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Residues of Organohalogen Compounds in Various Dolphin Tissues

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Organohalogens, in particular chlorinated hydrocarbones (CHCs) and polychlorinated biphenyls (PCBs) are very toxic and of the most persistent environmental pollutants. Their continuous input in the Mediterranean basin from the surrounding countries and their unavoidable bioaccumulation in marine organisms are consequences of human activity and bad environmental management. Also, in recent years, attention has been focused on the toxicity of PCBs, particularly on the congeners that elicit toxic responces similar to those of TCDD. Although PCBs are not a proven human carcinogen there is enough evidence of harm to animals (Cecilia and Koidu 1993). The lipophilic organochlorine compounds such as PCBs, DDTs and HCH isomers are known to accumulate in adipose tissues of terrestrial and aquatic animals. Marine animals such as seals and cetaceans are known to accumulate higher levels of organochlorine compounds in their subcutaneous fat or lipid fraction of tissues due to their occupation of high trophic levels in marine ecosystems (Kawai et al. 1988). The methods which are usually used for CHCs, PCBs and other chlorinated substances determination, are packed and capillary column GC-ECD, GC-MS but also HPLC with a Photodiode Array Detector (Krahn et al. 1994). Following the first dolphin deaths, approximately 130 confirmed by the Greek Authorities (News 1992), in several seas of our country, primary tissue samples of dolphins found in approachable areas, were sent to our laboratory for toxic residue determination. With the method we use routinely in our laboratory, several organohalogen compounds have been identified and quantified and their concentrations ranged from traces to 58 ppm on a wet weight basis and 1226 ppm on a lipid weight basis. These compounds are the following HCB, β-HCH, o-p DDE, p-p'DDE, o-p TDE, o-p DDT, p-p' TDE, p-p'DDT, Aroclor 1260. The mean recovery of this procedure is 85% ± 16 and the limit of detection is 10-20 ppb.

MATERIALS AND METHODS

Primary tissue samples coming from 11 dolphins were taken at the Regional Field Laboratories (RFLs) of the Veterinary Service of the Ministry of Agriculture, located

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in several parts of Greece (e.g.Chania-Creta island, Peloponnese - Gythion, Zakynthos and Kythira islands) and sent to the Residue Analysis Laboratory of Athens under frozen conditions. These dolphins of the Aegean Sea had been washed ashore and found dead All laboratory samples taken from the primary samples of liver, kidney, spleen, heart and subcutaneous fat tissue, just after their reciept, were kept in glass beakers sealed with aluminium foil prerinsed several times with n-hexane and stored at -35°C until the analysis.

Table 1. Concentrations of the working standard solutions (pg / μL).

HCB	20	o-p DDE	75
α-HCH	10	p-p'DDE	40
γ-HCH	10	Dieldrin	50
β-HCH	40	o-p TDE	80
Heptachlor	10	Endrin	80
Aldrin	20	o-p DDT	90
Hept.epoxide	30	p-p'TDE	80
γ-chlordane	75	p-p'DDT	100
Aroclor 1260		1000	

The solvents used were of pesticide residue grade. Stock solutions were the following: Chlorinated Hydrocarbones-CHCs Nanogen Analytical Standard 10.00 ± 0.05ng/μL and Aroclor 1260, CAS NO 11096-82-5, Polychlorinated biphenyl-60% chlorine in toluene. Working standard solutions were prepared in n-hexane at concentrations ranging from 10-100 pg/μL for CHCs and 1000 pg/μL for Aroclor 1260. The multi-residue method used for the determination of CHCs and PCBs was based on the methods of Venant et al. (1982) and Armour and Burke (1970) with our modifications. In table 1 we can see the concentrations of the working standard solutions of CHCs and of Aroclor 1260.

Weigh test sample of 200 g and grate finely. Place it in a mortar and grind well with anhydrous sodium sulfate and leave it overnight. (The amount of sodium sulfate required depends on the water content of the sample). Transfer the powder quantitatively into one or more extraction thimbles and extract with light petroleum in a Soxhlet apparatus. Concentrate the extract using a rotary evaporator with a vaccum distilation controler (vaccum 330 mbars & water bath temperature 40°C).

Weigh 0.5 g (±0.01g) of fat in a 10 ml centrifuge tube. Weigh fat in duplicate. Add 3 ml extraction mixture acetonitrile + dichloromethane 70+30 v/v, mix with vortex

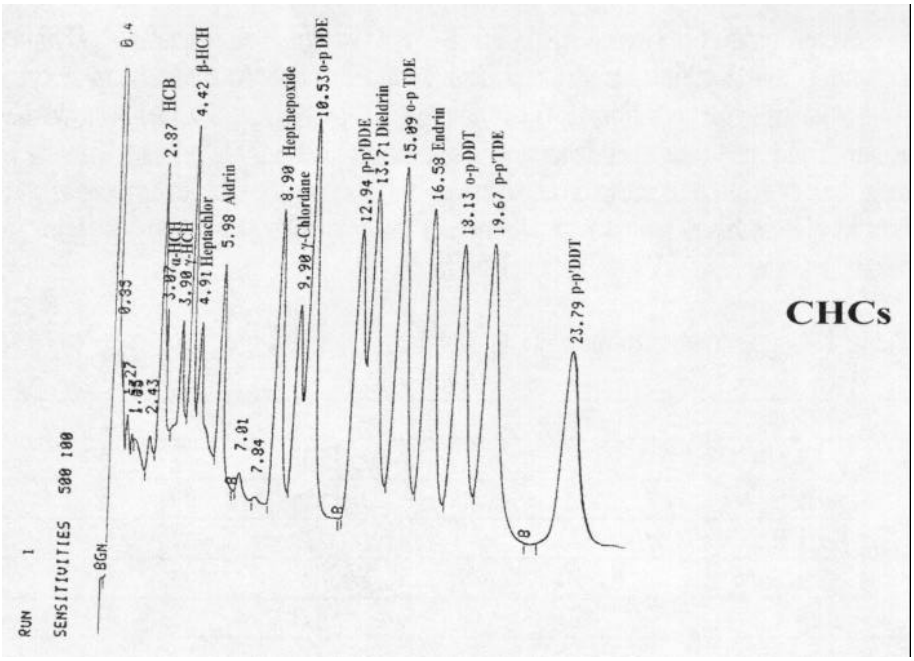

Figure 1. A gas-chromatogram of 16 chlorinated hydrocarbones.

and centrifuge at 3000 rpm for 20 min at -10°C. Transfer the organic phases into a test tube. Warm the remaining fat at the bottom of the centrifuge tube to melt, using a heating cabinet at 60°C, and repeat the extraction using 3 ml of same extraction mixture. Combine the organic phases and evaporate them at 40% using a gentle stream of nitrogen. Reconstitute the residue with 3ml n-hexane and procceed to florisil cleanup step. Prepare a florisil column using a glass chromatographic column and 5 g florisil which is topped with 1 cm anhydrous sodium sulfate. Prewet the column with 40 ml light petroleum + dichloromethane 80+20 v/v. Place a 150 ml round flask under the column to collect the eluate. Transfer the sample on to the column. Rinse the sample container twice with 3 ml n-hexane and pour rinsings on to the cohmm. Elute with 40 ml light petroleum t dichloromethane 80+20 v/v. Rotary-evaporate eluates at 40%, remove solvent residues using a gentle stream of nitrogen and reconstitute with 5 ml of light petroleum.

Weigh 5 g Celite and 20 g activated silicic acid . Combine in 250 ml beaker and shnry with 80 ml light petroleum. Pour shury into a chromatographic column and pack the cohmm using small portions of light petroleum. Place a 250 ml round flask under cohmm to collect the first eluate, and apply the sample (in < 5ml light petroleum). Elute with 250 ml light petroleum at a rate of 5 ml/min. Collect 250 ml exactly. Place a second 250 ml round flask under the column to collect the second

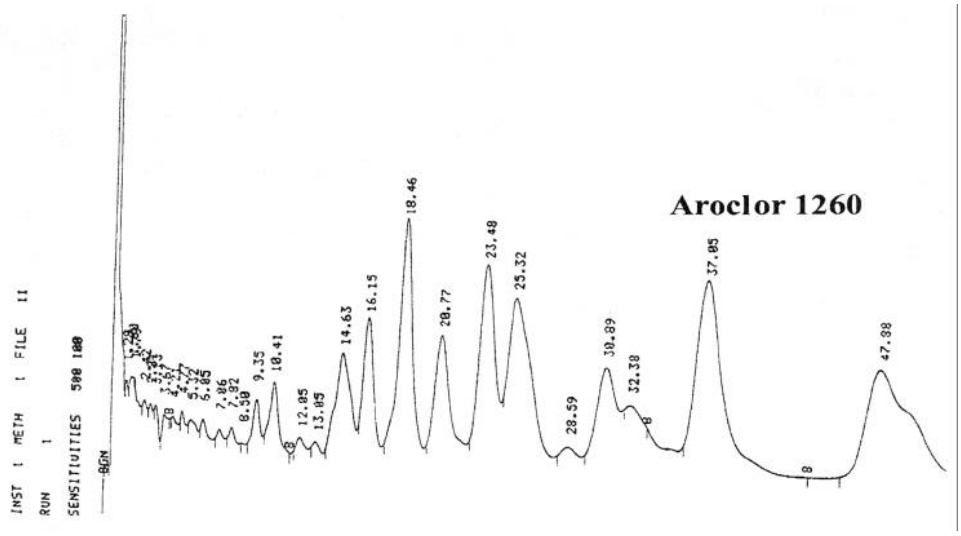

Figure 2. A gas-chromatogram of Aroclor 1260.

ehrate and elute with 200 ml eluting mixture acetonitrile + n-hexane + dichloromethane 1+19+80 v/v/v). Concetrate eluates to dryness and reconstitute to 5 mL with n-hexane. Inject 5 µL into the gas-chromatograph.

A Perkin-Elmer Gas chromatograph model Sigma 2000, equipped with an electron capture detector (^{63}Ni-ECD) was used with two-three packed glass columns:

1. 2.0m x 1/4" OD x 2mm ID containing 1.5% SP-2250 + 1.95% SP-2401 on 100/120 Supelcoport and
2. 3% Dexsil 300 on Supelcoport 100/120
3. 2.0m x 1/4" OD x 2mm ID glass containing 3% SP-2100 on 100/120 Supelcoport

Operating conditions were as follows:

a) temperatures : injector 270°C
column oven 200°C
detector 300°C

b) flow rate : 30 mL/min Ar/CH_4 (90/10) 99.999%

The identification of the CHCs peaks and of the Aroclor 1260 was done by comparison of the relative retention times(RRT) of the unknowns to those of the standards. For confirmation two more columns (n°2 & n°3) were used.

In figure 1 and 2 chromatograms of 16 CHCs and of Aroclor 1260 respectively are shown.

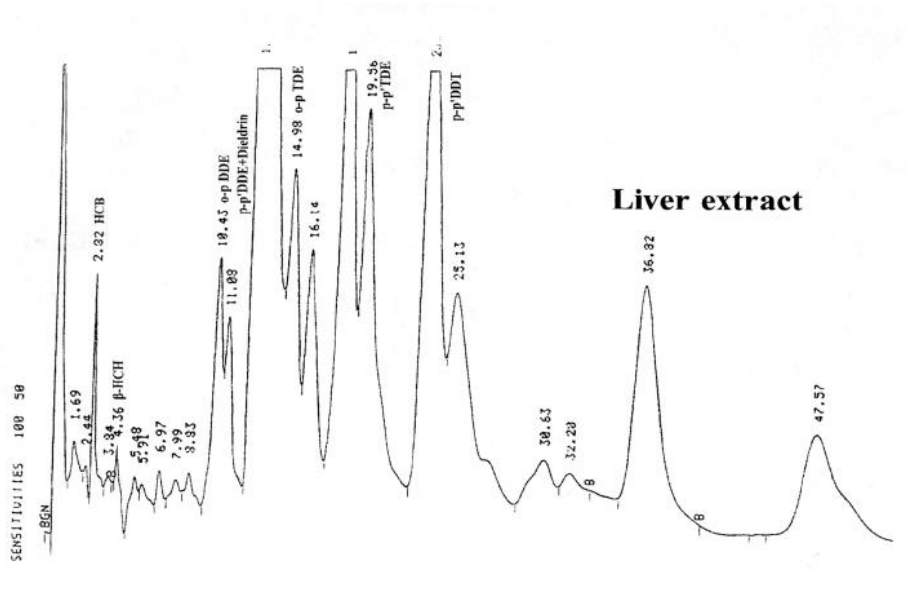

Figure 3. A gas-chromatogram of a liver extract before DDTs - Aroclor 1260 separation.

RESULTS AND DISCUSSION

Our study is original and unique for the Greek literature because such a study has never been done before. The Mediteranean Sea contamination and more precisely that of the Aegean Sea nowadays, is of high concern in Greece. Analytical data indicating similar levels of toxic substaces on marine organisms (fish and shelfish) from three Greek gulfs, have been published but never on dolphins (Kilikidis et al. 1981). Polychlorinated biphenyls are toxic substances, which produce, among other compounds, highly toxic dioxins and furans on thermal degradation. As a result of their chemical stability, temperature resistance, and adhesiveness, PCBs are used in condensers, transformers, adhesive agents and as a water repelling and flameproof impregnating material for fabric, paper and wood (Merck Spectrum 1996).

Studies of the 1987-88 Atlandic Coast mortalities and recent Gulf of Mexico die-offs suggest that immunosuppression due to morbillivirus infection may have been responsible for the tenfold increase in dolphin mortality. Believed to be the same in the Mediterranean in 1990 the toxic effects may be increased by immunosuppression due to high concentrations of PCBs and certain halogenated hydrocarbones.Supporting this, levels of PCBs observed in hundreds of striped dolphins (*Stenella coeruleoalba*) washed ashore along the Mediterranean Sea in Spain, Italy and France revealed PCB levels in the beached animals up to 10 times higher than levels previously reported in dolphins (Luke 1990).

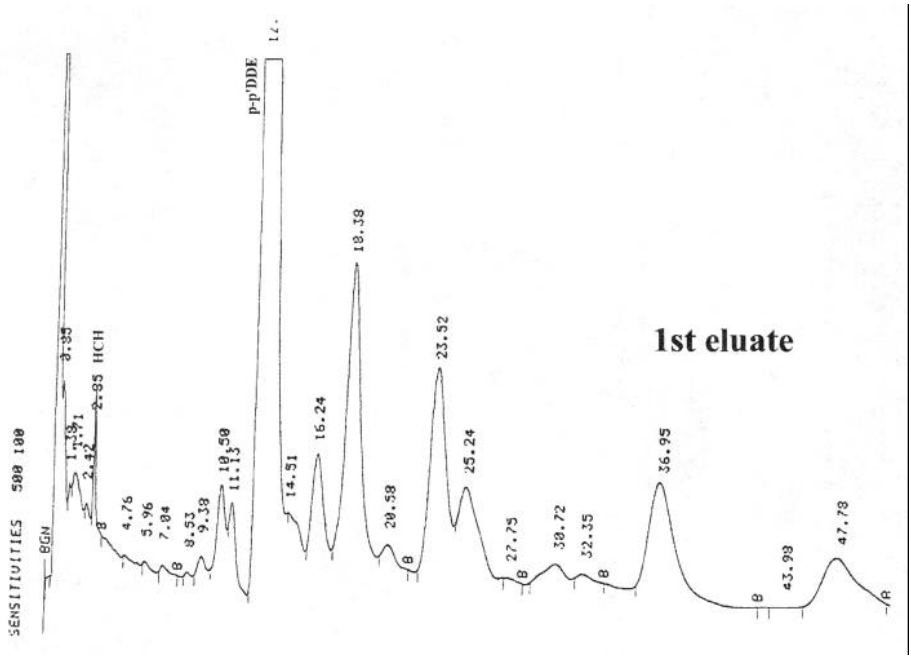

Figure 4. A gas-chromatogram of a liver extract after DDTs - Aroclor 1260 separation (1st eluate).

Considering the information on the occurrence, distribution and behaviour of PCBs in the ecosystems, the marine mammals are probably the most vulnerable and possible target organisms in forthcoming long-term PCB toxicity (Tanabe et al. 1988). A study on the PCB residues in a food chain which includes plankton, fish and marine mammals from western North Pacific clearly illustrates their preferential biomagnification from lower to higher trophic levels in which the highest predator, striped dolphins, revealed the bioconcentration factor (concentration ratio of PCBs in organisms to water) as high as 10^7 (Tanabe et al. 1988). In addition, higher chlorinated Aroclors such as 1260 and 1254 are of special concern, because they resist natural biodegradation and therefore tend to accumulate up into the food chain (Toepfer 1992). Aroclor 1260 for example contains penta 12%, hexa 42%, hepta 38%, octa 7% and nonachlorobiphenyls 1% (Doherty 1993).

Although the separation of Aroclor 1260 and p-p'DDE should have to be succeded by this method, p-p'DDE eluted in the first eluate together with Aroclor 1260. Trials to separate them by changing the chromatographic parameters were uneffective. That is because the margin of separation between them is small and is usually caused by an increase in the elution solvent polarity or the increased concentration of the substances to be eluted (Armour and Burke 1970 ; Griffin 1980). Therefore Aroclor 1260, HCB, Aldrin and p-p'DDE are eluted in the first eluate, while all the other

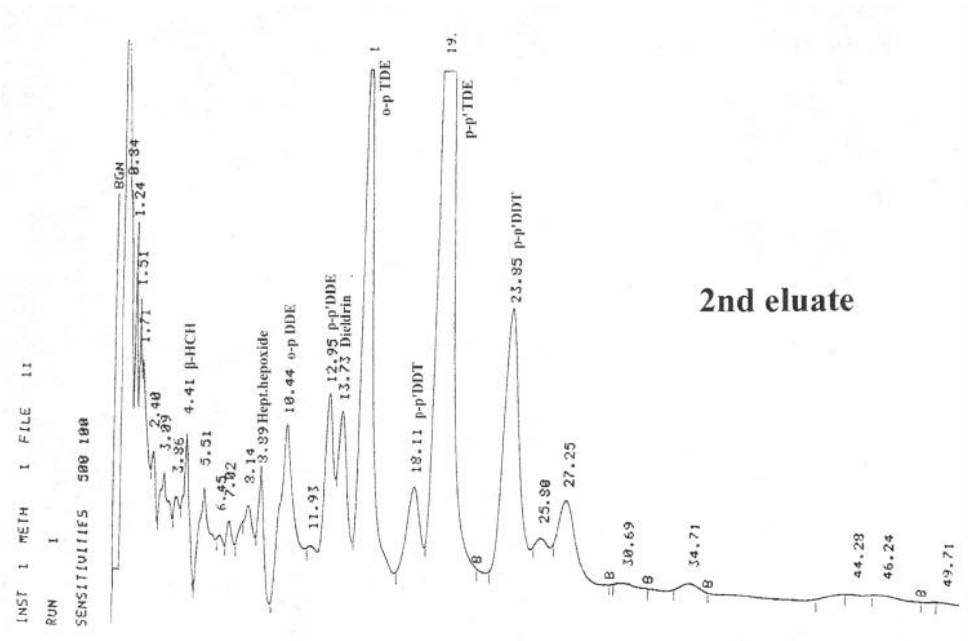

Figure 5. A gas-chromatogram of a liver extract after DDTs - Aroclor 1260 separation (2nd eluate).

organochlorine compounds (lindane, heptachlor, heptachlor epoxide, dieldrin, endrin, DDT and its analogs) are eluted in the second one. In figures 3,4 and 5 we can see chromatograms indicating a liver extract before the separation (figure 3) and after the separation of Aroclor 1260 - DDT and its analogs, 1st eluate (figure 4) and 2nd eluate (figure 5).

Table 2. CHCs in various dolphin tissues in ppm lipid & wet weight basis

	Spleen		Kidney		Heart		Liver		Subcut. fat	
	l/wt	wet/wt	l/wt	wet/wt	l/wt	wet/wt	l/wt	wet/wt	l/wt	wet/wt
1	1.0	.007	2.6	.027	.886	.007	2.2	.034	2.8	.199
2	.527	.004	.376	.004	traces	traces	1.3	.020	1.9	.132
3	1.1	.066	16.5	.168	23.9	.174	3.1	.047	19.9	1.4
4	324	2	500	5.1	495	3.6	1226	18.4	828	58
5	12.7	.079	11.7	.120	21	.150	16.8	.260	15.1	1.1
6	8.2	.051	13.2	.136	15.2	.109	4.1	.063	29.3	2.1
7	15.9	.098	15.1	.155	26.3	.189	32.1	.490	37.1	2.6
8	18.1	.112	21.1	.216	9.2	.066	15.5	.240	56.2	3.9
1.HCB, 2.β-HCH, 3.op DDE, 4.pp'DDE, 5. op TDE, 6. op DDT, 7. pp'TDE, 8. pp'DDT										

Table 3. Aroclor 1260 in ppm lipid & wet weight basis

Spleen		Kidney		Heart		Liver		Subcutan. fat	
l/wt	wet/wt	l/wt	wet/wt	l/wt	wet/wt	l/wt	wet/wt	l/wt	wet/wt
175	1.1	194	2	353	2.5	288	4.4	227	15.8

Table 4. pp'DDE in ppm lipid & wet weight basis

Spleen		Kidney		Heart		Liver		Subcutan. fat	
l/wt	wet/wt	l/wt	wet/wt	l/wt	wet/wt	l/wt	wet/wt	l/wt	wet/wt
324	2	500	5.1	495	3.6	1226	18.4	828	58

Table 5. Total DDTs in ppm lipid & wet weight basis

Spleen		Kidney		Heart		Liver		Subcutan. fat	
l/wt	wet/wt	l/wt	wet/wt	l/wt	wet/wt	l/wt	wet/wt	l/wt	wet/wt
380	2.41	577.6	5.9	591	4.29	1298	19.5	986	69

All samples have been found positive (100% positiveness) for the substances mentioned previously with concentrations ranging from traces to 58 ppm on a wet weight basis and to 1226 ppm on a lipid weight basis (Table 2). These high concentrations are referring to p-p'DDE (Table 4) which is known to result from the breakdown of DDT under aerobic conditions in the environment; it is therefore not unusual that DDE is the predominant DDT analyte found (Salata et al. 1995). On tables 3 and 5 we can see Aroclor 1260 and total DDTs concentrations found respectively.

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