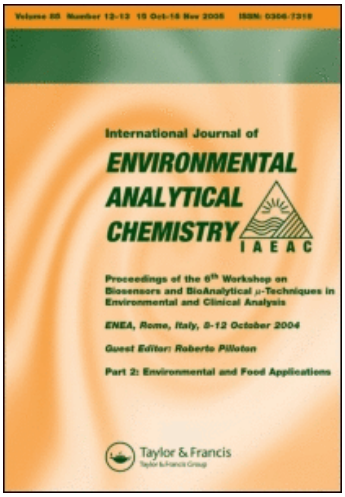

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Analysis and Fate of Dibenzothiophene Derivatives in the Marine Environment

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Dibenzothiophene (DBT) and related methylated derivatives are known to be among the most persistent and probably the most toxic PAH in the marine environment. Their analysis and their fate by photo-oxidation and biodegradation were studied.

The methylated DBT isomers, provided that they are resolved by high resolution GC, were used as organic markers of oil pollution in oysters. The determination of the relative distribution of the four monomethyl DBT allowed to characterize the source of pollution in an oyster-area in the North Brittany (France).

The fate of methylated DBT compounds was studied in a controlled sea-water enclosure where Arabian light oil was spilled. Analysis of the weathered oil showed that: (i) oil was degraded by photo-oxidation at a rate of 0.004% day; (ii) the half-lives of photolysis of methylated DBT was dependent upon the number of methyl groups on the aromatic nucleus: 8 days for DBT, 20 days for methyl-1 DBT and more than 2 years for trimethylated DBT. Compounds solubilized in the water column were identified as methyl-substituted dibenzothiophene sulfoxides and sulfones by HPLC with synchrofluorescence and GC-flame photometric detection.

The metabolic pathway of DBT was established *in vitro*. Rat microsomes transformed this substrate to DBT-5-oxide and subsequently to DBT-5-dioxide. Such an enzymatic S-oxidation was shown to be principally Cytochrome-P-450 dependent. It is suggested that the mixed-function oxidase (MFO) activity of marine species could be evaluated by this S-oxidation test in addition to the usual aryl hydrocarbon hydroxylase.

KEY WORDS: DBT, methylated DBT, GC analysis, organic markers, oil pollution, photo-oxidation, half-life, biological oxidation.

INTRODUCTION

It has been observed that polyaromatic sulfur heterocycles (PASH), especially dibenzothiophene (DBT) and related alkylated derivatives from fossil source, may be the most persistent PAH in the marine environment.[1-3] This observation was emphasized while monitoring the fate of oil spilled from the *Amoco-Cadiz* near the Brittany coasts.[2,4] Friocourt *et al.*[5] and Ogata *et al.*[6] suggested the use of alkylated dibenzothiophenes as organic markers of oil pollution in shellfish.

The DBT derivatives have not been characterized as extensively as the PAH in oils and in the environment. Owing to the multiplicity of possible isomers, which depends on the number of methyl substituents (4 isomers for one methyl group but 16 for two methyl groups), the complete resolution of all isomers was very difficult.[7] However complete resolution and relative quantification of each isomer is needed because the geochemical significance and/or the toxicity is often related to a specific position of the methyl substituent on the aromatic nucleus. This study highlights the use of methylated DBT derivatives as organic markers of oil pollution in the marine environment.

Moreover, if the weathering of petroleum has been extensively investigated,[8] the fate of DBT derivatives during the photo-oxidation process in the marine environment has not received much attention. However, such compounds are common to most, if not all, oils and oil products. Their occurrence in sediments and many marine organisms after the *Amoco-Cadiz* spill in Brittany raised questions about the fate of about 6,000 tons estimated[9] spilled in the Channel sea on March 1978.

The persistence of DBT derivatives in the marine environment[4] suggests that much attention must be given to their biological fate and activity. So the metabolism of DBT model pollutant entering aquatic systems was studied.

Three kinds of results are reviewed in this article:

— chromatographic separation of methyl and dimethyl DBT isomers and their use as organic markers of oil pollution;
— fate of DBT and methylated derivatives in a controlled marine environment under natural irradiation;
— *in vitro* metabolism of DBT by rat microsomes.

The major objective of this study is to provide the necessary base line data for proper evaluation of the fate of DBT and related alkylated derivatives in the marine environment.

EXPERIMENTAL SECTION

GC analysis of DBT derivatives in oil

Four oil samples spilled in the sea were analyzed: Ekofisk blow-out oil; *Amoco-Cadiz*; *Tanio* and *Gino* tanker spills. The oil samples were fractionated and prepared by liquid chromatography on aminosilane phase as previously described.[10] The DBT purified fraction was analyzed by capillary GC. The pure methylated DBT were kindly provided by Lee and Douglas.

GC was carried out on a glass capillary column, 40 m × 0.3 mm, laboratory coated by *in situ* gummification of methylphenylsilicone prepared by Verzele *et al.*[11] The GC apparatus was a Carlo-Erba model 4160 fitted with a flame-photometric detector (FPD) in the sulfur mode with a 394 nm filter. Samples were injected via the cold on-column injector at oven temperature of 66°C. The oven was then balistically heated to higher temperature and finally temperature programmed.

Analysis of photo-oxidized oil

The fate of DBT derivatives contained in the Arabian light oil (1 liter) spilled in a controlled unrenewed sea-water was studied.[12] The acidic compounds solubilized in the sea-water column were analyzed by HPLC with fluorescence detection (Kontron fluorimeter SFM 25). HPLC was carried-out on a Vydac-201-TPC-18, 5 μm 20 cm × 4.6 mm. Gradient elution was performed with 1.47% (v/v) acetic acid in water (solvent A) and acetonitrile (solvent B) in a 25 min linear gradient from 30% to 90% solvent B. Synchronous excitation-emission fluorescence scans (SEES) were obtained on the HPLC peaks by stopping the flow-rate, using a 25 nm offset over the 250–500 nm range.

The acidic extracts were analyzed by GC-FPD as previously described[13] after methylation derivatization.

The DBT fraction of the oil surface was analyzed by GC-FPD as described for the DBT derivatives.

Analysis of bio-oxidized DBT

Dibenzothiophene was used as a model substrate of hepatic microsomal mixed-function oxidases in order to study its metabolic fate in living organisms.

After incubation of pure DBT with rat hepatic microsomal suspension, the incubation mixture was analyzed by HPLC on a Radial-Pak-C-18 (Waters-Millipore) with UV detection. All the details were previously described.[14]

RESULTS

1. Analysis of DBT derivatives in the marine environment

The majority of GC PAH analysis in different environments have been carried-out on apolar type OV-1 or moderately polar type SE-52 or OV-73 stationary phases. Because of the complexities of PASH-rich fractions, recent efforts have focussed on high-resolution GC with sulfur-selective detection. High-efficiency capillary columns coated with selective stationary phases have been found to be essential for resolving the numerous isomers. Lee and his group[15] synthesized a biphenyl siloxane phase capable of resolving the four monomethyl DBT isomers. Simultaneously our laboratory described a methylphenylsilicone phase immobilized *in situ* that allowed to achieve the same separation.[10]

Retention indices of pure methylated DBT are listed in Table I; they are given according to the linear retention index proposed by Lee *et al.*[16] Figure 1 shows a chromatogram of the pure methylated DBT derivatives; four monomethylated, eight dimethylated for 16 possible isomers and five tetramethylated DBT isomers were completely resolved.

Such a selective stationary phase must bring an enhancement in the resolution of the numerous isomers present in various samples such as oils and polluted oysters. For illustration, analyses of four oils spilled in the sea and three oyster samples collected on the coasts of North-Brittany (France) were studied in order to help in tracing sources of oil pollution. The relative distribution of the four methyl-DBT is reported in Table II. For petroleum products, the quantitation showed a remarkably low proportion of the 2-methyl

TABLE I

Relative retention indices of methylated dibenzothiophenes on methylphenylsilicone (=RSL-500) with programmed temperature.

DBT	295.96	1,6-di-methyl-DBT	324.93
4-methyl-DBT	308.49	3,4-di-methyl-DBT	326.80
2-methyl-DBT	311.07	1,7-di-methyl-DBT	327.63
3-methyl-DBT	312.17	2,4,8-tri-methyl-DBT	336.59
1-methyl-DBT	317.99	2,4,6,8-tetra-methyl-DBT	341.81
4,6-di-methyl-DBT	318.12	2,4,7,8-tetra-methyl-DBT	358.18
2,6-di-methyl-DBT	319.13	3,4,6,7-tetra-methyl-DBT	365.25
3,6-di-methyl-DBT	319.65	3,4,6,8-tetra-methyl-DBT	365.74
2,8-di-methyl-DBT	321.62	3,4,7,8-tetra-methyl-DBT	367.05
1,8-di-methyl-DBT	324.54	3,4,8,9-tetra-methyl-DBT	373.07

TABLE II

Relative ratios of the four monomethyl DBT isomers in different oils spilled and in polluted oysters (see text). Determination by GC-FPD.

Sample	Peak height relative ratios	
Isomers	4-methyl/2-methyl	3-methyl/1-methyl
Gino	6.98	1.91
Amoco-Cadiz	8.42	1.33
Arabian light	9.33	1.11
Iranian light	10.6	1.56
Tanio	9.0	0.46
Ekofisk	28.6	0.92
Aber-Benoit oysters	6.52	1.30
Carantec oysters	6.48	1.40
Binic oysters	6.68	1.30

isomer relative to the other isomers. In addition, whereas the relative distribution of 4- and 3-methyl isomers seemed to be quite constant in all the examined samples, this was not the case for the 2-methyl and 1-methyl isomers (Figure 2). It is suggested that the relative ratio of 4-methyl/2-methyl and 3-methyl/1-methyl isomers could be useful in tracing sources of oil pollution in oyster samples.

Our prime idea was that Aber-Benoit and Carantec oysters were still polluted on July 1980 by *Amoco-Cadiz* oil because they were

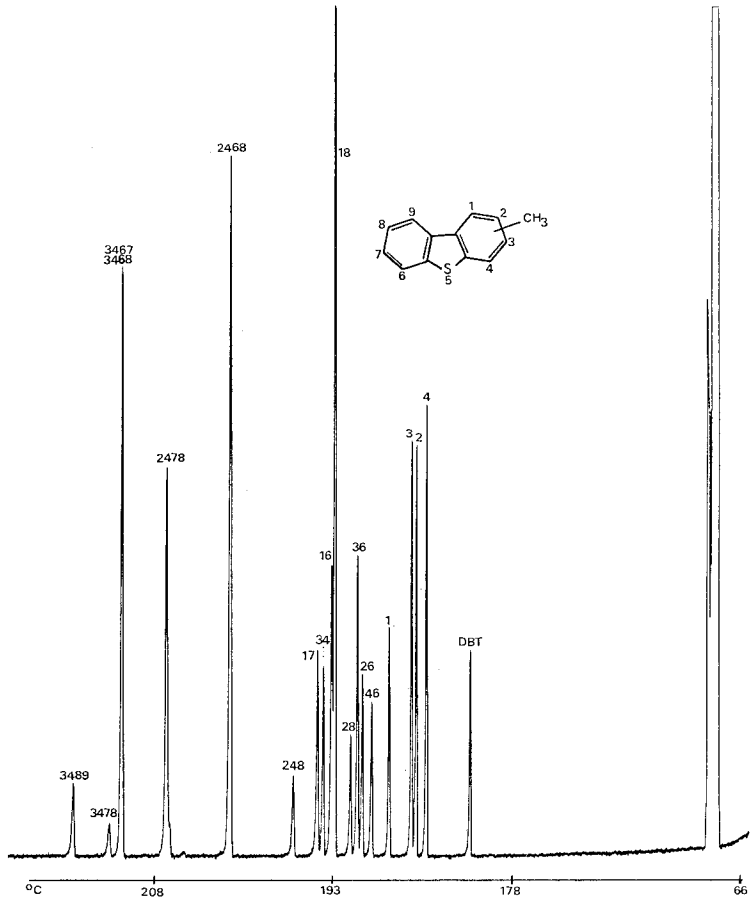

FIGURE 1 GC separation of pure methylated DBT isomers on a methyl phenylsilicone stationary phase (40 m × 0.3 mm). Detector FPD. Peaks are numbered according to the position of the methyl group on the aromatic nucleus.

collected from sites heavily polluted since March 1978. On the other hand, Binic oysters would be considered as controls in July 1980. If Aber-Benoit and Carantec samples exhibited the presence of methylated DBT isomers in proportions similar to the source of pollution, the same result was also observed for the Binic samples (Table II). It was suggested that the Binic site has been polluted by the *Amoco-Cadiz* oil contrary to the first observations[9,17] established.

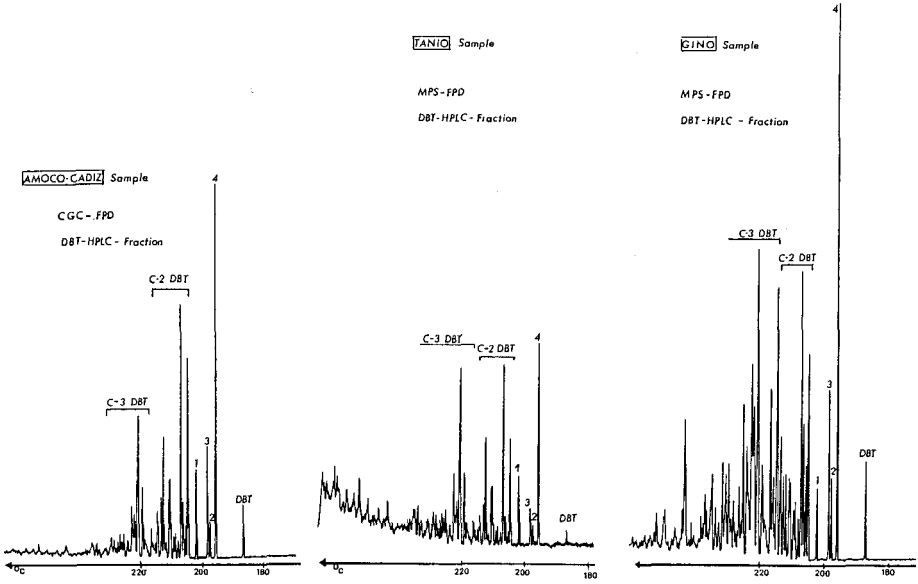

FIGURE 2 GC chromatograms of the DBT-ring fraction isolated from Amoco-Cadiz, Tanio and Gino oils spilled in the Channel-sea. Detector FPD. C-2 DBT and C-3 DBT = dimethyl- and trimethyl-DBT isomers.

Many results suggested a high degree of sulfur-selective accumulation in fish[18] and oysters.[19] So the alkylated dibenzothiophenes must be considered as a definite proof of oil pollution. The complete resolution of the four methyl DBT isomers and, tomorrow, of the 16 dimethyl DBT isomers opens attractive prospects in the monitoring of petroleum marine pollution and probably in geochemistry.

2. Photo-oxidation of DBT derivatives

When crude oil or petroleum products are released into an aquatic environment, immediate changes in their chemical and physical properties occur as a result of several simultaneous weathering processes. The importance of photochemical reactions was recently reviewed.[8,20] Although such an oxidation process was probably insignificant quantitatively in Brittany after the *Amoco-Cadiz* wreck[9] and was evaluated under natural conditions to degrade only 0.07%/

day,[21] it must not be neglected from a qualitative point of view. So compounds oxidized by photo-oxidation were dissolved in the sea-water column. This transformation yielded pollutants of potential toxicity probably higher than that of parent products.[22] Furthermore, such oxygenated compounds became bioavailable to marine organisms.[22]

The fate of Arabian light oil (1 liter) spilled in a controlled sea-water enclosure of 320 liters was studied under natural irradiation during 70 days.[12] The formation of sulfur oxygenated water-soluble compounds was investigated. Owing to the experimental conditions, it was suggested that such compounds present in the water column derived from crude oil via photochemical reactions.[13]

During the 70 days of the experiment the sea-water was not renewed, making the biological oxidation insignificant. So the photo-oxidized compounds having an enhanced water solubility were removed from surface slicks and diluted in water. From the results previously reported,[13] it has been calculated that a 0.45 mm surface oil film could be photochemically degraded under sunlight at a rate of 0.004%/day. Such a rate was about 16 times inferior to that calculated by Hansen.[21] This discrepancy may be due to the differences in experimental conditions: source of light, temperature, turbulent conditions, oxygen content of water.

By monitoring the disappearance of DBT derivatives in oil surface, the half-life of photo-oxidation reaction could be evaluated, provided that kinetics of such a reaction is a first order equation. Table III presents measured half-lives of photo-oxidation of some methylated DBT. Although these results suffer from several limitations, the values obtained appeared to be reasonable. So Mill *et al.*[23] reported for DBT a value of 8 days and showed that the rate constant of this

TABLE III

Estimated half-lives of photo-oxidation of mono- and dimethylated DBT under natural conditions (spring, sunlight, 48° 25′ N latitude).

DBT	8 days	dimethyl-DBT	80 days
4-methyl-DBT	14 days	dimethyl-DBT	70 days
2-methyl-DBT	12 days	tri-methyl-DBT	>2 years
3-methyl-DBT	16 days		
1-methyl-DBT	20 days		

reaction conducted in sunlight increased by a factor of 5 between winter and summer. Furthermore, the more the DBT nucleus bears methyl groups, the more compounds were resistant to photo-oxidation process. Such a result was in agreement with the observations that polymethylated DBT were persistent in the marine environment. So during the monitoring of *Amoco-Cadiz* pollution, traces of tri- and tetramethylated dibenzothiophenes could be detected 4 years after the spill.[24]

Patel *et al.*[25] photo-oxidized both an Arabian medium crude oil and oil from the *Amoco-Cadiz* tanker. They characterized a variety of DBT oxides and alkyl substituted DBT oxides. But these authors reported that the dibenzothiophenes oxides were decomposed during GC/MS analysis, so much so that the compounds detected were deoxygenated DBT. Such a decomposition was shown to be dependent on the column injector. So by using the cold on-column injector, no degradation products occurred.[26] Figure 3 presents chromatograms of acidic water-soluble fraction at 20, 45 and 70 days of natural irradiation. Compounds detected by FPD showed retention times longer than the parent methyl DBT. They were identified as DBT sulfoxide, monomethyl DBT sulfoxides and dimethyl DBT sulfoxides in the 20 days sample. As the sulfones were no completely separated from sulfoxides on the methylphenylsilicone stationary phase, positive identification of methyl DBT sulfones could not be carried-out by GC. Furthermore, the 70 days sample contained tri- and tetramethylated oxygenated dibenzothiophenes.

Since Wakeham[27] has shown that considerable improvement in resolution of spectra fluorescence was achieved by SEES fluorescence, this technique was shown to be very useful for the analysis of aromatic hydrocarbons in the environment. Synchronous fluorescence from 275 to 500 nm showed that maximum value was obtained for pure DBT, DBT-5-oxide and DBT-5-dioxide at 335, 350 and 375 nm respectively. Identification of methylated DBT sulfoxides and sulfones was attempted on this basis. The maximum intensity of the three-aromatic ring zone (330–390 nm) of the spilled crude oil was at 335 nm. The maximum intensity of this spectral zone was progressively displaced towards 375 nm for the 70 days sample (Figure 4), indicating the formation of DBT-sulfones rather than the dissolution of three- and tetra-ring PAH in the water column.

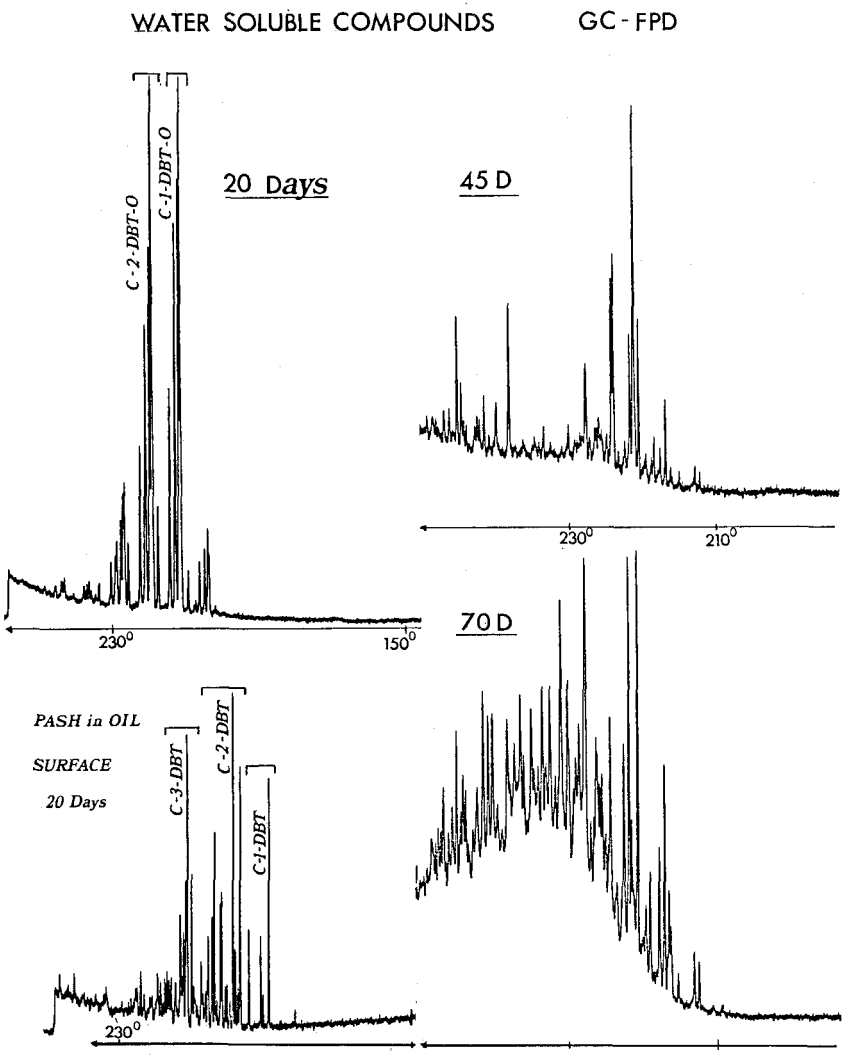

FIGURE 3 FPD-gas chromatograms of the 20-, 45- and 70-days samples extracted at acidic pH from the sea-water column, GC as in Figure 1. Methylated extracts. For comparison is shown the FPD-GC profile of aromatic fraction of the surface oil at 20 days.

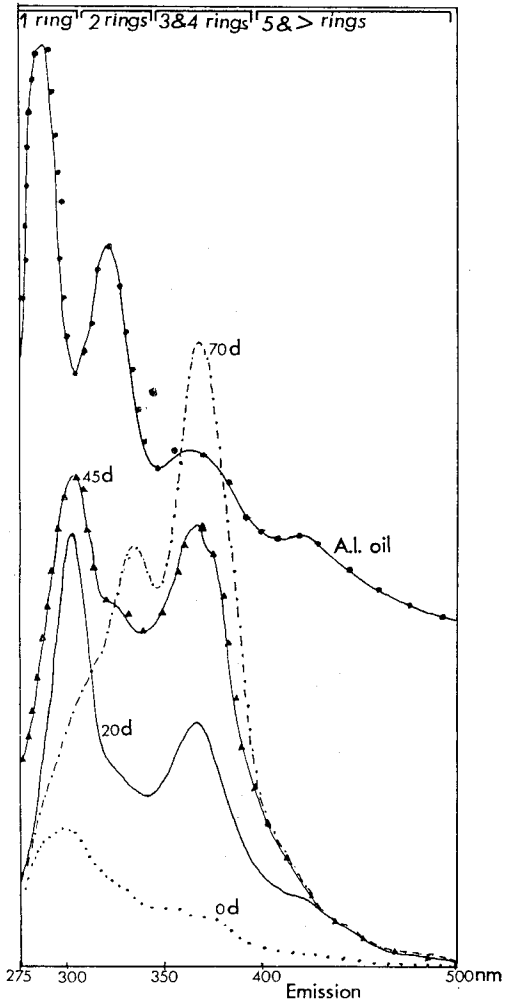

FIGURE 4 SEES fluorescence profiles of the acidic extracts from sea water at different times. Also indicated the respective fluorescence regions for different size PAH. SEES profile for Arabian light (A.l.) is included for comparison.

SEES fluorescence of HPLC peaks confirmed this result (Figure 5). Therefore, many HPLC peaks presented SEES profiles superposable to that of DBT-sulfones (Figure 5).

3. Biodegration of dibenzothiophene

Eastmond *et al.*[28] reported that DBT was more toxic than analogous PAH in zooplankton. Furthermore, it was bioconcentrated about 600 times relative to water. As little is known about the metabolism of condensed thiophenes by living organisms[29] the metabolic fate of the most representative of PASH, i.e. DBT, was studied *in vitro*.

Liver microsomes from Aroclor-induced rats oxidatively transformed DBT to DBT-5-oxide and subsequently to DBT-5-dioxide (Figure 6). As such compounds are more soluble than parent nucleus, this sulfoxidation represents a mechanism of elimination. Metabolic S-oxidation represents a general metabolic pathway of drugs such as phenothiazines.

The apparent Michaelis constant Km of 85 μM indicates a good affinity of the mixed-function mono-oxygenase enzyme for the substrate DBT. This value is close to that of benzo(a)pyrene estimated between 2–16 μM.

The two metabolic pathways, sulfoxide and sulfone formation, were induced differentially by different xenobiotic substances injected to rats, suggesting that the mono-oxygenases involved were partially different.[29] Furthermore, these two S-oxidations were *in vitro* inhibited by CO, *n*-octylamine, benzo-7,8 flavone or SKF 525 A.[30] Accordingly, contribution of Cytochrome P-450 dependent mono-oxygenase was estimated to be essential in the DBT S-oxidation. However, contribution of microsomal flavin-containing mono-oxygenase could not be totally excluded.[30]

The PAH substrate used most frequently for studies of mono-oxygenase activity (MFO) in aquatic species is benzo(a)pyrene.[31] Several procedures of enzymatic oxidation of this substrate have been reported; the results are expressed as aryl hydrocarbon hydroxylase (AHH). However, there is evidence that benzo(a)pyrene is only a minor constituent of crude oils. So the *Amoco-Cadiz* oil contained trace levels of this PAH whereas sediments contaminated by PAH from pyrolytic source contained relatively high concentra-

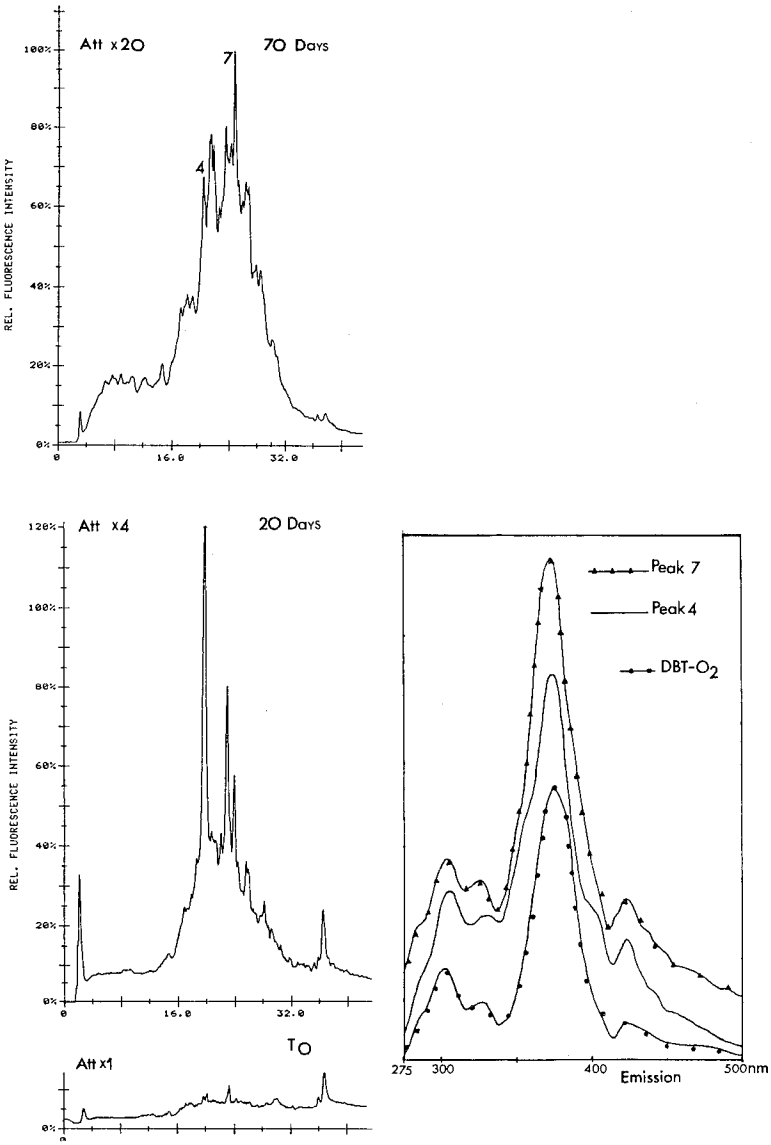

FIGURE 5 HPLC profiles of acidic extracts of sea-water at different times of sunlight irradiation. Fluorescence detection: excitation at 270 nm, emission at 380 nm. Gradient elution as indicated in the text. On the right-hand panel synchro-fluorescence spectra of pure DBT-sulfone and peaks 4 and 7 of the 70 days sample.

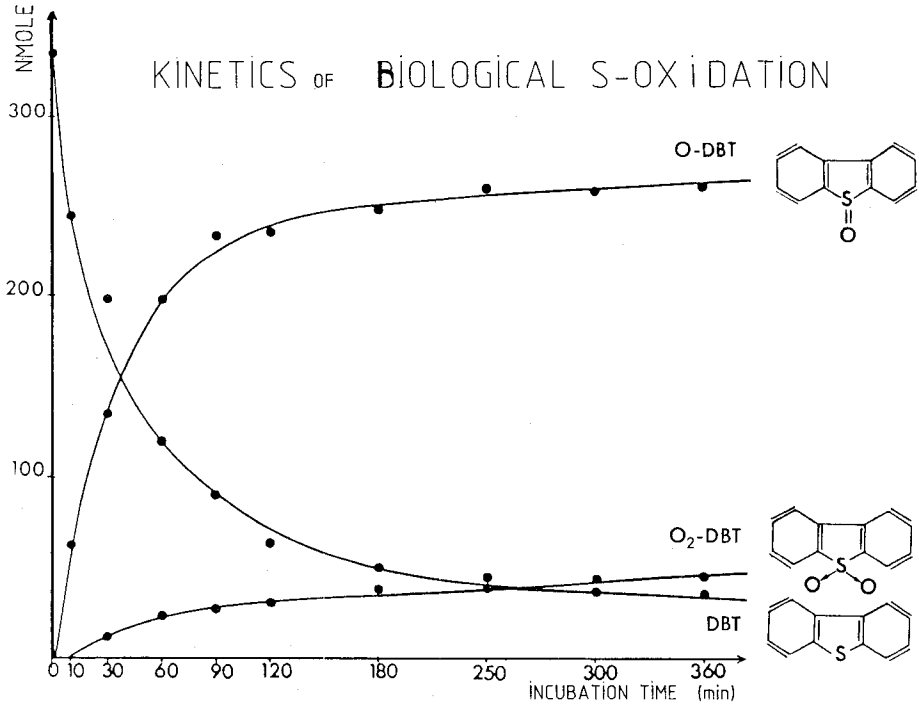

FIGURE 6 Kinetics of biological S-oxidation of DBT incubated with rat liver microsomes.

tions (ppm levels).[32] Accordingly benzo(a)pyrene cannot be considered as the most appropriate substrate for determining mixed-function oxidase activity in marine species exposed to petroleum pollution.[33]

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