Biological and toxicological contamination of cetaceans in the Pelagos Sanctuary: assessment, origin, monitoring and mitigation

Final report

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September 2020

Table of contents

List of Figures

List of Tables

List of Appendixes

ADMINISTRATIVE DETAILS

Biological and toxicological contamination of cetaceans in the Pelagos Sanctuary: assessment, origin, monitoring and mitigation. Final report

Study required and financed by

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Reference of the project

Convention No. 2018-03

Photography credit

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This report should be quoted as:
Berio Enrica, Grattarola Carla, Berny Philippe, Keck Nicolas, Jourdan Julie and Labach Hélène, 2020, Biological and toxicological contamination of cetaceans in the Pelagos Sanctuary: assessment, origin, monitoring and mitigation. Pelagos Secretariat Convention No. 2018-03. 31 pages + 25 appendices

NARRATIVE PART

1. CONTEXT OF THE PROJECT

The Pelagos Sanctuary is a marine area of 87,500 sq. km subject to an agreement between Italy, Monaco and France for the protection of marine mammals, which live in it. Eight cetacean's species occur regularly in this area. The Pelagos Sanctuary aims to mitigate the threats which can affect cetaceans' individual health and their population conservation status.

As apex predators with long life spans, Cetaceans are particularly sensitive to contaminants, in particular to PCBs, PBDEs and DDTs. They are considered as sentinels, their study provides a great source of information on the marine environment and can provide early warnings of acute perturbation (Moore 2008). Previous studies on cetaceans in the Pelagos Sanctuary have shown that animals were highly contaminated by chemical pollutants (Marsili & Focardi 1997; Fossi *et al.* 2000; Marsili *et al.* 2001; Tapie *et al.* 2012). So far, most research has been focused on PCBs, PBDEs, DDT and some trace elements (Hg). There is evidence indicating that some of these contaminants are declining (PBDEs, DDT), while others are still at toxic levels (Law *et al.*, 2012). PCBs can be considered as a general marker of human activity and because of their toxicity, the complete ban of these compounds as well as the elimination of all human sources in Europe, should result in a decrease in contamination in the blubber/liver of cetaceans in the Mediterranean area. Trace elements should also be considered as general indicators of the environmental contamination by human activities: mercury, cadmium and lead are mostly human sources and have well-known toxic effects in mammals. Pesticides (including insecticides, herbicides and fungicides) based on current available compounds have never been investigated in these species, although marine contamination (Brumovsky *et al.* 2017, Salvat *et al.* 2016) and exposure of mammals (dugong/manatee) have already been described in several areas (Romero-Calderon *et al.* 2016, Hermanusen *et al.* 2008, Kunito *et al.* 2008, Harino *et al.* 2007). Increased infectious disease susceptibility, immunosuppression, endocrine disruption, and neoplasia have been linked to increased levels of contaminants in marine mammals (Bossart *et al.* 2011), highlighting the necessity of multidisciplinary collaborative research to assess the impact of pollution on cetaceans.

Additionally, recently emerging terrestrial pathogens, such as *Toxoplasma gondii, Sarcocystis neurona, Listeria monocytogenes, Salmonella spp*. and *Erysipelothrix rhusiopathiae*, have been recorded in stranded cetaceans. Moreover, several studies have shown the association between extreme weather events and outbreaks of waterborne diseases, due to the presence of wildlife and livestock faeces that have been washed into the sea (Funari *et al.* 2012). Severe floods have occurred along the Pelagos Sanctuary coasts in recent years (Genova 2014, Nice 2016, Livorno 2017) and it can be expected that water fluxes from land could have carried pathogens in the sea water and increased the incidence of diseases and the occurrence of new ones. Understanding the pathogenesis of a given disease, as well as its epidemiology, is paramount to understand the potential effects of such disease on cetaceans' populations.

Nowadays, little is known about the origin of these contaminants and how they impact cetacean populations. In particular, few data are available on cetacean populations of the Pelagos Sanctuary. The examination of stranded marine mammals conveys a valuable source of biological and scientific data that can be used to assess the impact of human activities on the marine environment, and of pathogens on animal and human health (Giorda *et al.* 2017). Not only they can be sampled to quantify contaminants

levels in tissues, but they can alert researchers on the circulation of pathogens in the living populations, in which they are challenging to be detected. In this context, long-term monitoring programs and epidemiological studies are needed to assess the impact of diseases and pollutants on marine mammal populations. Such programs should be multidisciplinary and transboundary, to encompass the whole area frequented by a cetacean population. Information on marine mammal health and environmental pollution are most efficiently and accurately acquired through collaborative, international and interdisciplinary baseline research.

This project aims to contribute to the assessment and mitigation of the impact of biological and chemical pollution on cetaceans in the Pelagos Sanctuary. The main objectives were:

a) To evaluate the origin and the impact of pathogens and chemical pollutants of terrestrial origin on cetacean health in the Pelagos Sanctuary;

b) To develop a transboundary health monitoring network on cetaceans stranded in the Pelagos Sanctuary;

c) To promote the mitigation of biological and toxicological contamination in the Pelagos Sanctuary area.

2. ACTIVITIES CARRIED OUT DURING THE PROJECT (SEPT 2018- SEPT 2020)

Objective 1. Evaluate the origin and the impact of terrestrial pathogens and chemical pollutants on cetaceans in the Pelagos Sanctuary

Activity 1.1. State-of-the-art on chemical and biological pollution affecting cetaceans in the Pelagos Sanctuary.

The review on chemical and biological pollution affecting cetaceans in the Pelagos Sanctuary and the western Mediterranean was conducted by GECEM and the output is available in Appendix 1.

Activity 1.2 Standardization of protocols for samples collection and analysis on terrestrial pathogens

Project partners decided to focus sampling and analyses on two dolphin species:

- Bottlenose dolphin (*Tursiops truncatus*) is the only "coastal" species in the North Western Mediterranean Sea and so may be highly exposed to pollution from terrestrial origin. They have the potential to reflect terrestrial pollution in the Pelagos Sanctuary
- Striped dolphin (*Stenella coeruleoalba*) is the most abundant species in the Pelagos Sanctuary and consequently in very well represented in stranding data. Striped dolphin lives mainly in the pelagic

area but is also frequently encountered above the continental shelf. It can also be a good indicator of the Pelagos area's pollution.

The pathogens of terrestrial origin taken into consideration included both bacteria and protozoa, some having a zoonotic potential. CReDiMa and GECEM/LDV34 were in charge of performing necropsies and collecting samples according to its territorial competence (CReDiMa for the Italian side, GECEM/LDV34 for the French side), as well as of carrying out pathogen detection, isolation and characterization. The partners agreed to include *Escherichia coli* sampled from intestine, as it can be considered as a good indicator to evaluate the spread of antimicrobial resistance in the marine environment.

A shared protocol was established between CReDiMa and LDV34 in order to standardize sample collection, and pathogens detection and characterization, in function of the conservation status of the cetacean carcasses (cf. Appendix 2 Protocols for samples collection and analysis on terrestrial pathogens).

Activity 1.3 Standardization of protocols for samples collection and analysis on chemicals contaminants

A common protocol for samples collection and preparation for toxicological analyses has been established (cf. Appendix 3 Protocol for samples collection and preparation for toxicological analyses). All samples were collected and frozen until further analysis.

A methodology was developed to test several pollutants as organochlorinated pesticides, organophosphate insecticides, PCBs, trace elements (Hg, Pb) and 160 different pesticides (mostly fungicides and herbicides). A specific Liquid chromatography-Mass spectrometry (LC-MSMS) method was developed to investigate 160 different pesticides (mostly fungicides and herbicides. See Appendix 3). These substances have been selected on the basis of their current use in Europe (60%) or as former pesticides used in Europe still in use in many countries around the world (and especially in the Mediterranean area) and known to be persistent or potential endocrine disrupters (ED). Briefly, liver samples are extracted using QuEChERs techniques, followed by filtration and direct injection in the LC-MSMS. Each pesticide standard is identified by means of its retention time and 3 fragmentations (parent and two daughter ions). The method is optimized for all substances by LC-MSMS and is undergoing validation following recommendations of various authorities (USEPA, EMA, Society of Forensic testing, EU directive for the validation of analytical results). The limit of quantification is optimized at 0.01 mg/kg sample (10 µg/kg).

Activity 1.4 Sample collection on stranded cetaceans.

In this study, we aimed to collect 30 samples on cetaceans stranded in the Pelagos Sanctuary, 15 along French coast and 15 along Italian coast.

CReDiMa examined 24 carcasses of cetaceans stranded on Italian Pelagos coast, among which 18 were sampled for laboratory analyses. GECEM intervened on 25 cetaceans stranded on French Pelagos coast that have been sampled for laboratory analyses.

Due to the conservational status or adverse field conditions, it was not always possible to perform a complete sampling for each carcasses. Consequently, some animals were only sampled for toxicology while others for a complete set of samples for toxicology and pathogen analysis (cf. Table 1).

Table 1. List of stranded animals and samples collected by CReDiMa and GECEM along Pelagos coastline for toxicological and biological analysis.

	ID	species	age	date	State	place	code	TOXICOLOGY	PATHOGEN DETECTION
1	42906/18	*Tt*	newborn	13/05/2018	IT	Albisola (SV)	3		x
2	50099/18	*Sc*	juvenile	02/06/2018	IT	Imperia	2		x
3	68097/18	*Sc*	newborn	16/08/2018	IT	Framura (SP)	2		x
4	75722/18	*Sc*	newborn	23/09/2018	IT	Varazze (SV)	4	x	x
5	87558/18	*Sc*	calf	29/10/2018	IT	Arenzano (GE)	2	x	x
6	619/19	*Sc*	adult	03/01/2019	IT	Alassio (SV)	2	x	x
7	4561/19	*Sc*	adult	17/01/2019	IT	Pegli (GE)	4	x	
8	18013/19	*Tt*	calf	23/02/2019	IT	Diano Marina (IM)	3	x	x
9	21724/19	*Sc*	adult	05/03/2019	IT	Savona	2	x	x
10	33212/19	*Sc*	juvenile	04/04/2019	IT	Savona	4	x	
11	42472/19	*Tt*	juvenile	07/05/2019	IT	Isola Palmaria (SP)	4	x	x
12	44599/19	*Tt*	adult	15/05/2019	IT	Sestri Levante (GE)	3	x	x
13	59260/19	*Tt*	adult	04/07/2019	IT	Genova	2	x	x
14	62877/19	*Sc*	adult	21/07/2019	IT	Andora (SV)	3	x	x
15	63558/19	*Sc*	calf	23/07/2019	IT	Bordighera (IM)	4	x	x
16	81694/19	*Sc*	juvenile	12/10/2019	IT	Imperia (IM)	2	x	x
17	89879/19	*Gm*	adult	07/11/2019	IT	Imperia (IM)	2	x	x
18	11557/20	*Sc*	juvenile	01/02/2020	IT	Celle Ligure (SV)	4	x	x
19	SCGC20	*Sc*	adult	10/03/2020	FR	Six Fours les Plages	1		x
20	SCGC19	*Sc*	adult	04/03/2020	FR	Cannes	2		x
21	SCGC18	*Sc*	adult	27/02/2020	FR	Villeneuve Loubet	2		x
22	SCGC17	*Sc*	juvenile	16/02/2020	FR	Cagnes sur mer	1		x
23	SCGC13	*Sc*	adult	04/12/2019	FR	Hyères	2	x	x
24	SCGC04	*Sc*	adult	20/10/2019	FR	Le Lavandou	4	x	x
25	SCGC10	*Sc*	juvenile	19/10/2019	FR	Ste Maxime	4	x	
26	SCGC16	*Sc*	juvenile	23/09/2019	FR	Ramatuelle	1		x
27	SCGC09	*Sc*	calf	20/09/2019	FR	Fréjus	2	x	x
28	SCGC03	*Sc*	adult	05/09/2019	FR	Marseille	2	x	x
29	SCGC02	*Sc*	calf	11/08/2019	FR	Ramatuelle	2	x	x
30	SCGC08	*Sc*	adult	15/07/2019	FR	Bormes les Mimosas	1	x	x
31	SCGC11	*Sc*	adult	16/06/2019	FR	Cap d'Ail	1	x	x
32	SCGC12	*Tt*	adult	15/06/2019	FR	St Raphaël	1	x	x
33	SCGC01	*Sc*	adult	20/04/2019	FR	Hyères	1	x	x
34	SCGC15	*Sc*	adult	22/12/2018	FR	Hyères	3		x
35	SCGC05	*Sc*	adult	13/12/2018	FR	Menton	2	x	x
36	SCGC14	*Sc*	adult	20/11/2018	FR	Ramatuelle	3		x
37	P2018 3704	*Sc*	newborne	10/09/2018	FR	St Laurent du Var	1	x	x
38	SCGC06	*Sc*	adult	12/05/2018	FR	St Cyr sur Mer	3	x	
39	P2018 1843	*Sc*	juvenile	27/04/2018	FR	Saint Jean Cap Ferrat	2	x	x
40	P201874	*Sc*	adult	07/01/2018	FR	Saint Jean Cap Ferrat	1	x	
41	P201875	*Sc*	juvenile	06/01/2018	FR	Cagnes sur Mer	2	x	x
42	P2017 5048	*Sc*	calf	04/11/2017	FR	Saint Jean Cap Ferrat	1	x	x

Legend: Tt: *Tursiops truncatus*, Sc: *Stenella coeruleolaba*, Gm: *Globicephala melas.*

Activity 1.5 Pathogens detection (bacterial culture and PCR).

The common analytical protocol for pathogen detection was performed on 37 samples, 16 by CReDiMa and 21 by LDV34. The results of the analysis are presented in table 2.

Table 2. Results of diagnostic activity for pathogen detection

	ID	state	species	*Salmonella* spp.	*Listeria* spp.	*E.coli*	*E. rhusiopathiae*	*T. gondii*	Cause of death
1	42906/18	IT	*Tt*	neg	neg	n.e.	neg	neg	Antrophogenic (ship collision)
2	50099/18	IT	*Sc*	neg	*L. welshimeri* (spleen)	n.e	neg	POS (LN Ps, LN Tb)	Antrophogenic (by catch with underlying *T. gondii* systemic infection)
3	68097/18	IT	*Sc*	neg	neg	NEG	neg	neg	Perinatal pathology
4	75722/18	IT	*Sc*	neg	neg	n.e.	neg	neg	Perinatal pathology
5	87558/18	IT	*Sc*	neg	*L. innocua* (CNS)	POS	neg	neg	DMV infection
6	619/19	IT	*Sc*	neg	neg	n.e.	neg	neg	DMV infection
7	18013/19	IT	*Tt*	neg	neg	POS	neg	neg	ND
8	21724/19	IT	*Sc*	neg	neg	POS	neg	neg	DMV infection
9	42472/19	IT	*Tt*	neg	n.e.	n.e.	neg	neg	Antrophogenic (by catch with underlying DMV infection)
10	44599/19	IT	*Tt*	neg	neg	n.e.	neg	POS (heart)	ND
11	59260/19	IT	*Tt*	neg	neg	n.e.	neg	POS (muscle)	DMV infection
12	62877/19	IT	*Sc*	neg	n.e.	n.e.	neg	neg	DMV infection
13	63558/19	IT	*Sc*	neg	neg	n.e.	neg	POS (liver, LN Ps, CNS, heart, spleen, musde)	*T. gondii* systemic infection
14	81694/19	IT	*Sc*	neg	neg	n.e.	neg	neg	ND
15	89879/19	IT	*Gm*	neg	neg	n.e.	neg	neg	*C. perfringens* systemic infection
16	11557/20	IT	*Sc*	neg*	n.e.	n.e.	neg	neg	ND
17	SCGC13_20012700489	FR	*Sc*	neg	neg	neg	neg	neg	
18	SCGC04_20012700489	FR	*Sc*	neg	neg	neg	neg	neg	
19	SCGC16_20012700489	FR	*Sc*	neg**	n.e.	n.e.	neg	neg	
20	SCGC09_20012700489	FR	*Sc*	neg	neg	neg	n.e.	neg	
21	SCGC03_20012700489	FR	*Sc*	neg**	neg	n.e.	neg	neg	
22	SCGC02_20012700489	FR	*Sc*	neg	neg	neg	neg	neg	
23	SCGC08_20012700489	FR	*Sc*	neg**	n.e.	n.e.	neg	neg	
24	SCGC11_20012700489	FR	*Sc*	neg	n.e.	neg	neg	POS (liver, heart, muscle)	
25	SCGC12_20012700489	FR	*Tt*	neg	n.e.	neg	neg	neg	DMV infection?
26	SCGC01_20012700489	FR	*Sc*	neg	neg	neg	neg	neg	
27	SCGC15_20012700489	FR	*Sc*	neg**	n.e.	n.e.	n.e.	POS (liver)	DMV infection?
28	SCGC05_20012700489	FR	*Sc*	neg**	n.e.	n.e.	neg	neg	
29	SCGC14_20012700489	FR	*Sc*	neg**	n.e.	n.e.	neg	neg	
30	P2018 3704_20012700489	FR	*Sc*	neg**	n.e.	n.e.	neg	neg	
31	P2018 1843_20012700489	FR	*Sc*	neg**	neg	n.e.	neg	neg	
32	P2018 75_20012700489	FR	*Sc*	n.e.	n.e.	n.e.	neg	n.e.	DMV infection?
33	P2017 5048_20012700489	FR	*Sc*	neg**	neg	n.e.	n.e.	neg	
34	SCGC20_200311001480	FR	*Sc*	neg	neg	neg	neg	neg	
35	SCGC19_200311001480	FR	*Sc*	neg	neg	neg	neg	POS (CNS, liver, muscle, heart)	*T. gondii* systemic infection ?
36	SCGC18_200311001480	FR	*Sc*	neg	neg	neg	neg	neg	DMV infection?
37	SCGC17_200311001480	FR	*Sc*	neg	neg	neg	neg	neg	

Legend: Tt: *Tursiops truncatus*, Sc: *Stenella coeruleolaba*, Gm: *Globicephala melas,* DMV*: Dolphin Morbillivirus,* ND*:* not determined*,* NEG: negative, POS: positive, n.e.: not evaluated, CNS: central nervous system, LN Ps: prescapular lymphnodes

CReDiMa, as the National Reference Center for diagnostic investigations on stranded Marine Mammals in Italy is in charge to proceed to complete examination of stranded cetaceans, including full necropsies and complementary laboratory analyses. Thus, it was able to systematically provide the cause of death of the animals sampled.

Thirteen *E. coli* isolates from two striped dolphins and one bottlenose dolphin were isolated by CReDiMa, then shipped to LDV34 for antimicrobial susceptibility testing by disk diffusion. The results are presented in table 3. They were also tested for MIC determination on Vitek (results are presented in Appendix 4). No E. coli isolates could be obtained from French samples as they were frozen prior to testing which compromised the recovery of bacterial cultures.

Table 3. Results of antimicrobial susceptibility testing (disk diffusion) on *E. coli* strains.

	87558/18	18013/19	21724/19
Tested antimicrobics	*E. coli*	*E. coli*	*E. coli*
Streptomycin	3/3	5/5	5/5
Neomycin	3/3	5/5	5/5
Gentamicin	3/3	5/5	5/5
Florfenicol	3/3	5/5	5/5
Sulfonamides + Trimethoprim	3/3	5/5	5/5
Cefoxitin	3/3	5/5	5/5
Cefalexin (C1G)	3/3	5/5	5/5
Ceftiofur (C3G)	3/3	5/5	5/5
Cefquinome (C4G)	3/3	5/5	5/5
Amoxicillin + Clavulanic acid	3/3	5/5	5/5
Amoxicillin	3/3	3/5	5/5
Colistin	3/3	5/5	5/5
Nalidixic acid	3/3	5/5	5/5
Enrofloxacin	3/3	5/5	5/5
Tetracycline	1/3	1/5	1/5

For each animal, the denominator represents the number of E.coli tested for AST and the numerator represents the number of isolates which were sensitive to the tested antibiotic.

Listeria monocytogenes was not isolated from any sample, but 2 minor species of *Listeria* spp. were identified from 2 animals, in particular *L. welshimeri* from a spleen and *L. innocua* from a brain. For one isolate (*L. innocua* ID 87558/18) an antimicrobial test was performed, and results are shown in table 4.

Table 4. Antimicrobial susceptibility testing (MIC determination) for *L. innocua* isolate

	87558/18	
tested antimicrobics	result	MIC (µg/ml)
ampicillin	sensitive	0.5
clindamycin	sensitive	0.25
gentamicin	sensitive	4
teracyclin	sensitive	1
tylosin	sensitive	4

MIC: Minimum inhibitory concentration

Activity 1.6 Neuropathological and immune-histochemical investigations

Among the 18 individuals sampled on the Italian coastline, only 10 were suitable for neuropathological investigation, due the too poor conservational state. From the French side, CReDiMa examinated 7 brains. The details of the examined individuals are listed in table 5 and the results are presented in table 6.

Table 5. Animals sampled for neuropathological investigations at CReDiMa.

ID	species	age	date	State	place	code
50099/18	*Sc*	juvenile	02/06/2018	IT	Imperia	2
68097/18	*Sc*	newborn	16/08/2018	IT	Framura	2
87558/18	*Sc*	calf	29/10/2018	IT	Arenzano	2
619/19	*Sc*	adult	03/01/2019	IT	Alassio	2
18013/19	*Tt*	calf	23/02/2019	IT	Diano Marina	3
21724/19	*Sc*	adult	05/03/2019	IT	Savona	2
44599/19	*Tt*	adult	15/05/2019	IT	Sestri Levante	3
59260/19	*Tt*	adult	04/07/2019	IT	Genova	2
81694/19	*Sc*	juvenile	12/10/2019	IT	Imperia	2
89879/19	*Gm*	adult	07/11/2019	IT	Imperia	2
TTGC01_87211/2019	*Tt*	juvenile	12/09/2018	FR	Marseille	1
SCGC01_87186/2019/1	*Sc*	adult	20/04/2019	FR	Hyères	1
SCGC02_87186/2019/2	*Sc*	juvenile	11/08/2019	FR	Ramatuelle	2
SCGC03_87186/2019/3	*Sc*	adult	05/09/2019	FR	Marseille	2
SCGC04_87186/2019/4	*Sc*	adult	20/10/2019	FR	Le Lavandou	4
SCGC13_103972/2019	*Sc*	adult	04/12/2019	FR	Hyères	2
SCGC17_17217/2020	*Sc*	juvenile	16/02/2020	FR	Cagnes sur Mer	1

Legend: : Tt: *Tursiops truncatus*, Sc: *Stenella coeruleolaba*, Gm: *Globicephala melas. IT: Italy, FR, France. Code corresponds to the conservation status of the carcass*

Table 6 Results of neuropathological examination: histology, molecular biology and eventually immune-histochemistry detailed-study.

ID	species	neuropathology	IHC *T. gondii*	PCR *T. gondii*	IHC *S.neurona*	IHC DMV	PCR DMV	Cause of death
50099/18	*Sc*	Non-suppurative meningoencephalitis	POS	n.e.*	n.e.	n.e.	neg	Antrophogenic (by catch with underlying T. gondii systemic infection)
68097/18	*Sc*	Absence of neuropathological lesions	neg	neg	n.e.	n.e.	neg	Perinatal pathology
87558/18	*Sc*	Non-suppurative meningoencephalitis (compatible viral origin)	neg	neg	n.e.	POS	POS	DMV infection
619/19	*Sc*	Non-suppurative meningoencephalitis (viral origin)	neg	neg	n.e.	POS	POS	DMV infection
18013/19	*Tt*	Absence of neuropathological lesions	neg	neg	n.e.	n.e.	neg	ND
21724/19	*Sc*	Non-suppurative meningoencephalitis (viral origin)	neg	neg	n.e.	POS	POS	DMV infection
44599/19	*Tt*	Absence of neuropathological lesions	neg	neg	n.e.		neg	ND
59260/19	*Tt*	Autolysis, no evidence inflammatory lesions	n.e.	neg	n.e.	POS	POS	DMV infection
81694/19	*Sc*	Absence of neuropathological lesions	n.e.	neg	n.e.	n.e.	neg	ND
89879/19	*Gm*	Absence of neuropathological lesions	n.e.	neg	n.e.	n.e.	neg	*C. perfringens* systemic infection
TTGC01_87211/2019	*Tt*	Focal gliosis	neg	\	n.e.	neg	\	
SCGC01_87186/2019/1	*Sc*	Multifocal gliosis, perivascular cuffing of mononuclear cells and severe menigitis. Neuropathological lesions are attributable to non-suppurative meningoencephalitis	neg	\	n.e.	neg	\	
SCGC02_87186/2019/2	*Sc*	Focal gliosis, mild perivascular edema, presence of bacteria in vascular lumen and rare inflammatory cells in meninges.	neg	\	n.e.	neg	\	
SCGC03_87186/2019/3	*Sc*	Perivascular cuffing of mononuclear cells, severe non-suppurative menigitis with presence of rare neutrophils.	neg	\	n.e.	neg	\	
SCGC04_87186/2019/4	*Sc*	The sample is not suitable for a toxic-metabolic diagnosis due to autolysis, however inflammatory lesions are excluded.	neg	\	n.e.	neg	\	
SCGC13_103972/2019	*Sc*	Absence of neuropathological lesions	neg	\	n.e.	neg	\	
SCGC17_17217/2020	*Sc*	Absence of neuropathological lesions	neg	\	n.e.	neg		

Legend: IHC: immunohistochemistry, PCR: polymerase chain reaction, DMV: *Dolphin Morbillivirus*.
* Positive for *T. gondii* in lymphnodes

One individual (50099/2018) was positive for *T. gondii* neurotropic invasion. Biomolecular investigation identified *T.gondii* presence also in lymph nodes. From these results we hypothesized a systemic infection by *T. gondii*. It is noteworthy that neurotropic invasion by *T. gondii* can cause neurological alterations, like disorientation and abnormal behavior. In this case, the neurological abnormalities induced by *T. gondii* may have led the individual to hit the propeller of a ship causing severe injuries (cf. Figure 1).

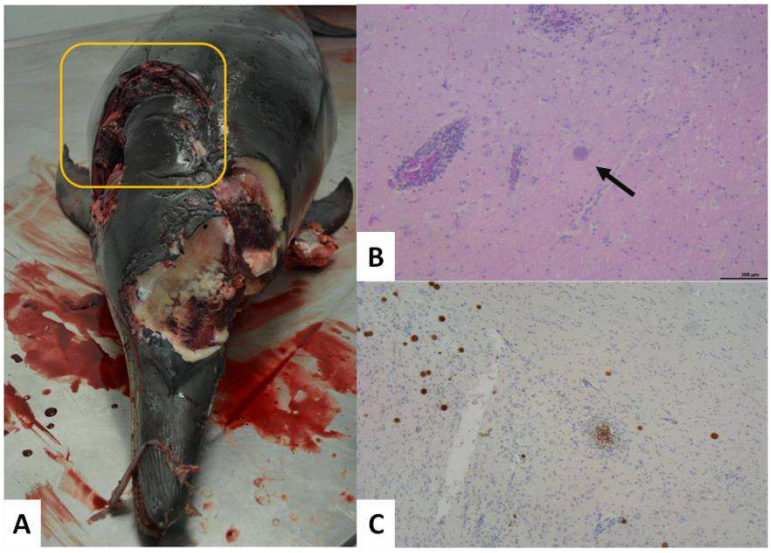

Figure 1. A) Striped dolphin ID 50099/2018 with a skull injury due to the impact with a propeller (yellow box). B) Occipital cortex with a *T. gondii* cyst (arrow), HE 20x. C) Several *T. gondii* cysts, IHC 10x.

Other samples were positive for *Dolphin Morbillivirus* that, even if it is not a pathogen of terrestrial origin, is an important infectious agent for cetaceans that should always be included in the diagnostic activity to identify the cause of death (cf. Figure 2)

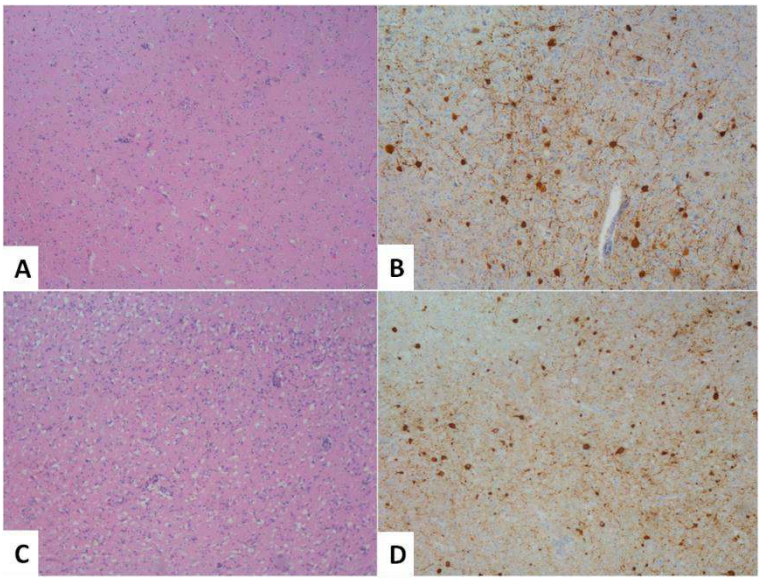

Figure 2. A) Frontal cortex of ID 619/2019, HE 10x. B) IHC for DMV on frontal cortex of ID 619/2019, 10x. C) Occipital cortex of ID 21724/2019, HE 10x.D) IHC for DMV on occipital cortex of ID 21724/2019, 10x.

Other individuals presented severe non-suppurative meningoencephalitis and perivascular cuffing of mononuclear cells (cf. Figures 3 and 4) indicating an infectious process. Unfortunately, it was not possible to identify the etiologic agents nor the hypothetical cause of death.

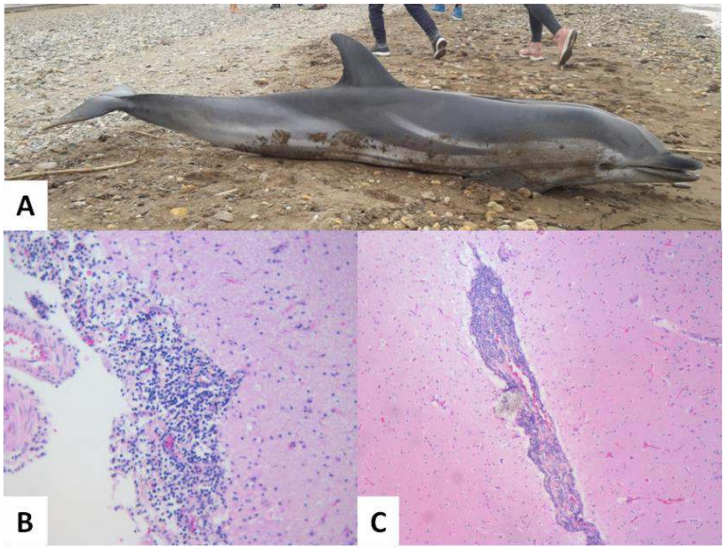

Figure 3. A) Striped dolphin ID SCGC01_87186/2019/1. B) Severe non-suppurative meningoencephalitis, 20x. C) perivascular cuffing of mononuclear cells, 10x.

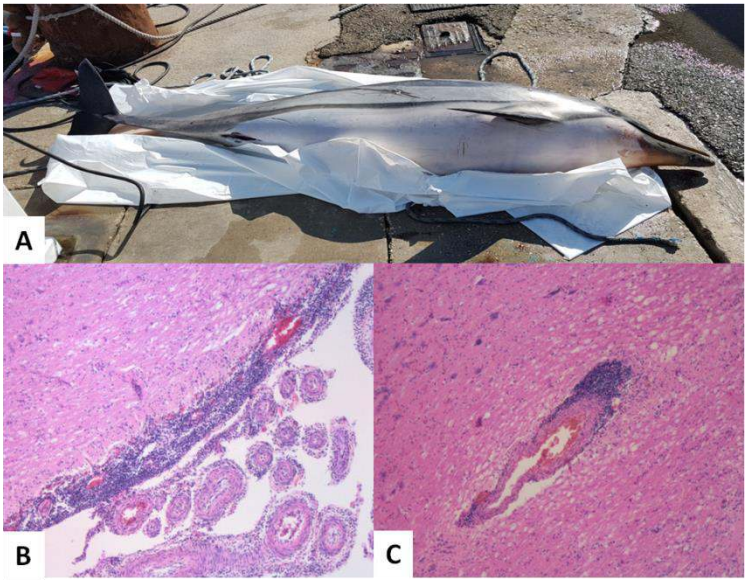

Figure 4. A) Striped dolphin ID SCGC03_87186/2019/3. B) Severe non-suppurative meningoencephalitis with presence of rare neutrophils, 10x. C) perivascular cuffing of mononuclear cells, 10x.

Activity 1.7: Toxicological analysis

Toxicological investigations were conducted on 32 animals (some received beginning of year 2020).
Table 7 displays all toxicological results which could be obtained on the stranded dolphins. Among the interesting results, it should be mentioned that all recent pesticides (including OP insecticides, pyrethroids, and a series of 160 different fungicides and herbicides) were not detected in the liver or blubber samples analyzed. Among pesticides, only DDE (the ultimate metabolite of DDT) was detected in high concentrations in blubber samples. The lack of DDT residues is consistent with the absence of recent use of DDT in the environment. On the contrary, high concentrations of Hg and PCBs were detected. Lead and Cadmium were at very low concentrations (close to or below the limit of quantification).

Table 7: Results of toxicological investigations.

ID	Species	Country	Hg (µg/g ww)	Pb (µg/g ww)	∑DDT (µg/g)		∑OC (µg/g)	OP	Pyre	Pesticide (µg/g)	PCBs (µg/g)
			liver		blubber	liver	blubber	liver	liver	**liver**	blubber
IDVETAGRO_ 75722/18	*Sc*	*It*	6.23	<1	0.15	<0.01	0.29	<0.1	<0.01	<0.01	
IDVETAGRO_ 87558/18	*Sc*	*It*	9.96	<1	1.30	<0.01	1.46	<0.1	<0.01	<0.01	
IDVETAGRO_ 619/19	*Sc*	*It*	>10	<1	2.49	0.07	3.09	<0.1	<0.01	<0.01	
IDVETAGRO_ 4561/19	*Sc*	*It*	>10	<1	0.01	<0.01	0.04	<0.1	<0.01	<0.01	1.32
IDVETAGRO_ 18013/19	*Tt*	*It*	0.98	<1	0.55	<0.01	2.38	<0.1	<0.01	<0.01	
IDVETAGRO_ 21724/19	*Sc*	*It*	>10	<1	0.36	<0.01	0.56	<0.1	<0.01	<0.01	11.23
IDVETAGRO_ 33212/19	*Sc*	*It*	3.5	<1	0.79	<0.01	0.99	<0.1	<0.01	<0.01	45.69
IDVETAGRO_ 42472/19	*Tt*	*It*	>10	<1	1.24	<0.01	2.04	<0.1	<0.01	<0.01	
IDVETAGRO_ 44599/19	*Tt*	*It*	>10	<1	0.45	<0.01	0.56	<0.1	<0.01	<0.01	
IDVETAGRO_ 59260/19	*Tt*	*It*	>10	<1	0.11	<0.01	0.16	<0.1	<0.01	<0.01	84.2
IDVETAGRO_ 62877/19	*Sc*	*It*	>10	<1	0.02	<0.01	0.06	<0.1	<0.01	<0.01	?
IDVETAGRO_ 63558/19	*Sc*	*It*	5.02	<1	0.16	<0.01	0.22	<0.1	<0.01	<0.01	
IDVETAGRO_ 81694/19	*Sc*	*It*	9.96	<1	0.44	<0.01	0.63	<0.1	<0.01	<0.01	
IDVETAGRO_ 89879/19	*Gm*	*It*	>10	<1	0.66	<0.01	1.22	<0.1	<0.01	<0.01	31.81
IDVETAGRO_ 11557/20	*Sc*	*It*	>10	<1	0.21	<0.01	0.27	<0.1	<0.01	<0.01	30.83
IDVETAGRO_ P2017 5048	*Sc*	*Fr*	7.34	<1	0.89	<0.01	1.06	<0.1	<0.01	<0.01	16.59
IDVETAGRO_ P2018 75	*Sc*	*Fr*	10.56	<1	1.53	<0.01	1.73	<0.1	<0.01	<0.01	56.55
IDVETAGRO_ P2018 74	*Sc*	*Fr*	>10	<1	4.84	0.26	6.39	<0.1	<0.01	<0.01	42.11
IDVETAGRO_ P2018 1843	*Sc*	*Fr*	5.99	<1	0.53	<0.01	0.71	<0.1	<0.01	<0.01	23.33
IDVETAGRO_ SCGC06	*Sc*	*Fr*	>10	<1	3.31	<0.01	4.49	<0.1	<0.01	<0.01	
IDVETAGRO_ P2018 3704	*Sc*	*Fr*	4.66	<1	0.25	<0.01	0.32	<0.1	<0.01	<0.01	
IDVETAGRO_ SCGC05	*Sc*	*Fr*	>10	<1	0.34	<0.01	0.40	<0.1	<0.01	<0.01	
IDVETAGRO_ SCGC01	*Sc*	*Fr*	31.85	<1	4.27	0.14	5.01	<0.1	<0.01	<0.01	62.23
IDVETAGRO_ SCGC12	*Tt*	*Fr*	>10	<1	0.08	<0.01	0.31	<0.1	<0.01	<0.01	
IDVETAGRO_ SCGC11	*Sc*	*Fr*	>10	<1	0.07	<0.01	0.11	<0.1	<0.01	<0.01	16.64
IDVETAGRO_ SCGC08	*Sc*	*Fr*	>10	<1	0.16	<0.01	0.24	<0.1	<0.01	<0.01	
IDVETAGRO_ SCGC02	*Sc*	*Fr*	5.48	<1	0.16	<0.01	0.21	<0.1	<0.01	<0.01	
IDVETAGRO_ SCGC03	*Sc*	*Fr*	>10	<1	0.27	<0.01	0.61	<0.1	<0.01	<0.01	16.39
IDVETAGRO_ SCGC09	*Sc*	*Fr*	5.03	<1	0.43	<0.01	0.54	<0.1	<0.01	<0.01	
IDVETAGRO_ SCGC10	*Sc*	*Fr*	6.72	<1	2.24	<0.01	3.46	<0.1	<0.01	<0.01	
IDVETAGRO_ SCGC04	*Sc*	*Fr*	>10	<1	0.44	<0.01	0.68	<0.1	<0.01	<0.01	13.53
IDVETAGRO_ SCGC13	*Sc*	*Fr*	>10	<1	0.42	<0.01	0.68	<0.1	<0.01	<0.01	29.36

Objective 2: Developing a transboundary health monitoring network on cetaceans stranded in the Pelagos Sanctuary

Activity 2.1: Identifying and prioritising the biological and chemical indicators in cetaceans:

Chemical pollution

Among the numerous toxicants that cetaceans can be exposed to, we selected only a few as general markers of human activity and contamination of the environment. The presence of high concentrations of DDE, but mostly Hg and PCBs is consistent with earlier reports on cetaceans from the Mediterranean showing that some contaminants are declining as a result of decreased use or ban (DDT, PBDEs). The ratio DDT/DDE is generally around 0.2, suggesting ancient contamination and lack of recent use of DDT around the Pelagos sanctuary. It is also generally agreed that PCBs are markers of human activity and they are still detected in all animals at very high concentrations, despite the total ban and the removal of PCB-filled electric appliances. PCBs are still very high and comparable to data obtained in 2013 in the same area (Fossi et al., 2013). Despite ban on production, these compounds are still around in electric material. Their high persistence combined with limited metabolism capacity are responsible for their prolonged persistence in the blubber or liver of dolphins. All individuals have PCB concentrations above the threshold of concern (ca 9000 ng/g lipid weight). Both PBT compounds should still be monitored as anthropogenic pollutants and endocrine disrupting chemicals. When considering the high concentrations of potential ED chemicals, it is also recommended to investigate biological markers of ED.

One original part of the contaminant screening was to focus on some strong markers of human presence and activity: pesticides. There is published (although limited) evidence of pesticide contamination of the oceans as well as of cetaceans (Brumowsky et al., 2017, Salvat et al., 2016). Focusing on these contaminants in the Pelagos sanctuary was indeed an interesting case-study: the sanctuary is surrounded by and close to coastal areas with agricultural activity. In such a situation, the stranded dolphins could be suspected to be exposed to the surrounding waters and (potentially) contaminated food. The absence of any residue of all the pesticides tested is, in that sense, a good news. It may reflect a lack of exposure or a rapid degradation / disappearance from the body. Our sampling protocol on stranded animals cannot help distinguishing between these two hypotheses. Low / undetectable concentrations of pesticides in liver/blubber of dolphins consistent with low environmental concentrations and low persistence (Romero et al., 2018), although there is recent evidence of contamination of Striped dolphins with pyrethroid insecticides, which were not detected in this study. It is possible that the limit of quantification was to high to detect all individuals but the study published by Aznar-Alemany et al. (2017) also revealed higher concentrations in some individuals (up to several thousands µg/kg which should have been detected). The limited sample size may bias the results, but it may also be that the Striped and Bottlenose dolphins stranded in the Pelagos sanctuary were less exposed / contaminated than their counterparts living in other parts of the Mediterranean.

Trace elements are also very interesting indicators. It is generally agreed that mercury is an indicator of human activity and of combustion (often associated with the burning of fossile fuels, coal, oil…). The oldest and most important Hg mine is located in Spain (closed in 2011 in Almaden, Spain) and this particular source may also increase the contamination levels of dolphins in the Mediterranean see. Many papers have described the high concentrations of Hg in top predators in the marine food web as a result of accumulation of methyl mercury. The concentrations detected in this project are lower than those reported in the same species in the same area 20 years ago (Monaci et al., 1998), which is also an interesting and positive news, considering all the toxic effects of Hg in mammals. Pb and Cd are mostly terrestrial

contaminants, which do not appear to represent strong threats for marine mammals. It does not seem necessary to monitor them on a regular basis.

Due to funding limitations and technical constraints, it was not possible to investigate newer compounds such as OP flame retardants and plasticizers. A recent study in the Mediterranean area showed that these flame retardants were present in all of 7 stranded dolphins analyzed. The highest concentrations were detected in the blubber, followed by the brain. It has been suggested (Sala et al., 2019) that these OPFR may be indicators of plastic contamination in dolphins. We strongly encourage to support the development of techniques to monitor these contaminants in stranded dolphins as part of the contaminant pack and up-to-date indicator of anthropogenic contamination.

Table 8 List of indicators of cetaceans' toxicological contamination

Indicators	**Organ**	**Relevance**	**Origin of contamination**
PCBs	Blubber	ED	Foodweb
DDT	Blubber	ED	Foodweb
Hg	Liver	Neurotoxicant	Foodweb
Others*	Liver	Exposure	Plastics

**including plastics and associated contaminants (OPFR)*

Biological pollution

By taking into account the state of the art and the experience of veterinary pathologists and toxicologists from both countries, the project allowed to defining indicators for monitoring the biological and toxicological contamination of cetaceans in the Pelagos Sanctuary. Recommendations are made on how they should be used overtime in the framework of a sustainable surveillance program (table 9). Indeed, the results obtained in this study should be considered as preliminary baseline values to be consolidated over time in order to identify some trends, provide early warning of emerging threats and, where appropriate, to guide policy makers and help them to apply and evaluate appropriate interventions.

Among the biological indicators which were identified, *T. gondii* was detected from 7/37 stranded animals. This pathogen is known to play a pathogenic role causing systemic infections and related neurological alterations as we could observe in one case, which presented *T. gondii* neurologic invasion that may have led to neurological alterations and secondary traumatic event.

Other biological contaminants such as *Salmonella* spp., *Erysipelothrix rhusopathiae* were not detected while for *Listeria* spp. some species were isolated from 2 individuals: *L. welshimeri* and *L. innocua*. These species are generally considered as low-pathogenic, but some case reports described fatal effects due to *L. innocu*a infection (Perrin *et al.*, 2003; Moura *et al.*, 2019). An interesting result is also the concomitant infection by *Listeria* spp. and other pathogens, such as dolphin morbillivirus (DMV) and *T. gondii*, since it is known that DMV can induce profound immunosuppression (Van Bressem *et al.*, 2014).

Antimicrobial resistance can also be viewed as a direct consequence of the contamination of the aquatic environment by pollution from land-based sources, and particularly anthropogenic activities. Our study on isolates from cetaceans showed resistance to certain antibiotics for which resistance is also very frequently observed in humans and animals (Bourély *et al.*, 2020), with lesser proportions in cetaceans for amoxicillin but very high resistance rate for tetracycline. Those data should however be consolidated, as they were obtained from a limited number of isolates due to methodological issues which should be solved in the future.

Together with data obtained from the examination of stranded animals in the frame of national stranding networks, those indicators will also allow to better identify the causes of death of stranded cetaceans. The most important cause of death for cetaceans in this study was infectious diseases, particularly due to the Dolphin Morbillivirus (DMV) which was detected in 9/37 stranded animals, although it is not considered as of terrestrial origin, but was included in the list of pathogens to be tested considering its high consequences on the health of cetacean populations. On this aspect, we can observe that although samples were taken in the same way in the two countries to meet the objectives of the project, the investigations carried out in France did not allow to determine in all cases the precise cause of the death of the animals. Indeed, the current organization of the French stranding network does not provide for systematic laboratory necropsy to carry out all the laboratory tests necessary to precisely determine the cause of death. The French system is however effective for collecting samples in the field, in the context of studies such as ours.

Table 9 List of indicators of cetaceans' biological contamination from terrestrial origin

Indicators	**Relevance**	**Protocol**	**Origin of contamination**
T. gondii	Protozoan globally distributed, allow for geographic comparison?	PCR and HE IHC analysis (CNS, heart, muscle)	Terrestrial. *T. gondii* cycle occurs in felids which excrete the oocysts. The oocysts became infectious in the environment and are very resistant to several conditions Animals infected with *T. gondii* oocysts can develop neurological signs, abortion, …
Listeria spp	Ubiquitous bacteria whith health consequences on cetaceans. Some species are zoonotic	Bacterial culture of brain after selective enrichment	*Listeria* has a worldwide distribution, but more frequently in temperate and colder climates, and has been isolated from soil, silage, sewage effluent, stream water, and over 50 species of animals, including ruminants, swine, horses, dogs, cats, and various species of birds. In some areas, up to 70% of humans are reported to be asymptomatic fecal carriers;
E. rhusiopathiae	Zoonotic bacteria	Bacterial culture of lesions, spleen and lymph nodes	*E. rhusiopathiae* is widely distributed in nature and is often recovered from sewage effluent, abattoirs, surface slime of fresh and saltwater fish, and soil. It has been recovered from over 50 species of mammals including swine, sheep, lambs, cattle, horses, dogs, mice, and rabbits and 30 species of wild birds such as turkeys, chickens, geese, pheasants, and pigeons. *E. rhusiopathiae* can be isolated from the tonsils and gastrointestinal tracts of apparently healthy pigs, considered the most prominent reservoir
Salmonella spp	Zoonotic bacterial species with consequences in human and animal health	Bacterial culture after selective enrichment of liver and intestine	The reservoir for members of the genus Salmonella is the gastrointestinal tract of warm- and cold-blooded animals. Sources of infection include contaminated soil, vegetation, water, and the feces of infected individuals. Birds, lizards and snakes are commonly infected with several serotypes.
Antimicrobial resistance in *E. coli* (used as a bacterial indicator)	Antimicrobial resistance in E. is an indicator of terrestrial pollution	Bacterial culture of small tract of intestine	*E. coli* is a commensal of animals and humans and can be isolated from soil, silage, sewage effluent, stream water.

Activity 2.2: Routinely exchange of information among the partners

Frequent communication among partners was assured through e-mail exchange and skype calls. Project meetings were held on May 30th 2018, June 29th 2018, December 18th 2018, May 10th 2019, as described in the mid-term report.
In the second half of the project, a meeting among GIS3M, GECEM, VETAGRO and CReDiMa occurred on October 4th 2019 during the workshop held at le Pole nautique de La londe-les-maures.
At the World Marine Mammal Conference in Barcelona on December 12th 2019, GIS3M, GECEM and CReDiMa had an informal briefing about the activities related to communication and dissemination to public. A skype call among all partners was conducted on January 30th 2020. On February 19th 2020 the second meeting among partners and stakeholders was the occasion to share updates about the project development. During a skype meeting held on May 6th 2020, the results were shared among partners. Thanks to the agreement extension, other virtual meetings were performed in July 13th and August 5th 2020 in order to finalize the report and the information material.

Activity 2.3: Sharing the results of the analyses conducted

- Results of investigations on Salmonella infection in Italy were published in a peer reviewed paper: Grattarola et al. (2019). First report of Salmonella 1, 4, [5], 12: i:-in free-ranging striped dolphins (*Stenella coeruleoalba*), Italy. Scientific reports, 9(1), 6061. (Appendix 5).

- Presentations at the **CONVEGNO NAZIONALE CReDiMa "Rischi emergenti per la salute dei cetacei in Italia"** Genova**,** April 5th 2018 (Appendix 6)
  - "La rete degli IZS sugli spiaggiamenti: successi e sfide future". Cristina Casalone, CReDiMa
  - "Cetacean strandings management and valorization in french Pelagos waters". Hélène Labach, GIS3M
  - "Patogeni emergenti in cetacei spiaggiati in Italia: attività diagnostica degli IZS nel 2017". Carla Grattarola, CreDiMa;

- Oral presentation at the 50° Congresso Della Società Italiana Di Biologia Marina - Livorno, June 10th-14th 2019: CETACEI SPIAGGIATI IN LIGURIA E CONTAMINAZIONE DA PATOGENI EMERGENTI E INQUINANTI IMMUNOTOSSICI: UNA MINACCIA PER IL SANTUARIO PELAGOS. C. Grattarola, M. Ballardini, A. Pautasso, B. Iulini, K. Varello, E. Bozzetta, F. Giorda, E. Berio, S. Gallina, A. Roman, M. Goria, S. Peletto, L. Masoero, L. Serracca, A. Dondo, S. Zoppi, F. Garibaldi, F.E. Scaglione, C. Di Francesco, L. Marsili, G. Garofolo, E. Ramon, G. Di Guardo, W. Mignone, C. Casalone. (Appendix 6).

- Oral presentations at the Workshop: Cetaceans contamination in the Pelagos Sanctuary- La Londe-les-Maures, October 4th 2019:
  - "Introduction and brief presentation of the project "Biological and toxicological contamination of cetaceans in the Pelagos Sanctuary: assessment, origin, monitoring and mitigation". Hélène Labach, GIS3M (Appendix 7)
  - "State-of-the-art on chemical and biological pollution affecting cetaceans in the Pelagos Sanctuary". Julie Jourdan, GECEM (Appendix 8)
  - "Results of diagnostic activities on stranded cetaceans, Ligurian coastline, 2017-2019". Carla Grattarola, CReDiMa (Appendix 9)
  - "Cetacean's chemical contamination in the Pelagos Sanctuary: issues and methodology". Philippe Berny, Vetagro Sup.

- Oral presentation at the WORKSHOP NAZIONALE CReDiMa 2019 "Esperienze, diagnosi e ricerca sui cetacei spiaggiati" Palermo, October 17th -18th 2019(Appendix 9). "Update sull'attività diagnostica degli IZS sui cetacei spiaggiati in italia (2018-2019)". Carla Grattarola, CReDiMa

- Poster at the World Marine Mammal Conference 2019. Barcelona. December 8th -12th 2019: EMERGING PATHOGENS AND IMMUNOTOXICPOLLUTANTS IN CETACEANS STRANDED ALONG THE COASTS OF LIGURIA, PELAGOS SANCTUARY, ITALY. E. Berio, M. Ballardin, A. Pautasso, C. Grattarola, B. Iulini, K. Varello, E. Bozzetta, F. Giorda, S. Gallina, A. Romano, M. Goria, S. Peletto, L. Masoero, L. Serracca, A. Dondo, S. Zoppi, F. Garibaldi, F.E. Scaglione, C. E. Di Francesco, G. Consales, G. Garofolo, E. Ramon, G. Di Guardo, L. Marsili, W. Mignone, C. Casalone. (Appendix 10)

- Oral presentations at the 2° Workshop: Cetaceans contamination in the Pelagos Sanctuary- Diano Marina, February 19th 2020 (Appendix 11):
  - "Introduction and presentation of the project: Biological and toxicological contamination of cetaceans in the Pelagos Sanctuary: assessment, origin, monitoring and mitigation". Hélène Labach, GIS3M
  - "Feedback on the 1st workshop (October 4th, 2019)". Léa Juret, GIS3M
  - "Contamination on the Italian coastline of Pelagos Sanctuary and stranding events of cetaceans during the project". Cristina Casalone, CReDiMa
  - "Biological and chemical contamination of cetaceans in the Pelagos Sanctuary - a review". Julie Jourdan, GECEM
  - "Sampling protocols for cetacean strandings: panel for microbiological, biomolecular and histological analyses". Carla Grattarola, CReDiMa
  - "Results of diagnostic activities in stranded cetaceans from Italian coast line of Pelagos Sanctuary". Enrica Berio, CReDiMa

- Poster at the XVIII Congresso Nazionale S.I.Di.L.V. Perugia, 7 - 9 Novembre 2018: Salmonella 1,4,[5],12:i: in 3 stenelle striate (Stenella coeruleoalba) spiaggiate in Italia. Mammiferi marini nuovi potenziali reservoir di Salmonella. C. Grattarola, A. Pautasso, S. Gallina, A. Romano, F. Giorda, M. Ballardini, B. Iulini, K Varello, E. Bozzetta, M. Goria, S. Peletto, L. Masoero, L. Serracca, A. Dondo, S. Zoppi, F. Garibaldi, F. E. Scaglione, L. Marsili,G. Di Guardo, C. Esmeralda Di Francesco,E. Ramon, W. Mignone, C. Casalone. (Appendix 12)
- OIE WORKSHOP Tblisi September 18th 2018. APPLICATION AS A CONSORTIUM FOR DESIGNATION AS OIE COLLABORATING CENTRE ON MARINE MAMMAL HEALTH. Poster Marine mammal strandings management in Italy C. Casalone (Appendix 13).

Activity 2.4: Organization of workshops involving partners and external stakeholders and experts.

The meeting "Workshop Cetaceans contamination in the Pelagos sanctuary" held in October 2019 and organized by GIS3M saw the intervention of Hélène Labach (GIS3M), Julie Jourdan (GECEM), Grattarola Carla (CReDiMa), Denis Ody (WWF France), Philippe Berny (Vetagro Sup), Maria Cristina Fossi (University of Siena), Mendez-Fernandez Paula and Caurant Florence (Observatory PELAGIS - UMS 3462, University of La Rochelle / CNRS) (cf. report in Appendix 14)

The second meeting was organized by CReDiMa on February 19th 2020 and was conducted with the interventions of Hélène Labach and Léa Juret (GIS3M), Letizia Marsili (Università di Siena), Julie Jourdan (GECEM), Alessandra Pautasso (ASL1 Imperiese), Cristina Casalone, Carla Grattarola and Enrica Berio

(CReDiMa) and Sabina Airoldi (Tethys Research Institute) (Appendix 12). Several stakeholders involved in the stranding network and in marine mammal research in Liguria Region participated to the meeting, such as Capitanerie di Porto, Carabinieri CITES, Menkab, Costabalenae coop., MarLab and others.

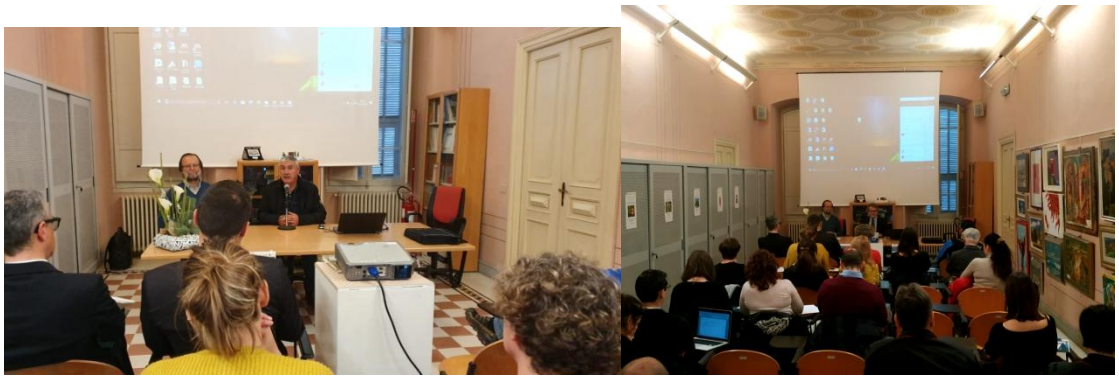

Figure 5. Second Workshop "Cetaceans contamination in the Pelagos sanctuary" held in Diano Marina (IT) on February 19th 2020.

Objective 3: Promoting the mitigation of biological and toxicological contamination in the Pelagos Sanctuary area

Activity 3.1: Realization of an itinerant exhibition on the impact, origin and mitigation of land-based marine pollution on cetaceans in the Pelagos Sanctuary.

GIS3M designed and produced, with the collaboration of CReDiMa, the set of panels to be used for the public events. The panels were presented at the meeting in Diano Marina (IT) on February 19th 2020 (Italian version: Appendixes 15-17; French version: Appendixes 18-20). The panels will be given to the Pelagos Sanctuary secretariat.

Activity 3.2: Organization of public conferences and exhibition in Pelagos ambassador cities

The first public event was held in France on 30/11/2019 in Nice. The remaining events were planned in the last months of the project, as previewed in the timetable of the project. In particular the plan was: 01/03/2020 Diano Castello (IT), 16/03/2020 Sanremo (IT), 20/03/2020 Monaco, 24/03/2020 Genova (IT), 27/03/2020 Saint Jean –Cap Ferrat (FR), 08/06/2020 Cannes (FR).

Unfortunately, the public events were cancelled because of the restriction of all public activities announced by the authorities in order to restrain the pandemic COVID-19 outbreak.

The partners in agreement with the Permanent Secretariat, decide to produce some educational videos about the main results and recommendations obtained during the project. Those videos are going to be shared by Pelagos on its website and social networks (Appendix 21-24), the modalities of the diffusion are presented in Appendix 25.

Activity 3.3: Presentation of project's results and elaboration of recommendations to Pelagos CST.

Cf. Part 5 achievements/results

Activity 3.4: Elaboration of recommendations to local authorities of the Pelagos ambassador cities

The research project "Biological and toxicological contamination of cetaceans in the Pelagos Sanctuary: assessment, origin, monitoring and mitigation" funded by the Permanent Secretariat of the Pelagos Agreement, held during 2018-2020, lead to some main conclusions about biological and chemical hazards in the Pelagos Sanctuary that may affect cetacean health and conservation.

BIOLOGICAL HAZARDS

The indicators identified in the Pelagos Sanctuary area among the biological hazards are represented by *Toxoplasma gondii, Listeria* spp., *Salmonella* spp*., Escherichia coli*, *Erisipelothyrix rhusiopathiae*. In cetaceans, *T. gondii* is responsible for neurological alterations that may lead to strand or collisions, the other pathogens are responsible for sepsis or systemic infections in the animal and represent a potential risk for humans.

The sources of these biological agents are summarized below:

Sewage: it is a major source of pathogenic microorganisms and it must be treated with high quality procedures. Unfortunately, not treated sewage can reach rivers and sea by several ways:

- Poor and old sewage systems that allow leakage of highly contaminated wastewater
- Accidental damage of the sewage system
- Inadequate sewage treatment plant
- Improper sewage treatment
- Heavy rain period or floods that flush untreated sewage into the environment

Rain off and floods: they carry the microorganisms from soil, overload sewers, dumps, street surface and rainwater collectors bringing into the coastal environment human and animal pathogens.

Rivers: rivers often carry high loads of pathogenic microorganisms due to high impact by run off, sewage and groundwater. Moreover, they can flush pathogenic microorganisms belonging to wild fauna.

Groundwater: Major contaminators of groundwater are households (with private sewers), livestock, and leaking sewage pipes.

Stray cats: not least it is to be considered the transport of *T. gondii* oocysts from sandboxes, litter boxes and gardening plots. A single infected cat can shed millions of oocysts within 1 week and oocysts can persist in the environment longer than 1 year. Flood-related natural events could drive the transmission of *T. gondii* especially in estuarine environments.

Ballast water: ballast water is essential for modern shipping operations, but it constitutes a serious ecological problem due to the transport of water and marine life across the seas. Indeed, ballast water includes bacteria, microbes, small invertebrates, eggs, cysts and larvae of various species that could be potentially dangerous for health and ecosystem where they are invasive. All ships must have a ballast water management plan that must include mechanical, physical, chemical, and biological processes, either singularly or in combination (i.e. UV treatment, ozone, sodium hypochlorite, inert gas) in order to prevent and minimize the transfer of harmful aquatic organism and pathogens.

MITIGATION FOR BIOLOGICAL POLLUTION

Several measures can be put in place in order to reduce the terrestrial pollution of the coastal environment:

- **Hygienic treatment of wastewater: restore** and **maintenance** of sewage system are **pre-requisite** to reduce the microbial pollution land derived. Detection of untreated wastewater and maintenance are fundamental to avoid the occurrence of accidents that frequently occurs during the touristic season and to avoid important leakage events.
- **Ballast water treatment: monitor and commit** shipping operators regarding a correct ballast water management. Particularly important for maritime authorities of cities with high naval traffic such as Vado, Genoa, La Spezia, Livorno, …
- **Restore the landscape** is an effective measure to reduce the transport of microorganisms, in particular protozoa (i.e. T. gondii), form land to sea.
- **Reforestation** and **renaturation** of rivers and wetlands improve the "filtering capacity" of the landscape, reduce erosion and the destructive potential of the floods. Those measures are useful not only for coastal and rural areas but also for the urban environment. Indeed, the reduction of concrete and asphalt surfaces in cities and the placement of green areas, like vegetated roof and gardens, will reduce the amount of stormwater and thus the load of microorganisms transported by run off. Moreover, this kind of intervention reduce the runoff of nutrients counteracting the eutrophication of fresh and marine waters that is highly related with microorganism's survival.
- **On-site treatment** of sewage: it means to treat the sewage as close as possible to the source and avoid the transport of untreated waters carrying microorganisms. Efficient procedures to reduce the load of pathogens in human and animal waste in a short period of time (one to a few days) are those that include a step with heating of the waste, such as composting (>60°C, due to microbial heating) and the thermophilic production of biogas by methanogenesis (> 50°C).
- **Preserve seagrass meadows**: they have been described to significantly reduce bacterial loads in coastal environment.
- **Stray cat management**: since cats are the definitive host of T. gondii and are responsible for the excretion of the oocysts that can induce toxoplasmosis in human and cetanceans, it is important to manage the stray cat population. Feline colony monitoring and population control through sterilization are the major activities to be done.

CHEMICAL HAZARDS

The indicators identified in the Pelagos Sanctuary area among the chemical hazards are represented by mercury, PCBs and plastic-associated contaminants. Chemical contamination is detrimental for cetacean health since it can induce negative effects on the immune and reproductive systems.

The sources of these chemical agents are summarized below:

Mercury: human activity is the main cause of mercury releases, particularly coal-fired power stations, residential coal burning for heating and cooking, industrial processes, waste incinerators and as a result of mining for mercury, gold and other metals.

PCBs: PCBs were included in the manufacturing process of many materials and incorporated into products during the recent past. They can be present in building caulking, window glazing, waste oil tanks, hydraulic equipment, oil-based paints, fluorescent light ballasts, thermal insulation, adhesives, large compressors, asphalt roofing materials, floor finishing, pesticides, printing inks, and wood treatment chemicals.

Plastic-associated contaminants: these contaminants originate from the terrestrial environment. Plastics are used in bags, industry and household products and in a large variety of products, thanks to their physical and chemical characteristics. Recent studies about cetaceans in the Mediterranean region suggest a high prevalence of contamination with organophosphate flame retardants.

MITIGATION FOR CHEMICAL POLLUTION

Several measures can be put in place in order to reduce the chemical pollution of the coastal environment:

Mercury: promote the use of clean energy sources (burning coal for power and heat is a major source of mercury); eliminate mercury mining and the use of mercury in gold extraction and other industrial processes;

guarantee a correct and safe disposal of mercury-containing products such as batteries, measuring devices (i.e. thermometers and barometers), electric switches and relays in equipment, lamps (including some types of light bulbs), dental amalgam (for dental fillings), skin-lightening products and other cosmetics, pharmaceuticals.

PCBs: several years ago, the EU started a longterm plan to counteract against PCBs pollution by banning their use in several industrial activities. Nowadays are still lacking regulation and references about soil and water contamination.

- Comply with European and national regulation
- Facilitate old building (private and public) renovation

Plastic-associated contaminants: Recent ban of disposable plastic bags and tableware is an important measure and more emphasis should be placed on the control of plastic wastes and recycling in order to reduce the amount of plastics released in the environment.

- Reduce use of disposable plastics
- Improve recycling and wastes collection

3. DIFFICULTIES ENCOUNTERED AND MEASURES TAKEN TO OVERCOME PROBLEMS

During the first half of the project, some difficulties were encountered about the shipping of biological samples across the Italian-French border. In order to overcome this problem cumulative expeditions were performed.

In the second half of the project similar problem about sample delivery occurred about formalin-fixed brains to be transported across the French-Italian border. The partners involved decided to transport the samples by themselves in the occasion of meetings or by planning appointments at IZSPLV in Imperia.

In CReDiMa 3 persons of the staff working on this project left the organization and one new veterinarian was in charged to pursue the objectives of the project.

During 2018-2019 only few stranding events occurred on the French side of the Pelagos Sanctuary, making difficult the collection of the previewed number of samples and consequently slowing down the analysis. For these reasons, an extension of 3 months has been allowed in order to collect more samples and get the results.

Another important issue was the occurrence of the pandemic outbreak of COVID-19 that obligated the partners to cancel the public events that were planned during March 2020 and determined the shutdown of the Vetagro Sup (ENVL) laboratories for 8 weeks. The partners for asked for a second 3 month extension and proposed to realize videos (one long and one short) in order to allow public awareness on the issue, despite the cancellation of the public events.

4. CHANGES INTRODUCED IN THE IMPLEMENTATION

In order to achieve a final diagnosis of the cause of death, the analysis for DMV was added to the protocol, by molecular biology and/or by immunohistochemical studies.

E. coli sampling protocol: during the project a negative effect of frozen storage of faecal samples was observed on *E. coli* viability. Then, refrigeration was recommended followed by a quick transport to the laboratory for analysis. Unfortunately, the field conditions or the difficulties of a prompt transport made this analysis challenging.

Public events were converted into educational videos for media and social networks. During the videos, partners describe their role in the project and the main obtained results.

5. ACHIEVEMENTS/RESULTS

Conclusion

The present study highlights the importance of the existence of a scientific network among neighboring countries and the need of strengthening the relationship among partners.

The number of collected samples was sufficient to screen the principal biological and chemical hazards that affect cetacean health. It is not surprising that “old contaminants” like PCBs are still present in the studied animals and that *T. gondii* is widely diffused in the dolphin population. Additionally, some species of Listeria spp. have been identified arising interest about its pathogenicity.

Proposals

Research

- To continue research on cetacean mortality causes in the Sanctuary.
- To pursue the studies on the impact of recent pesticides and biological markers of ED.
- To study the relationship between chemical contamination and infection/parasite occurrence, since pollution may affect immune system functions.
- To organize periodic scientific meetings where scientist and stake holders interested in cetacean biology, health and conservation may share and divulgate their result and improve professional network. The idea is to promote the Pelagos Sanctuary organization as a facilitator for scientific networking and to organize convivial meetings that may became an appointment “not be missed” for the scientific community, with the collaboration with ECS and other organizations related to cetacean studies. In the frame of this hypothetical appointment, organize a sort of “young researcher prize” to encourage the participation of young students or trainees that are approaching the research on cetaceans or a prize for “the best cetacean photo of the year” to be reproduced in postcards/calendars with the Pelagos logo and the name of the photographer. The Pelagos calendar with the best selected photos may be sell online or during meetings and activities in order to fund the “young researchers prize”. With these activities the sanctuary does not

intend to substitute scientific conferences or societies but to create new occasions of join up the cetacean community and celebrate the great natural reserve that the Sanctuary represents.

Monitoring

- To support the long-term monitoring of the indicators listed in this study
- To promote the systematic necropsies in France to improve the identification of the cause of death
- To promote the design and use of standardized protocols for samples collection and analysis on terrestrial pathogens and chemicals contaminants.

Those protocols should be however improved for further studies, especially on two aspects:

  - Samples for bacteriology testing should not be frozen in order to maximize the recovery rate of bacteria
  - Discussions should happen on the way to investigate inhibition of PCR reactions by degradation products of organic matter in samples from stranded animals which are sometimes in a poor state of conservation

- To lead a group of expert on the contamination issue
- To encourage scientists working on the impact of contaminants on cetaceans to share their results to publish regular reports and scientific papers together

Mitigation

- To promote the diffusion and implementation of the recommendations to the local authorities
- To push the local administration to promote sustainable tourism and environment restoration and requalification, particularly against land consumption and industrial pollution.
- To value the impact on the cetaceans health in the Pelagos Sanctuary to encourage authorities and professional to improve the mitigation of the release of contaminants, pathogens and waste in the marine environment.

Synthetic explanatory report

During the studying period, few biological pathogens of terrestrial origin were identified from stranded cetaceans on Pelagos coastline.

The most important cause of death for cetaceans on the Italian side of Pelagos, is infectious disease, in particular due to the cetacean pathogen *Dolphin Morbillivirus* (DMV) that was identified in 9/37 cases.

As already described in literature, a frequent pathogen of terrestrial origin is *T. gondii* that was often detected in our study. In particular, 7/37 cases were positive for *T. gondii* in at least one organ.

It is well known that *T. gondii* can play a clear pathogenic role causing systemic infections and related neurological alterations as we could observe in one case, which presented *T. gondii* neurologic invasion that may have led to neurological alterations and secondary traumatic event.

No relevant *Salmonella* serovars were identified and neither minor *Salmonella* strains. Nevertheless, since some carcasses were in a poor conservation status, we cannot exclude the possibility of *Salmonella* spp. contamination in some individuals. As a matter of fact, *Salmonella* Typhimurium infection in cetaceans stranded in Pelagos has already been described in literature (Grattarola *et al.*, 2019) and a general concern for public health arise regarding the increasing of antimicrobial resistance in many *Salmonella* spp. serovars.

Some *Listeria* strains circulate in the Sanctuary. *L. monocytogenes,* the most pathogenic strain, was not identified but other species were isolated from 2 individuals: *L. welshimeri* and *L. innocua*. These species

are generally considered low pathogenic but some case reports described fatal effects due to *L. innocua* infection (Perrin et al., 2003; Moura et al., 2019).
An interesting results is the concomitant infection by *Listeria* spp. and other pathogens, such as DMV and *T. gondii*, since it is know that DMV can induce profound immunosuppression (Van Bressem *et al*., 2014).
Considering the present results and previous literature data, we can also state that among the cetaceans of Pelagos Sanctuary exists the circulation of *Listeria* spp. An intriguing topic for future research would be to investigate the pathogenic potential of these strains in relation with concomitant infections.
In samples stranded in Italy, the most frequent neuropathological lesion was non-suppurative meningoencephalitis due to DMV infection. Moreover, one case of meningoencephalitis due to *T. gondii* was also described confirming the pathogenic role of this parasite. Seven brains collected from individuals stranded along the French coast were also analyzed. Similarly, a non-suppurative meningoencephalitis was a common findings. Unfortunately, it was not possible to identify the etiological agent. Furthermore, for these cases the microbiological investigations carried out on other organs did not provide additional information to identify a hypothetical cause of death.

Our study on isolates from cetaceans also showed resistance to certain antibiotics which can be viewed as a direct consequence of the contamination of the aquatic environment by pollution from land-based sources, and particularly anthropogenic activities. We observed resistance to antibiotics for which resistance is also very frequently observed in humans and animals, especially for tetracycline.

The levels of contamination of stranded dolphins with DDT but mostly PCBs are high. DDT concentrations are decreasing but PCB concentrations are similar to studies conducted in the area and the PCB concentrations measured in the livers are above the threshold of toxicological concern.
Hg residues are extremely high and compatible with neurologic / hematologic disorders.
All other pesticides were below the limit of quantification. We failed to detect any residue of pyrethroids, even though these pesticides have been detected in dolphins stranded in Spain (REF).
The presence of heavy loads of contaminants such as PBT (PCB, Hg) is known to have detrimental effects on the health status of birds and mammals. Reproduction, immunity may be impaired and explain contamination of dolphins with bacterias or parasites.
Because the number of animals is not very high, it is difficult to investigate the relationship between bacterial or parasite contamination and pollutant load. Nevertheless, high concentrations of PCBs, as detected in these animals, have already been associated with decreased immune response in pinnipeds.
Among the pollutants detected, some have ED properties. It is also suggested to monitor contaminants associated with plastics such as OPFR.

Considering the impact on the cetacean's population health and the important role of cetaceans as sentinels of environmental health, we strongly recommend to encourage the systematic monitoring of indicators of pathogens of terrestrial origin, as well as, ancient and recent chemical contaminants and to support authorities in their effort to mitigate contaminants and waste release in the marine environment.

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