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Polycyclic aromatic hydrocarbons (PAHs) in subcutaneous biopsies of Mediterranean cetaceans

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Abstract

The aim of the present study was to measure polycyclic aromatic hydrocarbon (PAH) levels in free-ranging Mediterranean cetaceans as they are likely to cause chemical stress in the organisms of this basin. Blubber samples were collected from live specimens of fin whales (*Balaenoptera physalus*) and striped dolphins (*Stenella coeruleoalba*) by means of biopsies, a non-destructive biological method. Fin whales were sampled in the Ligurian Sea, whereas striped dolphins were collected in the Ligurian and the Ionian Seas. A fingerprint of 14 PAHs was obtained for both species. In whales, the median value of total PAHs was 1970 ppb fresh weight (f.w.) while median carcinogenic PAH values were 89.80 ppb f.w.; in dolphins, the median values of total and carcinogenic PAHs were 29,500 and 676.00 ppb f.w., respectively. The different PAH values between the two species can be attributed to the different positions they take in the Mediterranean food web. The sampling period significantly influenced PAH concentrations of fin whales.

Keywords: Polycyclic aromatic hydrocarbons; Cetaceans; *Balaenoptera physalus*; *Stenella coeruleoalba*; Skin biopsy; Mediterranean Sea

1. Introduction

The most toxic family of hydrocarbons, polycyclic aromatic hydrocarbons (PAHs) are a large class of molecules with condensed benzene rings. These molecules have attracted much scientific interest due to their genotoxicity. Several lines of evidence indicate correlation between high levels of certain PAHs in the environment and the increasing incidence of carcinogenesis and mutagenesis in exposed organisms (NRCC, 1983). Their lipophilic nature enables them to cross biological membranes and accumulate in organisms, causing considerable damage. The United States Environment Protection Agency (EPA) and the World Health Organisation (WHO) identified 16 PAHs as priority pollutants. They are released into the environment by natural (pyrolysis, diagenesis, biosynthesis, natural seepage) and man-made processes (industrial processes, combustion of wood and fossil fuels, motor vehicles, incinerators, oil plants and refineries, oil spills). Environmental monitoring carried out under the United Nations Environment Program (UNEP, 1988) estimated an input of 635,000 t/yr of petroleum-derived hydrocarbons into the Mediterranean Sea. Of these, 330,000 t/yr are from tanker spills, loading, unloading and flushing of tanks. PAHs are highly photosensitive and thermolabile: in the presence of light and oxygen they are quickly degraded. Once they reach an

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aquatic medium they become less susceptible to solar radiation. In the water, they can be incorporated into sediments (especially anoxic sediments), where they may remain undisturbed for long periods of time. The PAH cycle in the aquatic environment is relatively simple: high molecular weight PAHs are quickly adsorbed on the surface of organic and inorganic particles and settle out. They may be remobilised into the water column by biological activity, bioturbation and mechanical processes, such as currents. Low molecular weight PAHs tend to remain in solution where they are readily available to marine organisms via ingestion or respiration. PAH solubility increases as temperature increases. For example, the solubility of anthracene ($C_{14}H_{10}$; molecular weight = 178.24) increases from 12.7 ± 0.4 to 55.7 ± 0.7 g/l from 5.2°C to 28°C (May et al., 1978). Therefore, the bioavailability of low molecular weight PAHs increases in warmer seasons. Because dissolved PAHs are more readily uptaken by biota than those adsorbed on sediment, low molecular weight PAHs are more toxic for marine biota (Neff, 1979; NRCC, 1983). Although much research has been carried out on the biological accumulation of these toxic compounds in marine biota, few studies focus on these fat-soluble contaminants in marine mammals (Neff et al., 1976; Geraci and St. Aubin, 1980; Hellou et al., 1990, 1991; Law and Whinnett, 1992; Loughlin, 1994; Martineau et al., 1994; Lake et al., 1995; Jenssen, 1996; Fossi et al., 1997a, b; Marsili et al., 1997a; Zitko et al., 1998; Holsbeek et al., 1999) and no data on Mediterranean cetofauna are available. Collecting ecotoxicological data on cetaceans is important for several reasons. First, cetaceans have no sweat/sebaceous glands nor gills, so they are relatively closed systems. Other than acting as a thermal barrier and an energy reserve, blubber isolates the skin from the rest of the body, which in turn reduces any exchange between the two. Fat-soluble contaminants usually build up in blubber, which is metabolised only during illness, pregnancy, lactation, migration or food scarcity; the stored contaminants are mobilised along with fat reserves. However recent studies on the metabolism of cetaceans indicate that these marine mammals' detoxifying capacity is limited (Watanabe et al., 1989; Tanabe and Tatsukawa, 1992; Fossi et al., 1992, 1997a, 1997b; Fossi and Marsili, 1997).

In the present study, PAH levels in Mediterranean cetaceans were investigated to determine whether these compounds can cause chemical stress. Many studies document the chemical stress related to organochlorine xenobiotics in stranded and free-ranging Mediterranean cetaceans (Aguilar and Raga, 1993; Aguilar and Borrell, 1994; Borrell et al., 1996; Marsili and Focardi, 1996, 1997; Marsili et al., 1996, 1997b, 1998; Marsili, 2000). This preliminary study involves two cetacean species of the Mediterranean: the planktophagous fin whale (*Balaenoptera physalus*) and the largely teutophagous striped dolphin (*Stenella coeruleoalba*). Because these mammals feed at different levels of the pelagic food chain, we aimed at determining whether a different PAH accumulation would follow. Dolphins from two different sections of the Mediterranean Sea were collected to detect correlation between PAH levels and habitat. We also investigated any relation between PAH levels and gender in fin whales as well as the three-year trend of PAHs from 1993–1996.

2. Materials and methods

2.1. Sampling methods

Twenty three fin whales were sampled in the summer of 1993 and 1996 in the Ligurian Sea, whereas 25 striped dolphins were sampled only in 1993 in both the Ionian and the Ligurian Seas (Fig. 1). Samples of subcutaneous blubber (about $1 \times 2\ cm^2$) were obtained from free-ranging whales using biopsy darts launched with a crossbow while biopsy tips mounted on a 2-m pole were used to sample bow-riding dolphins. A biopsy dart, a regular aluminium crossbow bolt with a modified stainless steel collecting tip and a floater were fired into the whale with a Barnett Wildcat II crossbow and a 150-pound test bow. To avoid infection, the bolt tip was sterilised with alcohol before shooting. Biopsy specimens were taken in the dorsal area between the dorsal fin and the upper part of the caudal peduncle. The procedure consisted of approaching the whale at low-to-moderate speed as it surfaced and firing the dart at a distance ranging from 10–30 m. Dolphins were sampled from the prow of the boat as they were riding the bow wave. Their reaction to sampling varied from a slight start to no reaction at all. Biopsy samples were immediately stored in liquid nitrogen and sent to the Department of Environmental Sciences for chemical analysis and other studies.

Fig. 1. Map of the Mediterranean Sea showing sampling sites.

2.2. PAH analysis

PAHs were analysed by HPLC/fluorescence system. Extraction was carried out according to Griest and Caton (1983) and Holoubek et al. (1990) with some modifications. About 0.1 g of fresh subcutaneous blubber was extracted with a mixture of KOH 2M/methanol (1:4) in a Soxhlet apparatus for 5 h at 70°C. This sample mixture was extracted by shakering in separatory funnels with 200 ml of cyclohexane. The liquid/liquid separation was performed to bring the PAH fraction in the supernatant part. The recovery liquid was successively concentrated in a Rotavapor system, resuspended with 5 ml of benzene and purified in a chromatographic column packed with 3 cm of Florisil, about 60–100 US mesh for chromatographic analysis, previously set at 110°C for 1 h. The elution in the column was carried out with 95 ml of benzene. The organic fraction was concentrated and suspended with 1 ml of acetonitrile. PAH separation was performed using a reversed-phase column (Supelcosil LC-18, 25 cm × 4.6 mm i.d., 5 μm particle size, pore size 120 Å) with an acetonitrile/water gradient from 60% acetonitrile to 100% for 20 min, and successively isocratic for 10 min. The flow rate was 1 ml/min. The mobile-phase was degassed with a helium stream. An external standard consisting of 16 PAHs from Supelco (EPA 610 PAH mixture, 100–2000 μg/ml methanol:methylene chloride, 50:50) was used. The working standard was prepared by diluting (1:100) the stock solution with acetonitrile. Fourteen PAHs (Table 1) were analysed. The results were expressed in ng/g or μg/g fresh weight (f.w.). Recoveries ranged between 80–98%. The detection limit, calculated at a signal-to-noise ratio of three, was 0.1 ng/g f.w. for all PAHs. Assay reproducibility was determined by five repeated analyses of one sample: the variation coefficient ranged from 1–3%, according to the compound. Blanks contained undetectable amounts of PAHs. Total PAH content was calculated as the sum of 14 PAHs, while the carcinogenic PAH level was expressed as the sum of five PAHs, both indicated in Table 1. Most of the PAHs detected were of low molecular weight.

2.3. Data analysis

Data was processed using Statistica 5.0 (Microsoft). Distribution normality was validated by the Shapiro–Wilks test: when W was significant ($P < 0.05$), distribution was considered to be not normal. Differences between groups of data were detected by ANOVA (Kruskal–Wallis test; significance level: $P < 0.05$) and the Kolmogorov–Smirnov test (significance level: $P < 0.1$). The Kruskal–Wallis test was applied to reveal any differences in variance. Because this test does not discriminate which groups differed or to what extent, the Kolmogorov–Smirnov test was used on pairs of samples. The ANOVA–MANOVA multivariate analysis (Scheffé test; significance level: $P < 0.05$) was used to compare PAH percentages on the total PAHs between the various groups.

3. Results and discussions

The first important result of this study was the presence of PAHs in subcutaneous blubber of both species.

The normality of data distribution was tested by the Shapiro–Wilk test. The data as a whole was not found to have normal distribution ($P < 0.05$). Non-normal distributions were also found in both year for fin whales (males plus females) ($P < 0.05$) and over the whole study period ($P < 0.05$). Separate distributions for male and female fin whales over the whole period ($P < 0.05$)

Table 1
PAHs analyzed (NRCC, 1983)

Compound name (IUPAC)	Abbreviation	Molecular formula	Molecular weight	Carcinogenicity (NRCC, 1983)[a]
Naphthalene	Naph	$C_{10}H_8$	128.2	0
Acenaphthene	Ace	$C_{12}H_8$	154.2	0
Fluorene	Fl	$C_{13}H_{10}$	166.2	0
Phenanthrene	Phen	$C_{14}H_{10}$	178.2	0
Anthracene	Ant	$C_{14}H_{10}$	178.2	0
Fluoranthene	Flt	$C_{16}H_{10}$	202.2	0
Pyrene	Pyr	$C_{16}H_{10}$	202.2	0
Benzo(a)anthracene	B[a]A	$C_{18}H_{12}$	228.3	+
Chrysene (93%)	Chry	$C_{18}H_{12}$	228.3	±
Benzo(b)fluoranthene	B[b]F	$C_{20}H_{12}$	252.3	++
Benzo(k)fluoranthene	B[k]F	$C_{20}H_{12}$	252.3	0
Benzo(a)pyrene	B[a]P	$C_{20}H_{12}$	252.3	+++
Dibenzo(ah)anthracene	D[ah]A	$C_{22}H_{14}$	278.3	+++
Benzo(ghi)perylene	B[ghi]Per	$C_{22}H_{12}$	276.3	0

[a] 0 = not carcinogenic; ± = uncertain or weakly carcinogenic; ++, +++ = strongly carcinogenic.

Table 2
Descriptive data (number of samples, arithmetic mean, median, standard deviation, minimum and maximum) on total (a) and carcinogenic (b) PAHs in the different species in function of the various parameters considered. (results are expressed as ng/g f.w.)

(a) *Total PAHs*	No	Mean	S.D.	Median	Minimum	Maximum
B. physalus	23	9052.5	21,304	1974.1	228.60	83,662
B. physalus males	7	12,173	27,019	1284.0	701.80	73,374
B. physalus females	11	11,377	24,602	1974.1	228.60	83,662
B. physalus 1993	9	21,989	32,621	4352.5	1094.9	83,662
B. physalus males 1993	3	26,274	40,882	4352.5	1094.9	73,374
B. physalus females 1993	4	28,549	37,361	13,201	4131.2	83,662
B. physalus 1996	14	1908.7	1344.0	1278.9	701.80	4798.6
B. physalus males 1996	4	1597.3	1057.2	1278.9	701.80	3129.7
B. physalus females 1996	5	1851.2	1522.8	1216.3	777.50	4439.5
S. coeruleoalba	25	36,205	41,107	29,455	199.40	198,368
S. coeruleoalba Ligurian Sea	20	36,205	42,970	32,504	199.40	198,369
S. coeruleoalba Ionian Sea	5	33,664	36,854	21,763	7680.0	97,795
(b) *Carcinogenic PAHs*						
B. physalus	23	306.5	857.0	89.80	6.570	4374
B. physalus males	7	192.4	174.7	126.2	6.570	481.3
B. physalus females	11	496.5	1290	62.60	9.100	4374
B. physalus 1993	9	714.3	1337	257.5	89.81	4374
B. physalus males 1993	3	316.0	178.8	340.4	126.2	481.3
B. physalus females 1993	4	1283	2061	267.3	224.4	4374
B. physalus 1996	14	75.96	88.96	50.43	6.570	282.3
B. physalus males 1996	4	99.70	114.1	63.10	6.570	265.9
B. physalus females 1996	5	31.10	22.40	27.50	9.100	62.60
S. coeruleoalba	25	938.0	927.8	676.0	13.11	3310
S. coeruleoalba Ligurian Sea	19	944.2	1002	599.6	13.11	3310
S. coeruleoalba Ionian Sea	5	913.3	629.4	908.8	220.0	1669

were non-normal, while data distribution on male and female fin whales taken separately year by year showed a prevalently parametric distribution, suggesting that inputs of PAHs were sufficiently different each year. Data on Ligurian dolphins also showed non-normal distribution ($P < 0.05$). On the other hand, data on Ionian dolphins was almost always parametric, which is presumably indicative of a single population respect to Ligurian specimens that occur between Cape Corso, the Cote d'Azur and La Spezia.

Descriptive data (number of samples, aritmethic mean, median, standard deviation, minimum and maximum) on total and carcinogenic PAHs in the two species in function of the various parameters considered, are listed in Table 2. PAH concentrations (total and carcinogenic) were analysed for significant differences by the Kruskal–Wallis and the Kolmogorov–Smirnov tests. Results are shown in Table 3. Comparison of total and carcinogenic PAH concentrations between fin whales and striped dolphins (Table 2) showed greater accumulation in subcutaneous blubber of dolphins than in whales. The same result was found comparing fin whales and striped dolphins sampled in the Ligurian Sea in 1993 (Table 2). Statistically significant differences in PAH

Table 3
Comparison between two groups of data using the Kolmogorov–Smirnov test[a]

	Total PAHs		Carcinogenic PAHs	
	P	Significance	*P*	Significance
B. physalus/*S. coeruleoalba*	<0.001	Yes	<0.001	Yes
B. physalus/*S. coeruleoalba* Ligurian Sea	<0.001	Yes	<0.005	Yes
B. physalus males/*B. physalus* females	>0.100	No	>0.100	No
B. physalus males/*B. physalus* females 1996	>0.100	No	>0.100	No
B. physalus males/*B. physalus* females 1993	>0.100	No	>0.100	No
B. physalus years 93/96	<0.025	Yes	<0.005	Yes
S. coeruleoalba Ligurian Sea/*S. coeruleoalba* Ionian Sea	>0.100	No	>0.100	No

[a] Significance was recognized for $P < 0.1$.

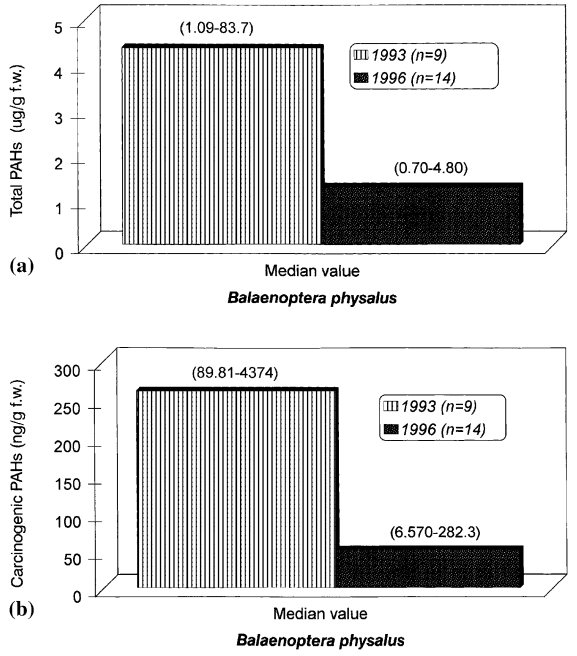

Fig. 2. Median value of total (a) and carcinogenic (b) PAHs (μg/g or ng/g f.w.) in specimens of *B. physalus* in relation to sampling period (minimum and maximum values in brackets).

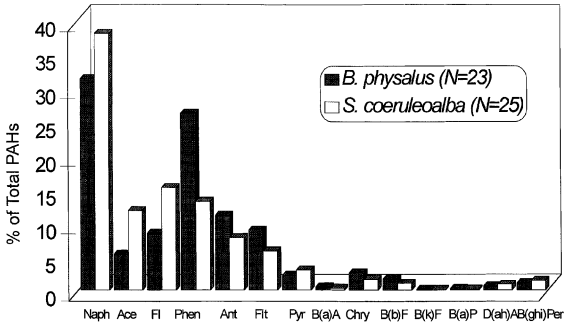

Fig. 3. PAH fingerprint in *B. physalus* and *S. coeruleoalba* specimens.

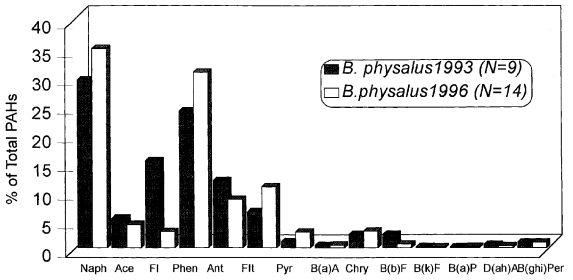

Fig. 4. PAH fingerprint in *B. physalus* in relation to sampling period (1993 and 1996).

concentrations between fin whales and striped dolphins (Table 3) can be related to differences in accumulating or metabolizing these compounds. Fin whales of the Ligurian Sea belong to a Mediterranean population, genetically different from the Atlantic species (Bérubé et al., 1994), and are characterized by the fact that they feed almost exclusively on the macroplankton *Meganjctiphanes norvegica*. Dolphins, being predatory mammals, are at the top of the food chain and have a fish diet of mackerel, sardines and small cephalopods.

Comparison of total and carcinogenic PAH concentrations between fin whales in 1993 and 1996 (Table 2 and Fig. 2) show that PAH levels were much higher in 1993. The significant differences found for fin whales between 1993 and 1996 (Table 3) could be due to the considerable amount of PAHs in the marine environment in 1993. In fact, the first sampling was carried out after two environmental disasters had occurred in 1991: the wreck of the tanker Haven in the Ligurian Sea and the collision between the ferry Moby Prince and the Agip Abruzzo oil tanker in the northern Tyrrhenian Sea. On 11 April 1991, the oil tanker Haven was carrying a charge of 144,000 t of "Iranian heavy 090" petroleum when it exploded and sunk in the Genova Harbor. The oil spill resulted in heavy damage to the local marine and coastal environments. Only 12 h after the Haven accident, another second disaster took place: the collision between the ferryboat Moby Prince and the oil tanker Agip Abruzzo. According to the Castalia Society, 11,000 t of oil were gathered in the sea, partly emulsive. The amount of burned oil was estimated to be 66–71%, while the evaporated fraction was about 9% and the fraction released in sea water was 17–23%; furthermore, the residual products of combustion ranged between 35,000 and 52,000 t (Relini, 1994).

No significant differences (Table 3) were found comparing fin whales in relation to sex; neither as a whole population, nor by separating data in relation to sampling year. Likewise, no significant differences were found between the Ligurian and Ionian populations of striped dolphins (Table 3).

To obtain the PAH fingerprint we calculated the percentage on total PAHs for each of the 14 PAHs analyzed in each specimen of striped dolphins and fin whales. Mean values of the following groups were then calculated: fin whales versus striped dolphins, fin whales in 1993 versus 1996, and Ligurian versus Ionian striped dolphins (Figs. 3–5). The result was that naphthalene peaked in both species (Fig. 3). In fin whales, naphthalene was followed by phenanthrene, anthracene, fluoranthene, fluorene and acenaphthene, while fluorene, phenanthrene, acenaphthene and fluoranthene followed in striped dolphins. Other PAH percentages were negligible. The most abundant PAHs were therefore of low molecular weight, which are also the most water-soluble

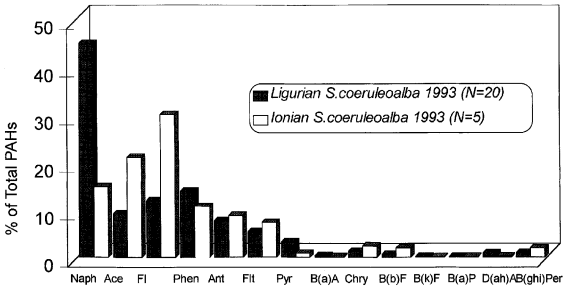

Fig. 5. PAH fingerprint in *S. coeruleoalba* sampled in summer 1993 in relation to sampling site (Ligurian and Ionian Seas).

and largely bioavailable. The six most abundant PAHs (naphthalene–fluoranthene in Table 1) accounted for nearly 90% of total PAHs in both species. The most abundant high molecular weight PAHs in both species only accounted for 3% of the total PAHs and were all carcinogenic (dibenzo[ah]anthracene, benzo[b]fluoranthene and chrysene). Although in both species the only significant differences according to the Scheffé test were the relative percentages of two low molecular weight PAHs, i.e., acenaphtene ($P = 0.0262$) and phenanthrene ($P = 0.0083$), the PAH fingerprint of the two species is however evident. In comparing fingerprints of whales sampled in 1993 and 1996 (Fig. 4), naphthalene and phenanthrene percentages peaked in both years. The only significant differences according to the Scheffé test were to be found in the relative percentage of fluorene ($P = 0.0107$). If a PAH fingerprint of the Haven oil were available, a possible link between contaminants in whales sampled in 1993 and the Haven disaster could have been drawn.

In comparing Ligurian and Ionian dolphins sampled in 1993 (Fig. 5) those from the Ligurian Sea accumulated mainly naphthalene, as in fin whales, followed by phenanthrene, fluorene, acenaphthene, anthracene and fluoranthene. Other PAHs in Ionian dolphins were fluorene, acenaphthene, naphthalene, phenanthrene, anthracene and fluoranthene in decreasing percentage. Significant differences in PAH percentages that resulted from the Scheffé test between Ligurian and Ionian dolphins were found for naphthalene ($P = 0.0063$), acenaphtene ($P = 0.0365$) and benzo(a)anthracene ($P = 0.0319$). Many other PAH percentages neared significant P values.

An attempt was made to compare our results with global data by an online bibliographic search. Very little data about these compounds in marine mammals is available (Hellou et al., 1990, 1991; Law and Whinnett, 1992; Martineau et al., 1994; Lake et al., 1995; Fossi et al., 1997a; Marsili et al., 1997a; Zitko et al., 1998; Holsbeek et al., 1999) and comparison was not fully accomplished for several reasons. With few exceptions, PAH concentrations were usually expressed in chrysene-equivalent or crude oil-equivalent according to the recommendations of the Intergovernmental Oceanographic Commission (IOC). Another reason was that PAHs were generally measured in muscle, often by sacrificing mammals (Hellou et al., 1991; Zitko et al., 1998). In some studies, PAHs were reported to be either low or not detectable at all (Law and Whinnett, 1992; Holsbeek et al., 1999). The literature, however, does confirm the presence of PAHs in many marine mammals' species and the overall predominance of low molecular weight PAHs.

To get an idea of PAH contamination in Mediterranean cetaceans, we compared our results with those on 63 sea lions (*Otaria flavescens*) sampled in the colony of Mar del Plata (Argentina), highly exposed to petroleum contamination. The median value of total PAHs detected in these specimens by subcutaneous blubber biopsies was 7.46 μg/g f.w. with the maximum value of 401 μg/g f.w. (unpublished data). In Mediterranean striped dolphins, the median PAH value was 29.5 μg/g f.w. and the maximum value was 198 μg/g f.w., which indicate that PAHs are toxicologically stressful for cetaceans living in our basin.

4. Conclusions

The following conclusions can be drawn from the results of this study:

- Besides other well-known types of chemical stress, Mediterranean cetaceans are also exposed to PAHs.
- Comparison of total and carcinogenic PAHs in biopsies of *B. physalus* and *S. coeruleoalba* relate to their different positions in the marine food chain. In fact, these species are representative of the two cetacean suborders, Mysticetes and Odontocetes, respectively.
- PAH inputs varied in relation to the year of sampling (1993 and 1996) in which total and carcinogenic PAH concentrations peaked in both fin whales and dolphins of the Ligurian Sea in 1993. This is presumably linked to the incident of the tanker Haven that spilled about 144,000 tons of crude oil in the Ligurian Sea in early 1991.
- The fingerprint of the 14 PAHs showed that naphthalene was the most ubiquitous compound, followed by other low molecular weight PAHs, which is a logical consequence of their major bioavailability in water.

Furthermore, we stress the importance of skin biopsies as a non-invasive method for obtaining biological material, as they can be used for ecotoxicological investigation on threatened animals (Fossi and Marsili, 1997). The recent discovery that Mediterranean fin whales form a small population that is genetically and geographically isolated from the ocean populations (Bérubé et al., 1994) makes the identification of risk factors a priority task in view of conserving this species' biodiversity.

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