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The Pelagos Sanctuary for Mediterranean marine mammals: Marine Protected Area (MPA) or marine polluted area? The case study of the striped dolphin (*Stenella coeruleoalba*)

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ABSTRACT

The concurrence of man-made pressures on cetaceans in the Mediterranean Sea is potentially affecting population stability and marine biodiversity. This needs to be proven for the only pelagic marine protected area in the Mediterranean Sea: the Pelagos Sanctuary for Mediterranean Marine Mammals. Here we applied a multidisciplinary tool, using diagnostic markers elaborated in a statistical model to rank toxicological stress in Mediterranean cetaceans. As a case study we analyzed persistent, bioaccumulative and toxic chemicals combined with a wide range of diagnostic markers of exposure to anthropogenic contaminants and genetic variation as marker of genetic erosion in striped dolphin (*Stenella coeruleoalba*) skin biopsies. Finally, a statistical model was applied to obtain a complete toxicological profile of the striped dolphin in the Pelagos Sanctuary and other Mediterranean areas (Ionian Sea and Strait of Gibraltar). Here we provide the first complete evidence of the toxicological stress in cetaceans living in Pelagos Sanctuary.

1. Introduction

Marine protected areas (MPAs) are not necessarily protected from man-made pollution. This is true for the only pelagic MPA in the Mediterranean Sea: the Pelagos Sanctuary (International Sanctuary for the Protection of Mediterranean Marine Mammals). The Pelagos Sanctuary hosts eight resident cetacean species (*Balaenoptera physalus*, *Physeter macrocephalus*, *Grampus griseus*, *Globicephala melas*, *Tursiops truncatus*, *Stenella coeruleoalba*, *Delphinus delphis*, *Ziphius cavirostris*) and was established in 1999. It is the first international high-seas MPA in the world and includes territorial waters of France, Italy and the Principality of Monaco. While the Pelagos Sanctuary represent a unique example of conservation in Mediterranean, without strong leadership and action, the risk of failure is ever-increasing (Notarbartolo di Sciara et al., 2008). Efforts to protect this valuable and complex ecosystem have not yet succeeded in blocking one of the major human impact: contamination by anthropogenic compounds.

Pressures on whales and dolphins in Mediterranean waters have dramatically increased in recent decades and have different origins (Notarbartolo di Sciara and Birkun, 2010; Van Bressem et al., 2009). The concurrence of different man-made pressures is potentially affecting cetacean population stability, community structure, food chain and marine biodiversity. Contaminants, infectious and immunosuppression diseases, bycatch, shipping, food depletion (by overfishing), noise pollution and climate change affect survival, recruitment, reproductive success, mutation rates and may play a significant role in the partitioning of genetic variation among populations exposed to high and less extreme stress (Whitehead et al., 2003).

The striped dolphin (*S. coeruleoalba)* is the most abundant cetacean species in the Pelagos Sanctuary and, indeed, the Mediterranean as a whole; although no overall population estimation for the entire region exists. The most recent survey estimates the abundance of striped dolphins in Pelagos Sanctuary population to be: in winter 19,462 (95% CI = 12,939–29,273) and in summer 38,488 (95% CI = 27,447–53,968) (Panigada et al., 2011). The Red

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List of the IUCN classifies Mediterranean striped dolphin subpopulation as *Vulnerable*. Striped dolphin, as other Mediterranean odontocetes, accumulates high concentrations of anthropogenic contaminants, many considered Persistent, Bioaccumulative and Toxic (PBT). There is still no evidence that PBT chemicals are causing direct mortality of marine mammals, however it is certain that lipophilic contaminants cause immune and reproductive dysfunction (Hammond et al., 2005; Ross et al., 1995). Polychlorinated biphenyl (PCB) loads in *S. coeruleoalba* that died in the 1990–1992 *Morbillivirus* epidemic were significantly higher than in surviving individuals. The well-known immunosuppressive effects of PCBs in mammals suggested that PCBs impaired immune responses and increased the severity of the outbreak (Aguilar and Borrell, 1994). Between 2007 and 2008 new cases of *Morbillivirus* infection (Raga et al., 2008), after the massive die-off of 1991–92, were detected in Mediterranean specimens. Contaminants such as Organochlorine Compounds (OCs) are also known to be endocrine disrupting chemicals (EDCs) (Fossi et al., 2003). Although OC contamination is decreasing, Polybrominated Diphenyl Ethers (PBDEs) and other emerging contaminants seem to be increasing in the environment, including the Mediterranean Sea (De Wit et al., 2010). PBDEs are lipophilic, persistent and toxic to wildlife and humans (Alaee et al., 2003; De Wit et al., 2010). The highest levels of PBDEs have been recorded in top marine predators, including Mediterranean odontocetes (Pettersson et al., 2004).

In this context international institutions, such as the International Whaling Commission (IWC) have encouraged research on panels of sensitive non-lethal biomarkers, combined with PBT detection in skin biopsies of free-ranging animals, to define the health status of cetacean species with respect to multiple threats, also supporting projects such as Pollution2000+(IWC). At the 2011 Scientific Committee of IWC held in Trømso, Hall et al. (2011) used two case study populations and species (*T. truncatus* and *Megaptera novaeangliae*) to describe and demonstrate an individual-based model that can be used to simulate a variety of pollutant impacts and their potential effects on population growth.

Cetacean skin biopsies are suitable for hazard assessment of free-ranging cetaceans (Fossi et al., 1992, 2010a) obtainable with minimum disturbance for animals. In this paper the markers measured in skin biopsies were general and specific diagnostic signals at different hierarchical levels. A brief description of the mechanisms of action and the link with the toxic compounds is described in Table 1. The biomarkers are subdivided in: (1) markers of exposure to anthropogenic contaminants, (2) markers of general stress; and (3) markers of genetic erosion.

The main aim of this project, supported by the Italian Ministry of the Environment within the ACCOBAMS (Agreement on the Conservation of Cetaceans in the Black Sea Mediterranean Sea and Contiguous Atlantic Area), was to apply a multidisciplinary diagnostic tool, using specific and general biomarkers in striped dolphin in order to verify the toxicological stress in cetaceans living in the Pelagos Sanctuary MPA. In skin biopsy we analyzed persistent, bioaccumulative and toxic chemicals (PBT) combined with a wide range of diagnostic markers of exposure to anthropogenic contaminants and genetic variation as marker of possible genetic erosion. Finally, a statistical model was applied to obtain a complete profile of toxicological status of the striped dolphin in the Pelagos Sanctuary and in two other Mediterranean areas (Ionian Sea and Strait of Gibraltar).

2. Materials and methods

2.1. Sampling areas

The study area was the Mediterranean Sea (Fig. 1C), where we selected three areas with different types of human pressure/stressors for cetacean populations: Pelagos Sanctuary (P) (Italy–France), Western-Ionian Sea (Italy) (I) and Strait of Gibraltar (G) (Spain). *Pelagos Sanctuary* (Ligurian Sea) – The Pelagos Sanctuary is a MPA of about 90,000 km^2 in the north-western Mediterranean Sea between Italy, France and the Island of Sardinia, encompassing Corsica and the Tuscan Archipelago. Compared to the rest of the Mediterranean, this marine area is characterized by high offshore primary productivity with a large biomass of highly diversified zooplankton, which attracts various levels of predators, marine mammals included, to the area. All eight regular cetaceans present in the Mediterranean sea can be found in this area. The remarkable cetacean faunal diversity in the Pelagos Sanctuary has coexisted with very high levels of human pressure such as: intensive maritime traffic, industry and agriculture run-off, military exercises, seismic prospecting, over-fishing, oil–gas exploration and whale watching. *Western-Ionian Sea* – This pelagic area lies between eastern Sicily and south-western Calabria and hosts the eight cetacean species commonly present in the Mediterranean Sea. The area has a medium anthropogenic impact. Threats to cetaceans include drift-nets, maritime traffic and pollution of terrestrial and marine origin. *Strait of Gibraltar* – Since the Strait is the only connection between the Mediterranean Sea and the Atlantic Ocean, maritime traffic is intense. The area is a critical habitat and migration corridor for Mediterranean and Atlantic cetacean species, and is the most diverse cetacean habitat in the Mediterranean Sea. Threats for cetaceans include drift nets, noisy shipping traffic and pollution of terrestrial and marine origin (Cañadas et al., 2005).

2.2. Biopsy collection and analyses

Integument biopsies were obtained from free-ranging striped dolphins in the Pelagos Sanctuary (P) (Italy–France) ($n = 20$, Males = 12, Females = 8) in Western Ionian Sea (I) (Italy) ($n = 12$, Males = 7, Females = 5) and the Strait of Gibraltar (G) (Spain) ($n = 15$, Males = 7, Females = 8) (Fig. 1) during spring–summer 2007, using biopsy tips mounted on a pole (CITES Nat. IT025IS, Int. CITES IT 007 issued to Accademia dei Fisiocritici and University of Siena). Sex was determined by PCR according to Bérubé and Palsbøll (1996). A set of major PBT chemicals (OCs and PBDEs) and a set of biomarkers (CYP1A1, CYP2B, CAT) were analyzed in skin biopsies of three striped dolphin populations and integrated into the statistical model together with the values of gene expression biomarkers (CYP1A1, Aryl hydrocarbon Receptor-AHR, Estrogen Receptor α-ERα, E2F-1 transcription factor, Heat shock Protein 70-HSP70) used as variables (Panti et al., 2011). A screening of the genetic variability was also performed.

2.3. Detection of anthropogenic contaminants

A set of PBTs were analyzed in skin biopsies of three striped dolphin populations. Organochlorine Compounds (OCs): the analytical method used for quantitative and qualitative analysis of hexachlorobenzene (HCB), dichlorodiphenyltrichloroethane and its metabolites (DDTs), and polychlorinated biphenyls (PCBs) is high resolution capillary gas chromatography with electron capture detector (^{63}Ni ECD)(AGILENT 6890/N), according to US Environmental Protection Agency (EPA) 8081/8082 modified (Marsili and Focardi, 1996). The GC has a SPB-5 bonded phase in a fused silica capillary column, 30 m long. Polybrominated Diphenil Ethers (PBDEs): fifteen brominated tri- to deca-substituted BDE congeners (# 17, 28, 47, 66, 85, 99, 100, 153,154, 183, 184, 191, 196, 197, 209) were analyzed by high resolution gas chromatography and low resolution mass spectrometry (HRGC–LRMS). A 6890N gas chromatograph coupled with a 5975 quadrupole mass spectrometer (Agilent, Palo Alto, CA, USA) operated in selected ion monitoring mode (SIM) with negative chemical ionization (NCI) was used.

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Table 1
Skin biopsy for diagnosis of anthropogenic threats. Analysis carried out in each skin biopsy at different hierarchical levels.

Marker	Nature	Estimator	Reference
Detection of anthropogenic contaminants			
HCB, DDTs, PCBs	Organoclorine contaminants (OCs)	HRCGC – Concentration in blubber	Marsili and Focardi (1996)
PBDEs	Flame retardants- Polybrominated Diphenil Ethers	GC/MS – Concentration in blubber	Pettersson et al. (2004)
Markers of exposure to anthropogenic contaminants			
CYP1A1 CYP2B	Protein induction, substrate-inducible and substrate-specific (OCs, PAHs, PBDEs, PCDDs)	WB – Protein concentration in skin biopsy	Fossi et al. (2010)
CYP1A1	Up regulation of mRNA CYP1A1 (PAHs, PCBs, PCDDs)	qRT-PCR – Gene expression in skin biopsy	Panti et al. (2011)
AhR	Up regulation mRNA of nuclear transcription factor (PAHs, PCBs, PCDDs)	qRT-PCR – Gene expression in skin biopsy	Panti et al. (2011)
Steroid hormone receptors (ERs)	Disregulated in the presence of endocrine disrupting chemicals (EDCs)	qRT-PCR – Gene expression in skin biopsy	Panti et al. (2011)
Marker of general stress			
HSP70	Stress induced transcription factor (Chemicals – General stress)	qRT-PCR – Gene expression in skin biopsy	Panti et al. (2011)
E2F-1	Stress induced apoptosis signal (Chemicals – General stress)	qRT-PCR – Gene expression in skin biopsy	Panti et al. (2011)
Catalase (CAT)	Oxidative stress signal (Chemicals – General stress)	Spectrophotometry – Enzyme activity in integument biopsies	Aebi (1984)
Markers of genetic erosion (Individual Heterozygosity)			
Population genetic variability	Within-individual genetic variation related to health/resilience. Calculation of heterozygosity by assigning a score to each locus, weighted by the average heterozygosity at that locus size/stability	Standardized heterozygosity observed (st.het_Obs)	Coltman and Slate (2003)

Comprehensive details of the analytical procedure can be found in Muñoz-Arnanz et al., 2011.

2.4. Detection of diagnostic markers

CYP1A1 and CYP2B proteins were detected by western blot: analysis was performed in integument biopsies by WB in duplicate for each sample, using goat anti-rabbit CYP1A1 and CYP2B4 (Oxford MI, USA). Semi-quantitative analysis was performed with Quantity One software (BioRad) (Fossi et al., 2010a).

Catalase activity (CAT) was evaluated in integument biopsies by spectrophotometry using the procedure of Aebi (1984) with some modifications in order to evaluate oxidative stress. Briefly, the assay mixture consisted of 0.97 ml phosphate buffer (0.05 M, pH 7.0), 1 ml hydrogen peroxide (0.019 M) and 0.03 ml 10% S9 in a final volume of 3 ml. The change in absorbance was recorded at 240 nm. CAT activity was calculated in terms of nmol H_2O_2 consumed/min/mg protein.

Detailed screening of the genetic variability of Mediterranean populations and/or subpopulations of *S. coeruleoalba*, related to multiple human impacts, was performed using neutral genetic co-dominant (DNA microsatellite). To examine whether within-individual genetic variation is related to health/resilience, we used 15 microsatellite loci and calculated individual genetic diversity using standardized heterozygosity observed (st.het_Obs). This measure of heterozygosity is based on a score for each locus weighted by the average heterozygosity at that locus (Coltman et al., 1999). The proportion of heterozygous typed loci/mean heterozygosity of typed loci was used. This method assigns equal weight to all loci examined, regardless of their allelic frequencies, and assumes a linear relationship between locus-specific heterozygosity and number of alleles.

2.5. Statistical analysis and statistical model elaboration

As a final aim of this work we elaborate a statistical classification model (named as *ST.R.E.S.S. – STatistical Risk Elaborating System in Stenella)* for ranking multiple-stress in different striped dolphin populations into Mediterranean area. The data (PBT and biomarkers) of the three populations analyzed in this study, were integrate in the model with the gene expression data (CYP1A1, ER α, HSP70, E2F-1, AHR) previously detected in the same samples used as variables (Panti et al., 2011).

The data was processed using non-parametric tests. Hierarchical cluster analysis by the minimum energy (E) distance method (Szekely and Rizzo, 2005) was applied to define clusters and measure heterogeneity between groups and homogeneity within groups. Canonical discriminant analysis on PCA factors was performed to find clustering variables and define a classification model for Mediterranean striped dolphins to apply to future observations (Dray and Dufour, 2007). The Montecarlo test of the sum of discriminant analysis eigenvalues (divided by rank) was used to test the quality of discriminant analysis (Manly, 1991). R version 2.10.1 (Development Core Team, 2009) (2009-12-14) software was used and the packages ade4 (Dray et al., 2007) and energy (Rizzo and Szekely, 2008).

3. Results

The three populations studied showed dramatic differences in PBT chemical levels and biomarker responses (Fig. 1) as described below.

3.1. PBT chemicals

Concentrations of PCBs, DDTs, OCs known as endocrine disrupting chemicals (OCs–EDCs) and PCB congeners concentration expressed as REP (relative effect potency) values relative to PCB126 (PCB-REP), were higher in Pelagos Sanctuary striped dolphins than in Ionian Sea and Gibraltar specimens (Fig. 1D–G). In particular PCBs were 1.7 and 1.5-fold higher in Pelagos than Ionian and Gibraltar respectively. PBDEs concentrations were higher in the two Mediterranean populations (Pelagos Sanctuary and Ionian

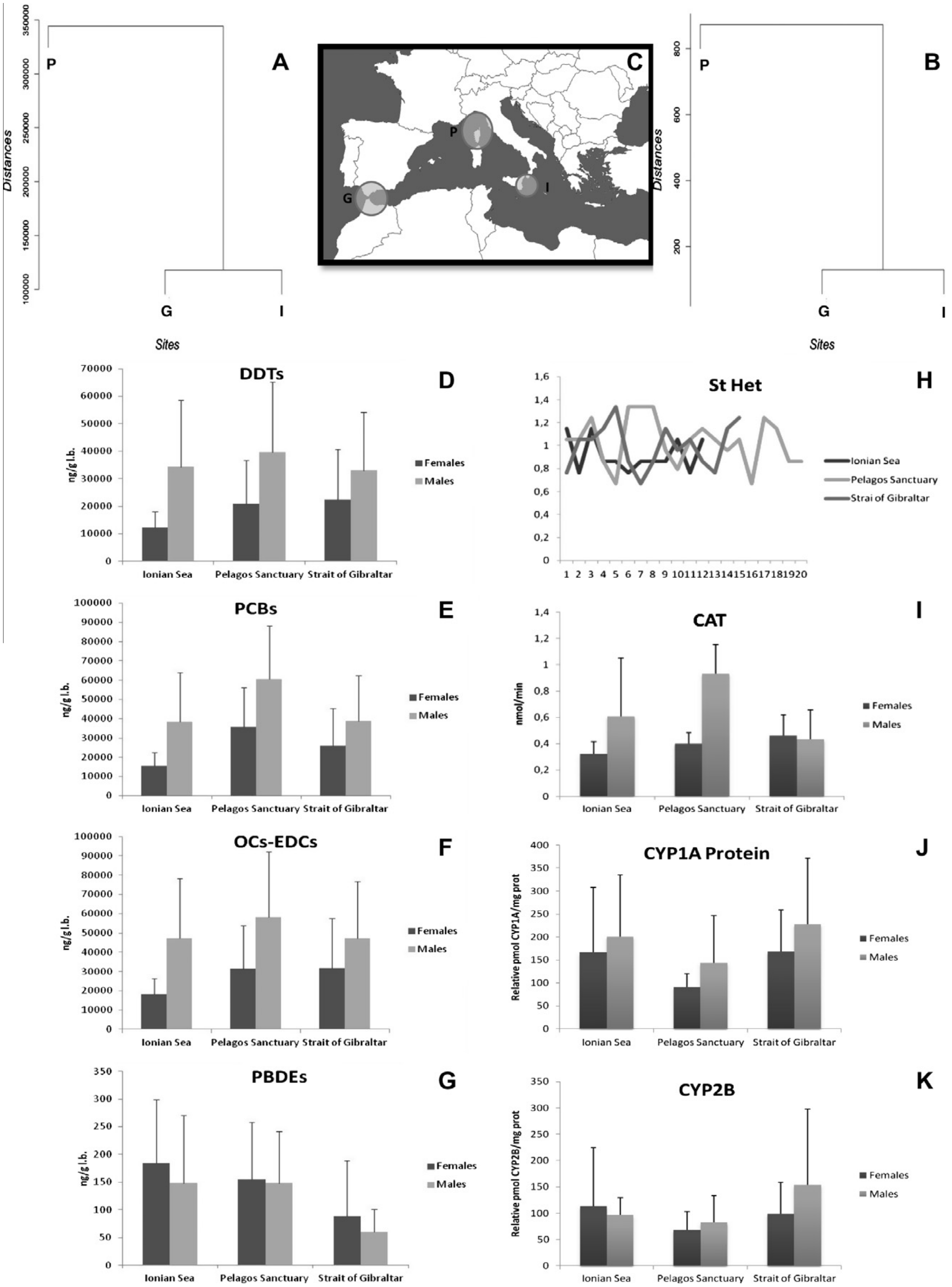

Fig. 1. Skin biopsy diagnostic tool applied to three Mediterranean striped dolphin populations. Dendrograms of classification of the three populations studied: (a) sites grouped by contaminants (OCs and PBDEs). (b) Sites grouped by biomarkers. (c) Study areas: Pelagos Sanctuary (P), in Ionian Sea (I) and the Strait of Gibraltar (G). PBT detection in subcutaneous blubber: (d) DDTs levels, (e) PCBs levels, (f) OC–EDCs levels and (g) PBDEs levels. Markers of genetic erosion: (h) st.het_Obs. Markers of general stress: (i) catalase activity (CAT). Markers of contaminants exposure: protein levels of (j) CYP1A1, (k) CYP2B. For all set of data mean value ± SD are reported.

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Sea), whereas Gibraltar samples contained lower average levels (2.2 and 2.0-fold respectively). This data confirmed that odontocetes in the Pelagos Sanctuary are exposed to high levels of PBT chemicals as previously reported by Fossi et al. (2010a).

3.2. Markers of exposure to anthropogenic contaminants

Surprisingly, CYP1A1 and CYP2B protein levels were higher in Ionian and Gibraltar striped dolphins (CYP1A1 0.69 and 1.69-fold respectively; CYP2B 1.39 and 1.67-fold respectively) than specimens living in the most polluted site, the Pelagos Sanctuary (Fig. 1J and K). Low CYP1A1 and CYP2B induction, despite high levels of lipophilic contaminants, was evident in male and female Pelagos dolphins suggesting dose-dependent down-regulation of CYPs in this population, as previously detected in the fin whale population of the same MPA (Fossi et al., 2010a). Further evidences on PBT chemicals exposure were previously shown by upregulation of CYP1A and AHR genes in Pelagos Sanctuary striped dolphins with respect to dolphins from the other two areas; male values were also higher than female values (Panti et al., 2011). The usefulness of dermal CYP1A1 expression as a biomarker of PHAH and PAH exposure in cetaceans is further supported by studies showing that CYP1A1 levels or activity in skin and liver correlate with blubber PCB concentrations in cetaceans (Fossi et al., 2003; Godard-Codding et al., 2011; Wilson et al., 2007; Hooker et al., 2008). Moreover mixtures of lipophilic contaminants are strong inducers of CYP1A1 mRNA and can produce a bell-shaped CYP1A1 protein dose response. This was evident in the Pelagos striped dolphin population, as previously reported by Fossi and collaborators from biopsy slices treated *ex vivo* (Fossi et al., 2010b). All these data suggest that the Pelagos population is exposed to high toxicological stress due to agonist for aryl hydrocarbon receptor such as the PBT compounds detected in the blubber of the same specimens.

3.3. Markers of general stress

Catalase activity was higher in the Pelagos population with respect to the other two populations (1.4-fold each, Fig. 1I) suggesting an effect of general stress in the Pelagos population due to the mixture of PBT pollutants as described in Section 3.1.

3.4. Markers of genetic erosion

Positive associations between individual genetic diversity (heterozygosity) and fitness-related traits are reported across many *taxa* (Coltman and Slate, 2003), including humans (Lie et al., 2009). Individuals have different capacities to respond to stress. Our results provided support for an association between genetic diversity and toxicological stress. Striped dolphins with greater st.het_Obs had significantly lower contaminant loads (Fig. 1H). In fact, the dolphins least affected by contaminants were those with greater genetic diversity, confirming that genetic variability is linked to resilience. Ecological resilience is connected to genetic diversity not only on the persistence of populations and species but also in influencing ecosystem function. Low genetic variability depresses individual fitness, resistance to diseases and flexibility in coping with environmental changes and challenges, such as the stress caused by pollutants. Additionally, genetic differentiations among pairwise populations (estimated by Fst, microsatellites) are not statistically significant, implying that the differences in the contaminant responses are not due to genetic differences between populations.

3.5. Statistical analysis and population differences

In order to evaluate the different ecotoxicological status of the three striped dolphin populations, we used hierarchical cluster analysis to classify samples ($n = 47$) by site. The analysis was performed separately on contaminant levels and biomarker responses. Fig. 1A and B shows the resulting classification dendrograms. Though based on different datasets, the contaminant (Fig. 1A) and biomarker (Fig. 1B) dendrograms coincided. In particular, the Ionian and Pelagos populations proved very distant. The Gibraltar samples were in an intermediate position.

3.6. Development of ST.R.E.S.S. classification model

As final outcome of the work we developed the *ST.R.E.S.S* statistical classification model (*STatistical Risk Elaboration System in Stenella)* for Mediterranean striped dolphins. The model was applied for the classification of the toxicological status of the three dolphin populations and ranks toxicological stress in Mediterranean cetaceans. In order to achieve this goal several steps were followed.

Step 1. A first goal in model implementation was construction of a "virtual control" based on contamination levels, using the whole data set of the three populations studied. Hierarchical cluster analysis was performed to identify groups of samples with homogeneous contaminant levels. Three clusters (named as low, moderate and high contaminant levels) emerged (Fig. 2 and Table 2). The data were consistent with average levels of the different classes of PBT compounds, referred to the most worldwide distributed dolphin species (*T. truncatus*), in pristine (polar regions of both hemispheres: DDTs < 10 mg/kg; PCBs < 10–30 mg/kg), moderate impact (NW Atlantic: DDTs 10–30 mg/kg; PCBs 30–100 mg/kg) and high impact areas (Mediterranean Sea: DDTs 100–500 mg/kg; PCBs > 500 mg/kg) (Aguilar et al., 2002).

Step 2. The second step was to assign to each virtual population data (low, moderate, high contamination levels) the trend of biomarker responses that best defined the different toxicological status of the dolphin populations. Canonical discriminant analysis was performed on the complete data set in order to determine the discriminating variables of the three groups. Fig. 3A shows the correlations between the variables and the discriminant functions. The analysis was significant: Monte Carlo test used 999 times by replicating the test is equal to 0.085 $p = 0.006$ showing the existence of a very strong discriminative component.

The three set of data ("Low group", "Medium group", "High group") are clearly characterized by different diagnostic markers signals. The "Low group" (low concentrations of PBT chemicals) appears to be defined and influenced by high values of St.het_Obs, and low values of ERα and AHR (as xenoestrogen and dioxin-like compounds marker of exposure, respectively). The "Medium group" (medium concentrations of PBT) appears to be defined and influenced by high values of CAT, CYP2B and CYP1A proteins. Finally the "High group" (high concentrations of PBT chemicals) is characterized by low values of St.het_Obs and high values of CYP1A, E2F-1 (as general stress signal), ERα and AHR. The different categories of parameters considered (PBT levels, markers of exposure, markers of xenoestrogen exposure, markers of general stress and markers of genetic erosion) showed different trends (Fig. 3A and B), describing three different levels of toxicological concern. The final outcome of this statistical process enabled separation of the three populations of data on the basis of toxicological concern, generated by bringing together overall PBT chemical levels, biomarker responses, and markers of genetic erosion (Fig. 3B).

Step 3. The proposed canonical discriminant model (*ST.R.E.S.S)* estimates the discriminant canonical scores by the linear relations reported in Supplementary data.

Step 4. Canonical discriminant weights made it possible to develop the described statistical classification model (*ST.R.E.S.S*) used to define the toxicological risk of the target species (striped dolphin) in the Mediterranean Sea and for a further application in other marine areas. The 42 complete observations were classified

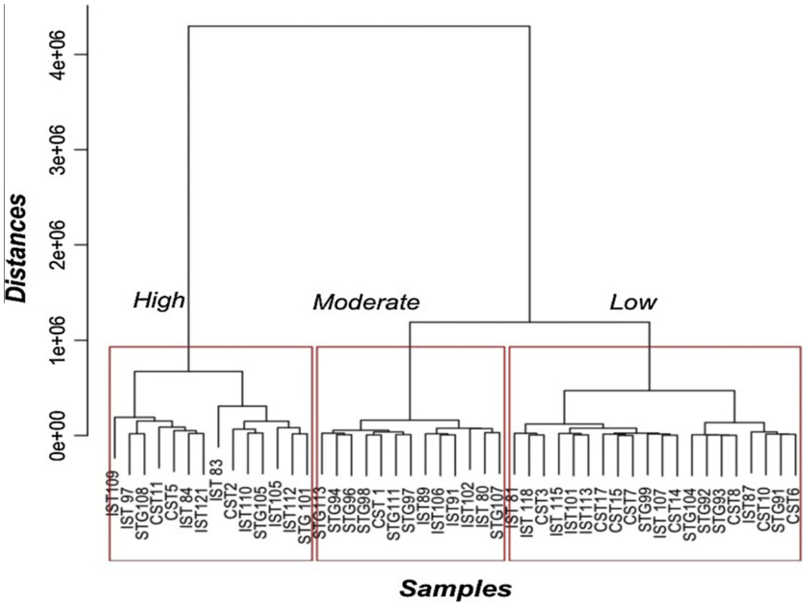

Fig. 2. Hierarchical cluster analysis. Identification of groups of samples with homogeneous contaminant levels. The low group is the "virtual control" group of the model. The low group was composed by 20 subjects (*P* = 7 (35%), *G* = 5 (33.3%), *I* = 8 (66.7%)) with low concentrations of contaminants. The moderate group was composed by 13 subjects (*P* = 5 (25%), *G* = 7 (46.7%), *I* = 1 (8.3%)) with intermediate concentrations of contaminants. The high group was composed by 14 subjects (*P* = 8 (40%), *G* = 3 (20%), *I* = 3 (25%)) with high concentrations of contaminants.

Table 2

Step 1. Mean ± standard deviations of contaminant concentrations in μg/g lipid basis for each cluster. Hierarchical cluster analysis was performed to identify groups of samples with homogeneous contaminant levels. Three clusters emerged : low, moderate and high levels of contaminants. The data was consistent with average levels of the different classes of PBT detected in bottlenose dolphins in pristine, moderate and high impact areas (Aguilar et al., 2002).

μg/g lipid basis	DDTs	PCBs	OCs	OC–EDCs	PCB-REP	PBDEs
Low *N* = 20	11830.2 ± 5300.7	17996 ± 7268.2	29926.3 ± 11723.8	17655.1 ± 7404.2	59.4 ± 25.4	100.7 ± 89.2
Moderate *N* = 13	25143.5 ± 3802.3	36670.9 ± 9195.7	61923.6 ± 8358.8	37435.8 ± 4456.9	125.2 ± 30.2	129.2 ± 94.8
High *N* = 14	56484 ± 18194.9	72810.4 ± 16417.3	129387.6 ± 27755.6	79719.4 ± 22837.7	253.5 ± 56.2	172.7 ± 113.4

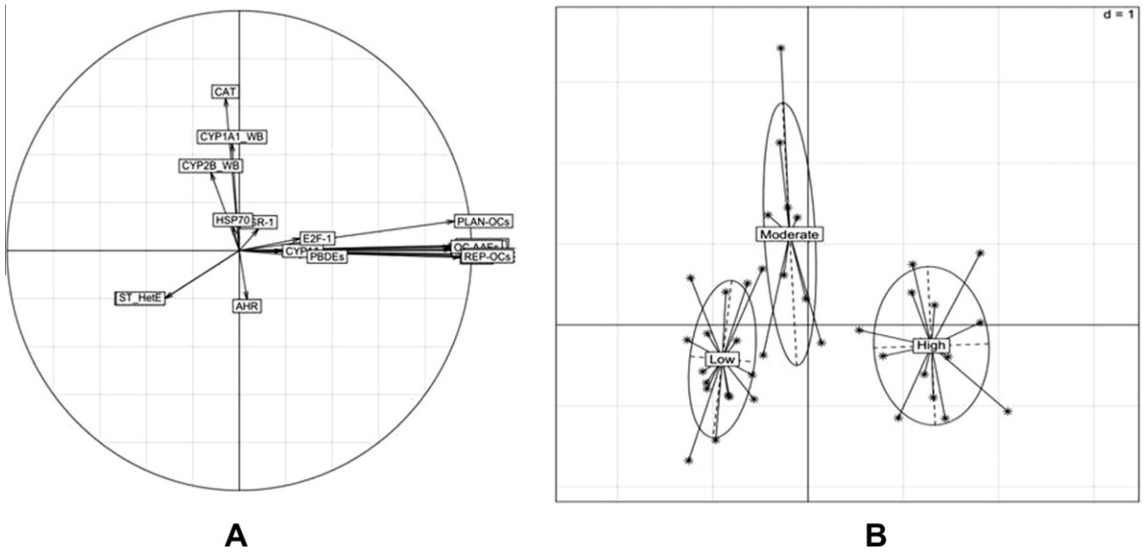

Fig. 3. Correlations between the variables and the discriminant functions. (a) Correlation between variables. Markers of exposure: protein levels of CYP1A1 (CYP1A1-WB), CYP2B (CYP2B-WB), mRNA levels of CYP1A1 (CYP1A1-PCR) and AHR (AHR-PCR)); markers of xenoestrogen exposure: mRNA levels of ERα (ERα-PCR); markers of general stress: (catalase (CAT), mRNA levels of HSP70 (HSP70 – PCR) and E2F-1 (E2F-1-PCR); markers of genetic erosion (st.het_Obs); PBT chemicals (OCs and PBDEs) in subcutaneous blubber. (b) Plot of discriminant scores. The three data sets, describing three different levels of toxicological concern (low, medium group, and high group), are characterized by different diagnostic signals.

as shown in Fig. 4A. Five incomplete observations (two clustered in the low group and three in the moderate group) were processed by the classification model and proved to be correctly classified (Fig. 4B). Moreover, we noted that 66.7% of the dolphins sampled in Ionian sea (I) fell in the low group, while 50% of Gibraltar samples (G) were classified in the moderate group. Surprisingly, 50% of

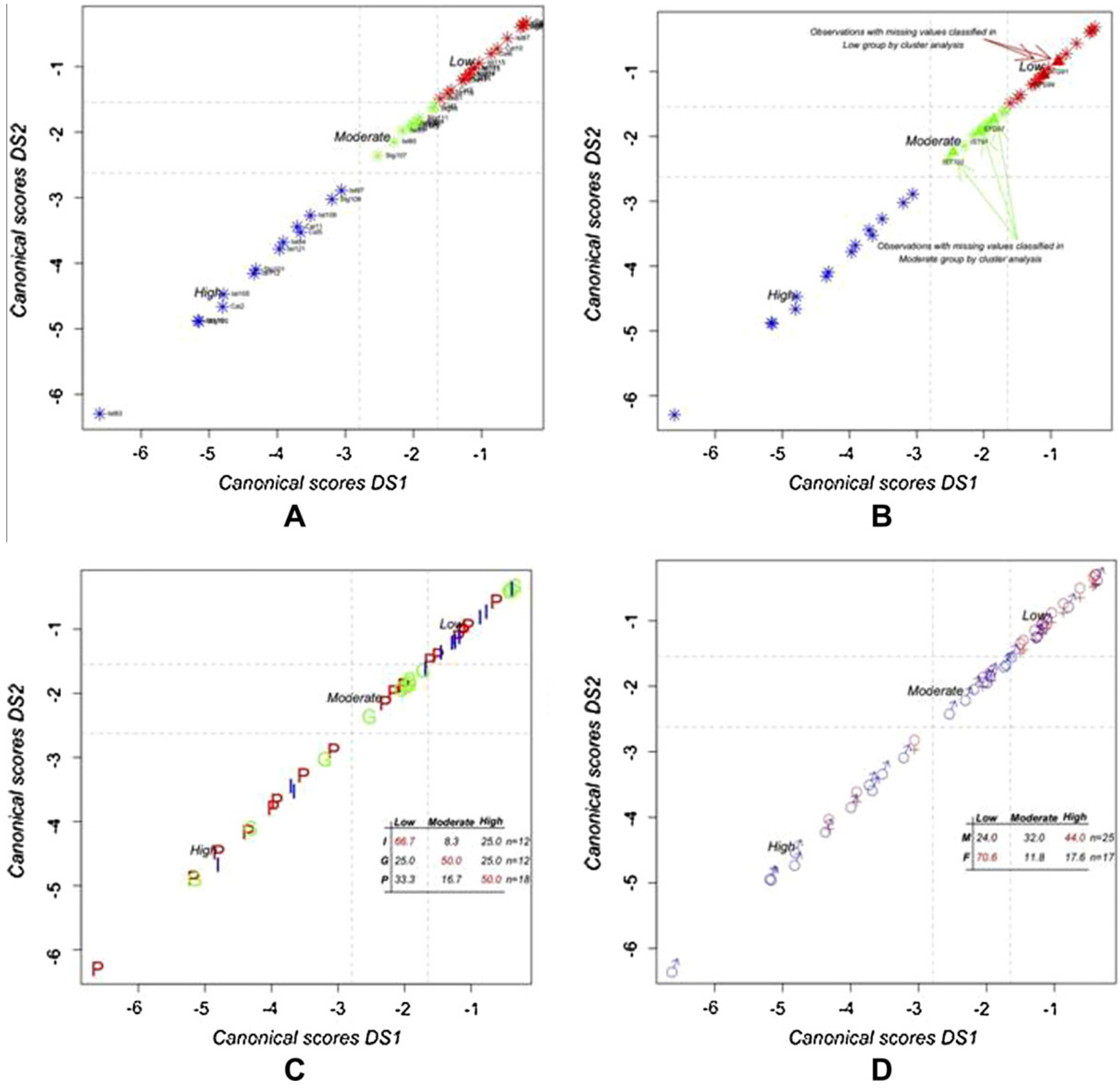

Fig. 4. *ST.R.E.S.S.*: STatistical Risk Elaboration System in Stenella. (a) Canonical discriminant weights used to develop the classification model (*ST.R.E.S.S.*) for ranking toxicological risk in striped dolphin in the Mediterranean Sea. (b) Reattribution of five incomplete observations into the model. (c) Reattribution of the 42 observations into the model in relation to the different sampling areas, Pelagos Sanctuary (*P*), Ionian Sea (*I*) and the Strait of Gibraltar (*G*). (d) Reattribution of the 42 observations into the model in relation to gender.

the Pelagos (P) dolphins fell in the high toxicological hazard group (Fig. 4C). Regarding male–female stratification, we observed that 70.6% of females were classified in the low group and 76% of males in the moderate (32%) and high (44%) groups (Fig. 4D).

4. Discussion

The proposed set of diagnostic tools in skin biopsies provided evidence of toxicological stress in striped dolphin living in the Pelagos Sanctuary, underlining differences in PBT chemicals and molecular biomarker responses in the three striped dolphin populations investigated. The highest toxicological stress in the Pelagos population was highlighted by high PBT chemical levels, combined with correlated biomarker responses. The results support an association between genetic diversity and toxicological stress, confirming that genetic variability is linked to resilience. Individuals with lower st.het_Obs reported significantly higher contaminant loads (50% of the dolphins of the Pelagos Sanctuary).

The final application of the classification model (*ST.R.E.S.S*) provides a outline of the toxicological status of striped dolphin populations and represents a potential tool for monitoring and conservation of cetacean biodiversity. The complete data set confirmed that striped dolphin in the Pelagos Sanctuary are subject to greater toxicological stress than other Mediterranean populations. Particular concern arise from the evidence that 50% of the striped dolphins from the Pelagos Sanctuary were classified in the high toxicological hazard group (Fig. 4C). The classification model can also be used to detect ecotoxicological gender differences; 70.6% of females were classified in the low toxicological risk group and 32% and 44% of males were classified in the moderate and high groups respectively (Fig. 4D), confirming that male cetaceans are subject to higher toxicological risk (Aguilar et al., 2002; Marsili et al., 2004).

Nevertheless, the variability and the complexity of the multiple response needs further investigations, in order to provide a clear description of the global health status of this specie and other cetacean species. Correlate the responses of specific biomarkers in *ex-vivo* and *in vitro* experiments with responses in wild animals, could confirm the specificity of the observations as described in Fossi et al., 2013.

In conclusion, by applying a set of diagnostic biomarkers in skin biopsies of striped dolphin elaborated in the classification model,

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we provide the first evidence of toxicological stress in cetaceans living in the only pelagic marine protected area of the Mediterranean Sea: the Pelagos Sanctuary.

Protecting Pelagos Sanctuary's marine biodiversity requires active management to prevent, control or eradicate environmental threats. This includes developing and delivering programs and activities that efficiently and effectively use available resources to achieve desired environmental outcomes as also underlined in the Nature Editorial (Troubled Waters, 2011) about the effectiveness of MPAs. In the 13 years since its establishment Pelagos has partially failed to fulfill its goal of significantly improving the conservation status of the area's cetacean populations. This weakness is particularly surprising in view of the current joint effort by the Barcelona Convention and the EU to establish a network of MPAs in the Mediterranean High Seas. In order to retain its crucial status of Specially Protected Areas of Mediterranean Importance (SPAMIs), the Pelagos Sanctuary must comply with the requirement of having "a management body, endowed with sufficient powers as well as means and human resources to prevent and/or control activities likely to be contrary to the aims of the protected area" (SPA Protocol, Annex I, D.6) (UNEP-MAP-RAC/SPA, 2010). With the present paper we aim to contribute to comply this requirement.

Moreover, the *ST.R.E.S.S* model estimates ecotoxicological risk on a statistical basis, providing different toxicological threshold levels in dolphin populations, and can be proposed as a tool to monitor the achievement of the Good Environmental Status in pelagic marine areas in line with the European Marine Strategy Framework Directive, in particular for the Descriptor 8 (concentrations of contaminants are at levels not giving rise to pollution effects).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.marpolbul.2013.02.013.

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