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Rapid Communication

## The diatoms test in veterinary medicine: A pilot study on cetaceans and sea turtles

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### ABSTRACT

Fishing activities are considered one of the most relevant threats for cetaceans and sea turtles conservation since these animals are sometimes found dead entangled in fishing gears. Currently, postmortem diagnosis is based mainly on the presence of nets and lines on the body and the related marks and injuries evident at gross examination. A more detailed and objective evidence is needed to clarify doubts cases and the diatoms technique, used in forensic human medicine, could support drowning diagnosis also in this field. Diatoms' investigation was implemented to be applied in marine vertebrate on 8 striped (*Stenella coeruleoalba*) and 1 bottlenose (*Tursiops truncatus*) dolphins and 5 sea turtles (*Caretta caretta*) stranded along the Italian coastlines with a likely cause of death hypothesized on necropsies carried out by veterinary pathologists. Diatoms were microscopically searched in the bone marrow collected from long bones implementing protocols used in human medicine and their presence was observed in 4 cetaceans and 2 sea turtles. Despite a clear relation between diatoms' presence and amount and the likely cause of death was not proved due to the poor number of samples, the higher burden of diatoms was found in 3 animals deemed to be death for the interaction with human activity. Despite more studied are necessary to identify the possible relation between the cause of death and diatoms' findings, the present study implemented this technique to be adapted to marine animals, confirming its possible application also in veterinary forensic medicine.

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### 1. Introduction

The most relevant causes of death of cetaceans are due to natural causes, to environmental changes, or can be caused by anthropogenic activities [1,2,3], including, but not limited to interaction with fishing activities [4,5,6], impact with acoustic sources [7], ship strikes [8,9] and pathological agents and persistent chemical contaminants from domestic, industrial and agricultural wastes [10,11].

Among the possible interactions with fisheries, the incidental capture and entanglement in fishing gears are frequently reported

[12,13], with gill-nets, drift-nets and trawls as the most frequent cause of by-catch for free-ranging odontocetes [13]. In these cases, marine mammals could drown because they cannot reach the surface and breath correctly. Furthermore, some individuals may accidentally ingest a hook, when they depredate catch from longline hooks [14,15,16]. These events may lead to internal injuries, infections, starvation, or even eventual death [17], but some hooked individuals may be unable to reach the surface to breathe, thus leading to a more immediate death by drowning [14]. Moreover, Gomerčić et al. in 2009 reported for the first time that ingestion of gill-net parts leads to larynx strangulation [3]. Similar considerations are reported also for sea turtles [18,19].

In both taxa the interaction with fisheries is generally assessed during external evaluation and/or postmortem examinations, considering direct (i.e. presence of gears and lines,

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cutaneous linear and regular impression and external marks) and indirect (i.e. hemorrhages, fractures, blunt traumas, absence of extremities, pulmonary edema) evidences as well as the presence of fresh food remains in the stomach chambers and the absence of any other predisposing disease ongoing [9]. Scant information are related to the mechanism of death in order to support the final diagnosis according to an evidence-based approach, except for gross and microscopic pulmonary features related to asphyxia [9]. The aim of present study is to investigate the possible application of the research and counting of diatoms used in human forensic medicine to marine vertebrates and consider if drowning could be considered as a possible mechanism of death in these cases.

Diatoms are ubiquitous unicellular, eukaryotic microorganisms living in many different environments, included the marine environment. The hallmark of the diatoms is their cell wall, that is highly differentiated and almost always heavily impregnate with silica ( $\text{SiO}_2 \cdot n\text{H}_2\text{O}$ ); they vary in size from 5 to more than 500  $\mu\text{m}$  [20]. Diatoms, for their features (siliceous envelope) and distribution (specific species are related to specific environment) have been considered one of the more suitable indicator of drowning. They also may be found in moist soil and the atmosphere. The type of diatoms found in a certain location is unique and specific of that area. The season also affects what type is found. Diatoms can enter the body in three different ways: through inhalation of airborne diatoms, ingestion of material containing diatoms, and aspiration of water containing diatoms. This last route is the foundation for forensic diatom testing, which in conjunction with other findings provides a diagnosis of drowning [21,22,23]. Diatoms enter within the airways during water inspiration and they are able to pass in the circulatory system through the alveolar walls. In still alive animals, they are distributed to several tissues following the bloodstream and here diatoms remain intact thanks to their morphological features.

These algae are able to pass the air-blood barrier also in dead animals but they are not disseminated in different tissues through the circulation. In forensic human medicine, drowning diagnosis using diatom-test based on microscopic observation could be reached respecting specific criteria. More in detail a correspondence of diatoms amount, type and morphology between the drowned body and the water were it has been found should be ascertain [24,25] with diatoms numbers in lungs and other organs which are respectively from 10 to 100 and from 100 to 1000 lower than into the water [26]. Among all analyzed tissues, bone marrow has been considered as the most suitable due to the low contamination and influence of external and autolytic factors [24]. Slides obtained from bone marrow usually show less than 10 diatoms with an average size less than 30  $\mu\text{m}$  and an ovoidal or fusiform shape [21].

## 2. Materials and methods

In this preliminary study, bone marrow samples were collected from the humerus of 1 bottlenose dolphin (*Tursiops truncatus*) and 8 striped dolphins (*Stenella coeruleoalba*) since other long bones were not available due the evolutionary absence of rearlimbs. In order to obtain a comparable amount of tissue, both femur and the humerus were collected from 5 loggerhead sea turtles (*Caretta caretta*), due to the little size in these species. The bottlenose dolphin and the loggerhead sea turtles stranded along the Northern Adriatic coastline (Italy) whereas striped dolphins were found stranded along the Northern Tyrrhenian shores (Liguria region, Italy) between November 2014 and March 2017.

During complete postmortem examinations carried out according to the national protocol based on international standards [27,28], long bones were collected from the carcass by using

scalpels. All the sampling and materials were accurately rinsed using fluids (distilled water or ethanol 96%) previously evaluated for the presence of diatoms by centrifugation and subsequent microscopic observation. The bones were fractured to sample bone marrow, according to protocols used in human medicine, which use from 5 to 50  $\text{cm}^3$  of tissue [21,26,29]. In detail, 50  $\text{cm}^3$  were initially obtained according to Pollanen [21] from the first animal (the bottlenose dolphin) in order to assess the methodology, while in the other animals (8 striped dolphins and 5 sea turtles) 15  $\text{cm}^3$  were used according to internal routine procedures.

All the samples were incubated 24 h in a bottle of glass containing 2 volume of 70%  $\text{HNO}_3$  per each volume of sample at the temperature of 50°. Subsequently samples were refrigerated till lipids solidification. The liquid phase was then centrifuged (2500 rpm for 25 min); 100  $\mu\text{l}$  of the obtained sediment was resuspended in 10 ml of distilled water and centrifuged again with the same parameters. 100  $\mu\text{l}$  of the sediment was then put on slides and dried at 50°. Diatoms were counted by microscopic observation at 400 $\times$  magnitude.

## 3. Results

The easiness in sampling and the good quality of the obtained material make the bones a suitable sampling site for this analysis. More in detail, sampling in striped dolphins cannot be carried out according to standard protocols due to the limited volume that could be obtained from their long bones (15  $\text{cm}^3$  in total, consisting of bone tissue and bone marrow): In fact, due to their specific features (i.e. reduced size and absence of the medullary cavity) bones had to be fractured and directly digested to collect properly the full amount of bone marrow.

In Table 1, results of the diatoms-test carried out on the 9 stranded dolphins and 5 sea turtles under examinations have been summarized, grouping examined animals according to species (cetaceans and sea turtles), likely manner of death (natural vs accidental) [23], possible causes of death according to 2018 ICD-CM classification ([www.cdc.gov/nchs/icd/icd10cm.htm](http://www.cdc.gov/nchs/icd/icd10cm.htm)) and their etiology. Positive results were reported in 4/9 cetaceans and in 2/5 sea turtles with the highest number found in the bottlenose dolphin deemed to be died for the interaction with human activity (Fig. 1) and in a striped dolphin affected by dolphin morbillivirus (DMV): In these individuals, the amount of diatoms found in the bone marrow was respectively 2 and 3 orders of magnitude greater than in animals died respectively for fungal infection and toxoplasmosis. In all these three cases of spontaneous disease breathing control could be affected to damages related to central nervous system as well as to airways, as reported in Table 1. Also for sea turtles, diatoms were found in higher number in those subjects deemed to be killed by fishing activities (Fig. 2).

## 4. Discussion

The interaction between marine vertebrates and fishing activities is a relevant menace for their conservation. Postmortem diagnosis is often supported by external evidences and eventually by the presence of fishing gears around the carcass. On the contrary, the absence of clear external findings consistent with this interaction do not exclude possible entanglement. In the case of marine mammals, Moore et al. [9] define the main pathological changes related to by-catch considering gross findings, but further diagnostic approaches are necessary to confirm the possible diagnosis. The present study was aimed to investigate the possible application of the diatoms-test used in human forensic medicine to marine animals as a support for the diagnosis of by-catch.

Diatoms were found in 6 out of the 14 examined marine vertebrates, but the limited number of examined animals and the

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