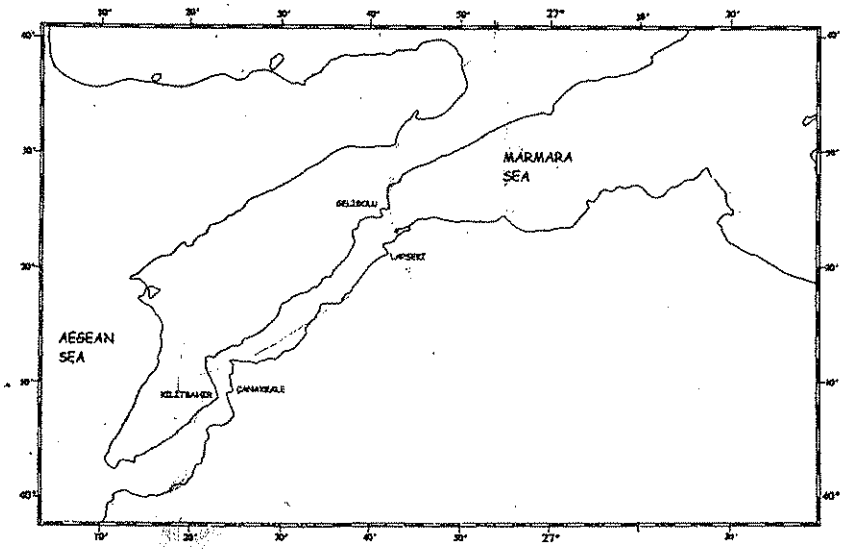

Fig. 1. Sampling stations

1-Oil determination

50 g Mussels were extracted with dichloromethane (DCM) in Soxhlet apparatus for 8 h. The extract was distilled at 40°C and the residue was saponified with 2% KOH in ethanol under reflux for 2h, then 50 ml distilled water added and re-extracted with pentane. The organic phase was distilled, the residue taken with hexane and the volume adjusted to 10 ml. It was measured by UVF (Fluorospectrophotometer, Shimadzu, RF-1501) as described in the previous work by Güven et al. (2002).

The calibration curve plotted by using crude oil of Arabia, Egypt, Libya, Syria and Russia which was transported in this strait. The crude oil samples were obtained from the Izmit Rafinery in 2001. The oil concentrations used for plotting of calibration curve were 0.2-2.4 μg/ml in hexane. The correlation equation was calculated from the standard curves of each oil (Güven et al., Unpublished data) This equation was used for oil measurement in mussels samples.

GC/MS analyses of were run on a HP 6890 capillary GC connected to a Hewlett Packard Mass Selective Detector (MSD) controlled by a HP ChemStation. Operating conditions were: 29.5m x 0.20 mm fused HP-5 MS, PMS, oven temperature programme: 110°C at 6 min, from 40-280°C at 10 °C/min, 280 °C at 10 min, splitless injector temperature 250°C; carrier gas helium, 29.4 psi, press.

The oil pollution in mussels was given as μg/g wet weight.

2-Phthalate derivatives

They were identifed by GC/MS on mussel extracted with DCM as mentioned above.

3-Bioassay

Bioassay was made on mussels collected from the stations of Dardanelles in 05.02.2002

3.1-Bioassay for PSP analysis

Outside of the mussels was cleaned with distilled water and opened by cutting addutor muscles. Inside of the mussels was rinsed with

distilled water to remove remaining sand and other foreign material. Meat is removed from shell carrefully without damaging the body of mollusc. It was collected circa 100-150 g meats in glazed dish. 100g material was taken and 100ml 0.1 N. HCl added and mixed, pH was adjusted to 2.5. The mixture was heated and boiled gently for 5 min and let to cool at the room temperature. The pH was arranged to 2-4 by 0.1 N NaOH. The mixture is transferred to graduate and diluted to 200 ml. It was taken in a beaker and stirred to homogeneity and let to settle until portion of supernate was decanted free of solid particles in tubes and centrifuged for 5 min at 3000 rpm then the clear solution was applied on mouse bioassay.

1ml clear supernatante phase is injected intraperitoneally 3 mice weighed 18-21 g and their death time were recorded.

3.2-Bioassay for DSP analysis

25 g hepatopancreas of mussel were homogenized in a mixer and its 20 g was taken and added 100 ml aceton, then stirred in a magnetic stires for 5 min. Supernatante phase was taken. This procedure was done twice. The extracts were collected and distilled at 50°C in rotary evaporator. 15 ml distilled water was added to the residue and extracted twice with 100 ml ether. Organic phase was distilled and the residue was suspended in 60ml 1% tween solution and then 1ml of the mixture was injected intraperitoneally to mouse.

Mouse test

For PSP: If a mice is not dead in 1 h or a mice dies while the others survive, the test is negative. These mussels are edible for humanbeing but when two or three mice die, the test is positive, therefore mussels are not suitible to consume.

For DSP: If two or three mice were dead in 24 hour, the test is positive; if no or only one mouse dies, the test is negative.

Results and Discussion

1-The oil pollution level in mussels is shown in Table 1.

Table 1. Oil pollution in mussels.

	µg/g (wet weight)								
S/D	*06/01*	*07/01*	*09/01*	*10/01*	*11/01*	*12/01*	*02/02*	*02/02*	*05/02*
G.	*17.11*	*6.19*	*5.16*	*5.57*	*10.51*	*6.84*	*17.02*	*17.02*	*17.26*
L.	-	-	-	*16.59*	*5.86*	*8.16*	*0.91*	*0.91*	*10.06*
Ç.	*7.53*	*2.73*	*1.31*	*12.31*	*3.56*	*21.82*	*1.24*	*1.24*	*28.36*
K.	-	-	-	*7.45*	*8.01*	*4.41*	*3.64*	*3.64*	-

S/D: Station/ Date; G.: Gelibolu; L.: Lapseki; Ç.: Çanakklae; K.: Kilitbahir.

The maximum oil pollution in mussels was found at Çanakkale station (exit of the strait) as 28.36 µg/g in May 2002 and in its entrance at Gelibolu in June 2001 and May. 2002 as 17.11µg/g and 17.26 µg/g, respectively. Their comparison shows that the Gelibolu samples are more polluted than the Lapseki samples.

Çanakkale samples are found to be more polluted than all other stations examined.

Petroleum components detected by GC/ MS in mussels are shown in Table 2 and their chromatograms in Figs. 2-5. They are divided into groups of aliphatic and aromatic. The former group contains C10-C22 homologous in mussel samples examined. Phytane, which in a oil hydrocarbon compound (C20) was detected in a sample. Prystane is also detected in the same sample. A cyclic aliphatic compound cyclohexadecane was also identified. The characteristic aromatic of oil components detected in the mussels are: phenanthrene, anthracene, indol, phenol and especially dibenzothiophene derivatives were detected.

Table 2. Oil components detected in mussels. Ç: Çanakkale, K: Kilitbahir, L: Lapseki, G: Gelibolu

1-Aliphatic groups

Compounds	***Station***
Cyclodecane	*Ç1, Ç2, G1, G2*
Cyclododecene	*G1*
Cyclohexadecane	*G1*
Cyclotetradecane	*Ç1, L1, L2*
Docacene	*L2*
Eicosane	*G1*
Heptacosane	*K1,L1*
Heptadecanol	*L2*
Heptadecene	*Ç3*
Hexadecane	*Ç1,Ç3, K1*
Hexadecene	*Ç1, Ç3, G1, G2, G3, L2*
Hexadecanol	Ç2, L2
Nonacosane	*K1*
Nonadecene	*L2*
Octadecane	*K1, Ç2, G3*
Octadecene	*Ç1, G3, L2, K1*
Pentadecane	*Ç1*
Pentadecene	*G3*
Pentadecanon 6, 10,14 trimethyl	*Ç1, L1*
Phytol	*G1*
Squalene	*L1, Ç2, G3*
Tetradecane	*K1*
Tetradecene	*Ç1, Ç3, K1, G3*
Tridecanol	*Ç1*

2-Aromatic groups

Compounds	***Station***
1 H Indole 3 methyl	*L2*
1 H Indole 3 methyl ethyl	*G3*
Anthrancene 9, 10 dimethyl	*Ç2*
Dibenzonthiophene 2, 8 methyl	*G3*
Dithiophene 2,5 methyl	*L2*
Naphthalen 1, 4, 5 trimethyl	*G3*
Naphthalene 2, 3, 6 trimethyl	*Ç2*
Phenanthrene	*G2,K1*
Phenanthrene 2, 5 dimethyl	*L2,Ç2*
Phenol 2.4 bis 1.1 dimethyl ethyl	*Ç2,L2, G2,G3*

Sampling dates:D1: 01.2002, D2: 05.2002 ,K1:02.2002,G1:02.2002 ,G2:03. 2002 ,G3:05.2002, L1: 02. 2002, L2:05.2002, Ç3: 09.2001

2-Phthalate derivatives identified in mussel are shown in Table3.

Table 3 .Phthalates detected (DEHP) in mussels.

Compounds	Stations
Bisethyl hexylphthalate	*G1,G2*
Dibutilphthalate	*G1, G3*
Disobutilphthalate	*L1,K1,G1*

(G: Gelibolu, L: Lapseki, K: Kilitbahir.)

Sampling date: G1: 02.2002, G2: 03.2002, G3: 05.2002, L1: 02.2002, K1:02.2002.

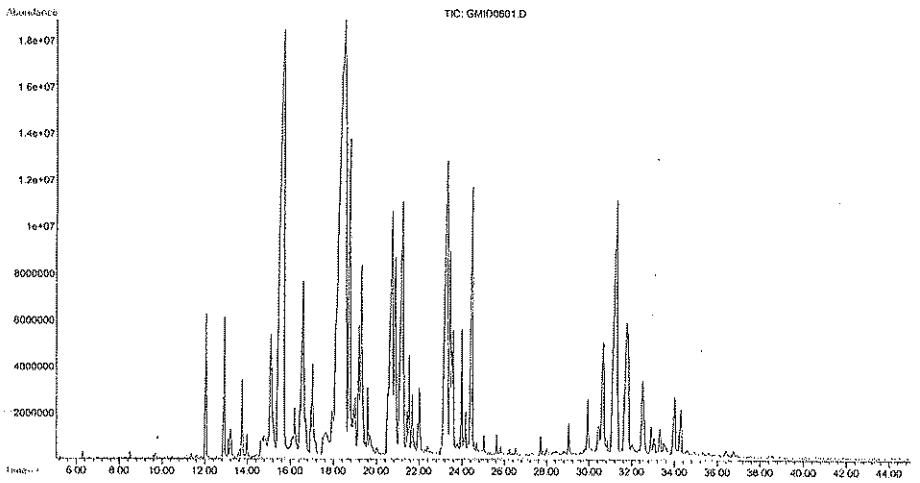

Fig.2.The GC/MS Chromatogram of mussels collected from Gelibolu in 26.06.2001

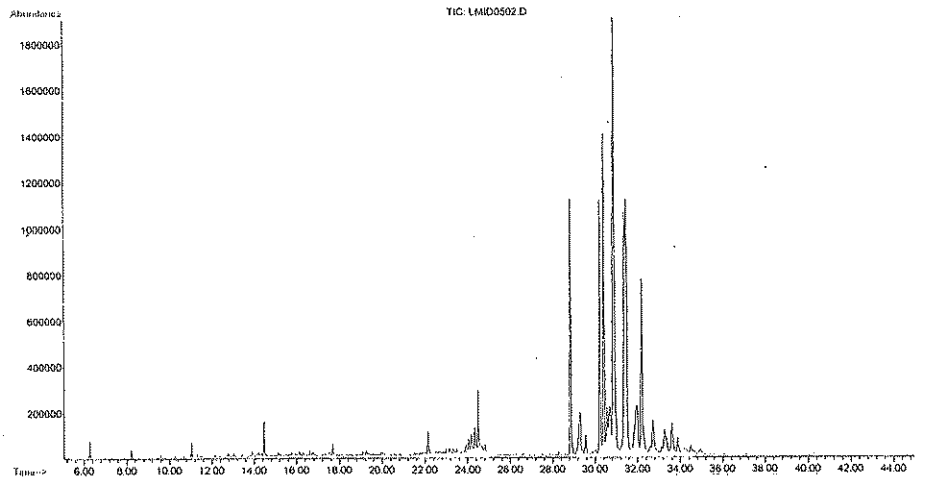

Fig. 3. The GC/MS Chromatogram of mussels collected from Lapseki in 05.2002

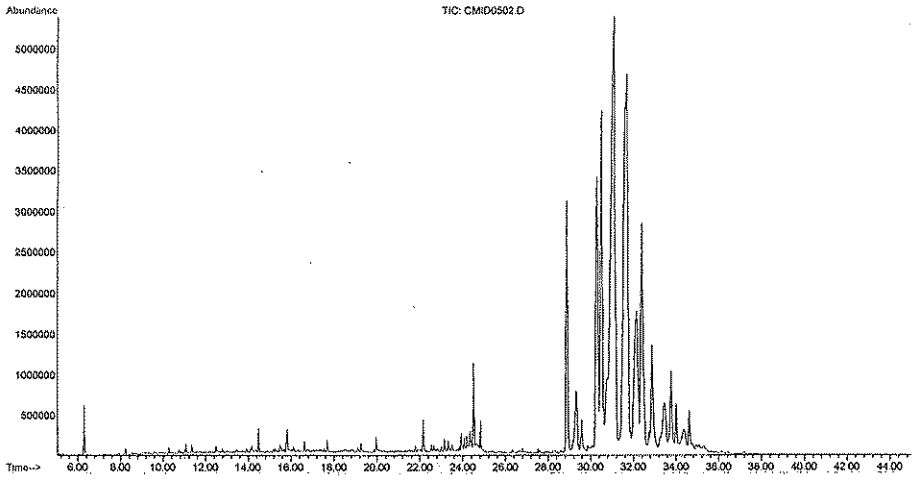

Fig.4. The GC/MS Chromatogram of mussels collected from Çanakkale in 05.2002

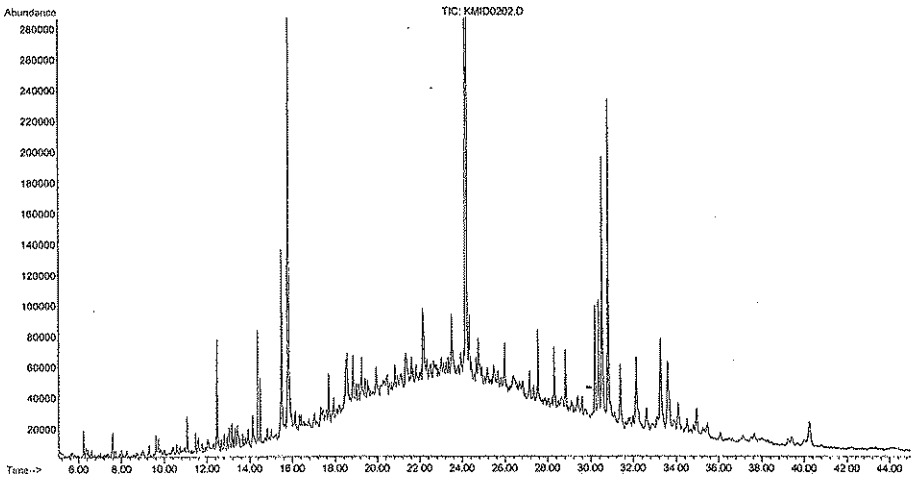

Fig. 5. The GC/MS Chromatogram of mussels collected from Kilitbahir in 02.2002

3-Results of biotoxin assay are: number of death mice; three in Lapseki, two in Gelibolu, two in Kilitbahir, three in Çanakkale for PSP and two for all of the samples for DSP. According to these results the tests are positive. Biological method is not sensitive but it is an official method for PSP and DSP (Annon, 1990;1996). Finally, mouse test involves significant errors, whereas the HPLC analyse has shown an error less than 5%. The mouse bioassay can be complemented by the HPLC method for the determination of sea food safety. Thus biotoxin assay of Dardanelles must be controlled by the HPLC method.

Özet

Bu çalışmada Haziran 2001 ile Mayıs 2002 yılı içinde Çanakkale Boğazı giriş (Gelibolu, Lapseki) ve çıkışından (Çanakkale, Kilitbahir) alınan midye örnekleri üzerinde petrol, ftalat ve biotoksin tayini üzerinde çalışıldı. En yüksek petrol miktarı Çanakkale örneğinde 28.26µg/g Gelibolu örneğinde 17.26µg/g (yaş ağırlık) bulunmuştur. Midyeler içinde alifatik petrol bileşikleri (C10-C20), aromatik gruptan fenol, naftalen, antrasen, fenatren,

indol, dibenzodiofen türevleri tespit edilmiştir. Ftalat derivelerinden dibütil, diisobütil, diethilhekzil, ftalat saptanmıştır. Petrol ve ftalat analizleri GC /MS de yapılmıştır. Biotoksin analizleri PSP ve DSP testleri positif sonuç vermiştir. Bu biotoksin analizine ait Türkiye'de ilk yayındır. Bunun HPLC metodu ile kontrol edilerek kesin sonuca varılabileceği belirtilmiştir.

References

Annon. (1990). Paralytic shellfish poison, Biological method. AOAC Official Method of Analysis.

Annon. (1996). Tarım ve Köy İşleri Bakanlığı, Su Ürünleri Kalite Kontrol Hizmetleri El Kitabı, Ankara.

Baumard, P., Budzinsk, H., Garrigues, P., Dizer, A. and Hansen, P.D. (1999). Polyclic aromatic hydrocarbon in recent sediment and mussels (Mytilus edulis) from the Western Baltic Sea: occurence, bioavailability and seasonal variations. *Marine Environmental Research* 47: 17-47.

Güven, K. C., Reisch, J., Kızıl, Z., Güvener, B. and Cevher, E. (1990). Dimethyl terephthalate pollution in red algae. *Phytochemistry*. 29: 3115.

Güven, K. C., Ünlü, S., Okuş, E. and Dogan, E. (1995). Oil contamination of Mytilus galloprovicialis after the Nassia accident in the Black sea, Bosphorus and the Sea of Marmara. T*urkish J. Mar.Sci.* 1:67-79.

Güven, K. C., Ünlü, S., Okuş, E., Doğan, E. and Gezgin, T. (1997). Identification of phthalate esters pollution in the Bosphorus and Dardanelles. *Turkish J. Marine Sciences*. 3: 137-148.

Güven, K. C., Okuş, E., Ünlü, S., Dogan, E. and Yuksek, A.(1998). Oil pollution of marine organisms after Nassia Tanker accident in the Black Sea, Bosphorus and the sea of Marmara, *Turkish, J. Mar. Sci.* 4:3-10.

Güven, K. C., Çetintürk, K., Alpaslan, M. and Tekinay, A. A. (2002). Oil and detergent pollution of sea water in Dardanelles in 2001-2002. *Turkish, J. Mar. Sci.* 8:2-121.

Hallegraeff, G.M., (1995). Harmful algae blooms. In: Manual of Harmful Marine Microalgae. (Hallegraeff, G. M., Anderson, D.M., Cembella, A.D.,eds.) . 10 C Manuals and Guides No. 33, UNESCO.

Igarashi, T. and Fujita, N. (1978). Identification of Dinophysis fortii as the causative organism of diarrhetic shell fish poisoning. *Bull. Jap. Soc. Sc. Fish.* 46: 1405- 1411.

Lee, J., Yanagi, T., Kenma, R. and Yasumoto, T. (1987). Fluorometric determination of diarrhetic shell fish toxins by high performence liquid chromatography. *Agr. Biol. Chem.* 51: 877-881.

Memorandum. (1941). Summary of hydrographical results concerning the Bosphorus and Dardanelles, Memoranda to accompany density an current Atlas to Bosphorus and Dardanelles, Hydrographic Dept. July.

Notar, M. and Leskovsek, H. (2000). Polycylic aromatic Hydrocarbons in mussels from the Northern Adriatic Sea. Fresenius *Invir. Bull.* 9: 427-434.

Ogata, M. and Fujisawa, K. (1985). Organic sulfur compounds and polycyclic hydrocarbons transferred to oyslen and mussel from petroleum suspension. *Water Res.* 19: 107-118

Piccardo, M. T., Coradeghini, R. and Valerio, F. (2001). Polycyclic aromatic hydrocarbon pollution in native and caged mussels. *Mar. Pell. Bul.* 42: 951- 956.

Sommer, H. and Meyer, K. F. (1977). Paralytic shell fish poisoning. *Arch. Pathol.* 24: 560-518.

Takagi, T., Hayashi, K. and Itabashi, Y. (1984). Toxic effect of free unsaturated fatty acids in the mouse assay of diarrhetic sell fish toxin by intraperitoneal injection. Nippon Suisan Gakkaishi 50: 1413- 1418.

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