TECHNICAL REPORT **SENTINELS OF THE SEAS** MARINE MAMMALS AS FLAGSHIPS FOR

MARINE MAMMALS AS FLAGSHIPS FOR OCEAN PLASTIC REDUCTION IN BRAZIL



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INTRODUCTION

ependent on the seas and oceans for all or most of their activities, marine mammals (MM) compose a very diverse group of animals, which are distributed across the globe in three taxa: Order Carnivora, Infraorder Cetacea, and Order Sirenia. Differences between orders concerning physiological, anatomical, and ecological adaptations for diving, swimming, temperature control, and foraging are the most diverse among groups. Cetaceans (whales and dolphins) and Sirenians (manatees and dugongs) are completely adapted to aquatic life, which led to the loss of their hind limbs and the development of a caudal fin. Pinnipeds (seals, sea lions) and aquatic mustelids (otters) use the terrestrial environ-

ment or ice platforms for reproduction, the birth of young, and molting. In this way, they preserved the four limbs with well-developed interdigital membranes. (Hoelzel, 2002).

Despite the marked differences between MM representatives, some similarities draw the attention of conservationists to these animals since the use of MM as environmental sentinels can provide indicators of ecosystem health (Tabor & Aguirre, 2004; Hazen et al., 2019). Some of the most prominent characteristics that give this title to the group are the high trophic level, long life expectancy, fat stores capable of accumulating anthropogenic toxins, and several representatives residing on the coast (Bossart, 2011).

Among the most significant threats reported to the group are hunting (Hovelsrud et al., 2008), bycatch (Hamilton & Baker, 2019), oil and gas exploration (Gales et al., 2003; Helm et al., 2014), persistent organic pollutants (Lavandier et al., 2016), habitat loss and marine litter (Panti et al., 2019). Marine litter is any persistent solid material manufactured or processed, discarded, or abandoned in the marine and coastal environment (Gomiero et al., 2019). Studies have revealed that plastic is the main component (Derraik, 2002). In addition, microplastics (MPs), in turn, make up one of the smallest and most abundant fractions of marine litter (Van Sebille et al., 2015) and comprise a very heterogeneous set of particles that vary in size, shape, color, density and chemical composition based on several polymers (Galgani et al., 2015). The usual definition of microplastics comprises particles smaller than 5 mm (Arthur et al., 2009), which are further divided into two categories according to the origin of the material. Primary MPs are produced in microscopic size to (i) be used as a raw material to manufacture most products made of plastic and (ii) be used as an abrasive agent, mainly in the cosmetics industry. Secondary MPs result from the fragmentation of larger plastics discarded in the environment called macroplastics (> 5mm) (e.g., fishing nets, bags, plastic bottles) (Olivatto, 2018).

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Due to the indiscriminate use of plastic associated with poor management (Worm et al., 2017; Sharma et al., 2017), plastic materials have become ubiquitous in rivers, coastal areas, and ocean basins (Borrelle et al., 2017). They represent up to 95% of the waste that accumulates on coastlines, in surface water bodies, and even at the bottom of the sea (Bergman et al., 2015). Plastic waste pollution is already recognized as a significant threat to marine life and the conservation of ocean habitats around the world. During the Basel Conference in 2019, held in the city of Geneva, approximately 180 governments identified plastics as hazardous waste due to their toxic capacity and ability to adsorb other pollutants (Lima et al., 2020).

Approximately 2,250 marine species have been documented to be affected by plastic debris (Tekman et al., 2019). For marine mammals, the immediate impacts of this encounter result from ingestion or entanglement (Gregory, 2009; Wilcox et al., 2015; Alexiadou et al., 2019). In the global scenario, it is estimated that 68% of cetacean species have already become victims of these interactions (Eisfeld-Pierantonio et al., 2022). The consequences of entanglements can be more easily understood since impaired locomotion can affect the ability to obtain food, leading to progressive weakness of the individual or immediate death by asphyxiation (Laist, 1997).

In contrast, the effects of plastic ingestion are still poorly documented, and its consequences are poorly understood (Simmonds, 2017). However, studies point out that ingestion causes ulcers and obstructions in the gastrointestinal tract, causing a feeling of satiety, blockage of digestion, and hunger leading to severe weaknesses, in addition to inflammation and acting as a vector of pathogens or pollutants (Fossi et al., 2020). Among cetaceans, ingestion of marine litter has been documented in 48 species, representing 56% of the diversity of this infraorder (Baulch & Perry, 2014; Kühn et al., 2015). From the ingested material, 46% of the composition was plastic items (Baulch & Perry, 2014).

Brazil is the fourth largest producer of plastic waste in the world (WWF, 2019), and as a developing country, it has inefficient waste management strategies (Margallo et al., 2019) that collaborate with the increase of plastic pollution in aquatic ecosystems. With 51 species of marine mammals, it presents itself as a vital country representing megadiversity (Costa et al., 2015). Mortality in the country as a result of plastic ingestion was reported in Guiana dolphins (Sotalia guianensis), rough-toothed dolphins (Steno bredanensis), beaked whales (Ziphius cavirostris) and manatees (Trichechus manatus) (Aquasis, unpublished data; Meirelles et al., 2007; Attademo et al., 2015; Bortolotto et al., 2016.)(Figure 1).



Figure 1: Reported cases of marine mammal's death linked to ingestion of plastic debris in Brazil.

However, to our knowledge, there are no studies documenting the ingestion of MPs in the group, although most scientific investigations on the subject in the Brazilian ecosystem between 2009 and 2017 have focused on analysis of microplastics associated with the biota (Castro et al., 2018). Globally, studies that precisely assess the ingestion of microplastics in cetaceans are still scarce; however, contamination has been documented in at least in fifteen species (Fossi et al., 2012; Besseling et al., 2015; Lusher et al., 2015; Van Franeker et al., 2018; Hernandez Gonzalez et al. 2018; Xiong et al., 2018; Nelms et al., 2019; Zhu et al., 2019; Nelms et al. 2019.; Moore et al., 2020) (Figure 2).



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Figure 2: Reported cases of microplastic ingestion by cetacean species around the globe.

Atlantic white-sided dolphin	Indo-Pacific
●Beluga whale	humpbacked dolphin
Bottlenose dolphin	Killer whale
Common dolphin	Pigmy sperm whale
●Cuvier's beaked whale	Risso's dolphin
Finless porpoise	Striped dolphin
Harbour porpoico	T

- True's beaked whale
 - White-beaked dolphin

Humpback whale

Given the relevant importance of Brazil regarding the diversity of MMs, the Brazilian scientific community must direct considerable efforts towards studies on threats to the group as an essential tool for outlining strategies for the conservation of specific species, as well as of the seas and oceans in general. In a minor cut, the state of Ceará, which has about 600 kilometers of coastline, is an essential region in the country for MMs, as it presents the occurrence of 25 species. Among these, the species with the highest number of records is the Guiana dolphin (Sotalia guianensis), the most common cetacean in much of the Brazilian coastal zone (Carvalho et al., 2020).

The Guiana dolphin is a small coastal dolphin (Figure 3), which measures up to 2.10 meters, and is distributed in Central and South America, from Nicaragua to the state of Santa Catarina, in southern Brazil. The species are preferably located in sheltered areas such as bays, estuaries, inlets, and even in port areas (Cunha et al., 2020)(Figure 4). Because of their coastal habitat, Guiana dolphins are exposed to several anthropic impacts, mainly in the more urbanized regions. The primary documented threats to the species are accidental capture in fishing nets (Figure 5), exposure to contaminants, boat traffic, noise pollution, and construction of coastal developments (Schiavetti et al., 2020).

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Figure 3: Guiana dolphin in Mucuripes bay, at Ceará State, Northeast Brazil.



Figure 4: Guiana dolphin using a Portuary area in Fortaleza, Ceará, Brazil.



Figure 5: Guiana dolphin found stranded on a beach from Ceará with clear evidence of entanglement with fishing nets.

Accidental capture in fishing nets represents Brazil's leading cause of death, but with a variable frequency between regions (Bertozzi et al., 2020). In Ceará state, Aquasis recorded more than 680 Guiana dolphin strandings over almost 30 years of effort (Figure 6). Of this total, at least 20% died due to asphyxiation due to capture. However, this percentage is undoubtedly underestimated since most of the carcasses are decomposed, making it impossible to assess the cause of death accurately.

Even for inhabits the coastal environment, the species is exposed to the impacts listed above, which can act synergistically and chronically. However, for many years the species' national and global conservation status was listed as Data Deficient. Only in 2014 was the Guiana dolphin listed as Vulnerable (VU) on the Brazilian red list (ICMBio, 2014) and in 2018, as

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Near Threatened (NT) by the IUCN (Secchi et al., 2018), which demonstrates the need for action of conservation aimed at the species in order to mitigate the risks of disappearance from nature (Meirelles, 2013). As a result of this sum of characteristics, the Guiana dolphin is a significant species for studies on the health of the seas and oceans, currently significantly impacted by the emerging contamination by plastic waste, considering that currently, these wastes are ubiquitous on beaches, bays, estuaries and various oceanic regions (Borrelle et al., 2017). Furthermore, to our knowledge, there are no specific studies evaluating the contamination of the species by MPs in South America.

Given this scenario, the Marine Mammals Program (PMM) of the Association for Research and Preservation of Aquatic Ecosystems has been carrying out since 2019 the Sentinels of the seas through the sponsorship of the National Geographic Society. The main objective was to evaluate and characterize contamination by plastic waste in the Guiana dolphin and the use of the species as a flagship species to demonstrate the effects of plastic pollution on the charismatic megafauna and alert society to the anthropic impacts on the marine environment.



Figure 6: Guiana dolphin found stranded on a beach from Ceará with clear evidence of entanglement with fishing nets resulting in strangulations on its body.

OBJECTIVES

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The objective of this study was to identify if the Guiana dolphins (S. guianensis) from the coastal area of Ceará state (Northeastern Brazil) are contaminated by plastic debris (microplastics and macroplastics) and to evaluate the patterns of plastic contamination (microplastics and macroplastics) of the specimens from the coastal zone of the Ceará state, northeastern Brazil, as well as their use as sentinels of environmental quality.

SPECIFIC OBJECTIVES

- To determine the frequency of contamination of Guiana dolphin by microplastic particles and to evaluate whether spatiotemporal variability is linked to microplastic ingestion.
- to characterize the particles according to the type, size, shape, color, and polymer type.

METHODS STUDY AREA

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The samples (strained individuals) were collected along the 573 km of the Ceará state (northeastern Brazil), located on the Southwestern Tropical Atlantic coast. The study area is characterized by an irregular rainy season, in which the highest volume (90%) of annual precipitation occurs in the first semester (January to June). In contrast, the second semester (July to December) is marked by the dry season combined with the presence of strong winds (up to 4m/s) (Campos et al., 2003). The Ceará state has an estimated population size of 9,240,580, with a greater concentration in its capital located in the coastal line (2,703,391 estimated population size) and its metropolitan region (IBGE, 2021). The coastal area monitoring was divided into three sectors (S): (1) Western coast (WC), (2) Fortaleza's Metropolitan Region (FRMS), and (3) Eastern coast (EC) (Figure 7).



Figure 7: Study area on the northeastern Brazilian coast (Ceará state) divided into Western, Fortaleza's Metropolitan Region (FRMS) and Eastern sectors.

SAMPLING

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During a decade, from 2011 to 2021, carcasses of S. guianensis were recovered during rescuing activities and beach monitoring on the coastline of Ceará state (northeast Brazil) by Aquasis (Figure 8 & 9), a nongovernmental organization (NGO) with efforts to promote research and actions focusing on the conservation of endangered species in Ceará state. Rescues were opportunistic since they depended on calls from the local community. Whenever a dead individual of S. guianensis was found, basic information was collected for registering the stranding, such as the geographic coordinate, date, sex, age classes, length, and stranding code according to Geraci & Lounsbury (2005) (2 - freshly dead, 3 - decomposed, 4 - advanced decomposition, 5 – mummified).



Figure 8: Sampling over the years (2011 to 2021).



Figure 9: Response to the stranding of a dead S. guianensis, collection of morphometric measurements, and biological samples (Photo: Aquasis).

If the animal was found freshly dead (Code 2) or decomposed (Code 3) (Geraci & Lounsbury, 2005), the specimen was submitted for a complete necroscopic examination at the Necropsy building of the Marine Mammal Rehabilitation Center of Aquasis. The stomach contents were stored without the stomach chambers for specimens that went through necropsy. For animals inappropriate to go under necropsy, stomach or stomach contents were removed in the field using a scalpel. When the specimen was in an advanced stage of decomposition (Code 4), The entire stomach or stomach contents were removed from the body cavity. The esophagus' terminal and the duodenum's initial portion were tied using a cotton wire to avoid stomach contents leaking, and then the stomach was cut away using a scalpel (Pugliares et al., 2007). Once collected, samples were stored in plastic bags and frozen (-20°C). This study comprises 40 individuals; among them, 23 were full stomachs (Figure 10), and 17 were stomach contents only.



Figure 10: Stomach sample of a S. guianensis (Photo: Aquasis).

LABORATORY PROCEDURES FOR MICROPLASTIC EXTRACTION

The protocol applied for microplastic extraction in the S. guianensis samples was adapted from Lusher & Hernandez-Milian (2018) and Justino *et al.*, (2021) (Figure 11). Firstly, samples were removed from the freezer, kept inside the bags on covered metal trays, and maintained at room temperature (30°C) for approximately 14 hours for thawing. Once thawed, the external stomach surface was rinsed with filtered (cellulose fiber filter, 8 μ m pore size, Whatman GR 40) distilled water to remove any particles attached and weighed (g). Stomach chambers were cut off using a scalpel and then were inverted directly on the beaker, and the excess attached to the mucosa was rinsed out into the beaker using filtered distilled water. When the sample was solely the stomach contents, they were transferred to the beaker and weighed.

Chemical digestion was used to extract microplastic particles from the samples. Stomach contents were digested in a filtered 10% KOH solution (Lusher & Hernandez-Milian., 2018; Moore et al., 2020; Zhu et al., 2019) in a volume three times greater than the sample and kept in an oven (60°C) for 24 hours (Justino et al., 2021). Beakers were covered with glass lids, and the solution was mixed two times during the process, using a glass stick, to homogenize the solution. If the sample had large bone parts, such as the skull and vertebral column, they were removed from the sample after the digestion step using steel forceps.

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After digestion, samples were filtered onto a cellulose fiber filter under a vacuum pump (Figure 12). Filters were transferred to covered Petri dishes and oven-dried at 60° C. After 24h, samples were observed on a stereomicroscope (Zeiss Stemi 508, using 40-50x magnification) coupled with a device camera (Axiocam 105 Color), with a detection limit of 20 μ m. Two observers inspected Petri dishes separately to avoid under or overestimation of particles (Figure 13).

Potential microplastic particles identified were photographed, measured (particle longest axis; Zeiss ZEN 3.2), counted, and characterized according to shape (Lusher et al., 2014) and color (black, blue, green, red, and white) (Board 1 & 2). The polymer composition of the sub-sample (6.8%) of particles detected was identified through Laser Directed Infra-Red analysis (LDIR) (Figure 14). The absorbance of the suspected microplastics was obtained using the Agilent 8700 LDIR Chemical Imaging System and compared with the reference spectrum of polymers from the Microplastics Starter 1.0 library. Each spectral curve resulted from at least ten scans performed in the wavelength ranging from 1800 to 975 cm-1 (Ourgaud et al., 2022). Then, the specific polymer was asserted when the analyzed particle registered above 60% of similarity with the reference spectrum.







Figure 12: Sample vacuum filtration process after chemical digestion process of *S. guianensis* food content (Photo: Aquasis).



Figure 13: Visual inspection of the filters using a stereomicroscope to identify plastic particles for particles identification (Photo: Aquasis)



Figure 14: Polymeric identification of plastic particles extracted from food content of *S. guianensis* using LDIR technique (Photo: Aquasis).



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Since the data did not meet parametric assumptions, Wilcox (factors with two levels) and Kruskal-Wallis (factors with three levels) tests were applied to determine whether there were any differences in the number and size of microplastic particles detected according to seasonal and sampling sector.

Whether the Kruskal-Wallis indicated significant differences, Dunn's test pairwise comparisons were used to investigate the source of variance. In addition, the number and size of detected particles were correlated with stomach contents weight using the Spearman correlation test. All analyses were carried out using R 3.6 (R Core Team, 2020) with a 5% significance level.

CONTAMINATION CONTROL

Workstations and equipment were cleaned with filtered 70% ethanol to prevent contamination and rinsed out with filtered distilled water before the analysis. Additionally, the 10% KOH solution was prepared using filtered distilled water, and the solution was also filtered before the digestion (8 μ m pore size: Whatman GR 40).

During laboratory procedures, 100% cotton lab coats, facemasks, and latex gloves were worn. The use of the room where the analysis took place was restricted to two or three people involved in the procedures.

In addition, a procedural blank (beaker filled with 50ml of 10% KOH solution) was implemented for each individual sampled, and blanks were submitted to the same methodological steps as samples.

RESULTS

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In total, 325 plastic particles were detected in the 40 dolphins analyzed (Figure 15 & 16), with a contamination frequency of 95% (38 out of 40 samples) (Figure 17). Most detected plastic particles were categorized as microplastics (319 particles < 5 mm), while only six were larger than 5 mm. Considering only microplastic particles, the overall mean was 7.97 ± 1.33 individual particles-1 (mean ± standard error). Our results revealed that the size of the MPs ranged from 0.018 mm to 4.24 mm, resulting in an average size of 0.36 ± 0.03 mm.







Figure 16: Microplastic particles extracted from S. guianensis (a) fragment, (b) pellets, (c) fragment, (d) filament, (e)foam, and (f) film.

Figure 17: Percentual of samples contaminated by microplastic particles.

Among the different particle formats detected in dolphins, fragment (56.1%) was the most predominant, followed by filament (15.7%), foam (10%), film (9.4%), and pellet (8. 8%) (Figure 18). White was the most representative color (41.1%), followed by black (38.5%), blue (12.8%), green (4.1%), and red (3.4%) (Figure 19).



Figure 18: Size distribution of microplastic into the shapes.

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Figure 19: Percentual of different colors of the microplastic particles extracted from food content of S. guianensis.

From the analyzed subsample (6.8% of particles detected), 55% were successfully identified as plastic polymers, 10% were biopolymers, and 35% did not match the cutoff point (below 60% of similarity with the reference spectrum); these were probably highly weathered plastic.

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Regarding the identified plastic polymers (Figure 20), polyurethane (PU), polyethylene terephthalate (PET), and ethylene-vinyl acetate (EVA) were the most prevalent (20% each), followed by styrene-butadiene rubber (SBR), polypropylene (PP), polyamide (PA), acrylonitrile butadiene styrene (ABS) and high-density polyethylene (HDPE) (10% each).



Figure 20: Polymer composition of microplastics extracted from the food content of S.guianensis.

Spearman's correlation analysis showed a moderate positive trend (r = + 0.43, p=0.006) in the number of MPs with the weight of stomach contents. The specimens sampled in the rainy season showed a higher number of particles (9.71 ± 2.45 parts. ind.-1) than in the dry season (7.03 ± 1.57 parts. ind.-1); however, there was no significant difference between stations and sectors (Figure 21).



Figure 21: Number of microplastics detected in S. guianensis from the northeastern Brazilian coast (Ceará state) according to the season.

On the other hand, the particle size was significantly smaller during the rainy season $(0.30 \pm 0.04 \text{ mm})$ than in the dry season $(0.40 \pm 0.04 \text{ mm})$ (p= 0.017) (Figure 22). In addition, the specimens from the West sector had the tiniest particles (0.28 \pm 0.03 mm; p = 0.037), followed by FMR (0.33 \pm 0.05 mm) and from the East sector (0.43 \pm 0.05 mm) (Figure 23).







Figure 23: Size of microplastics detected in S. guianensis from the northeastern Brazilian coast (Ceará state) according to the three sectors (Western, Fortaleza's Metropolitan Region, and Eastern).

DISCUSSION

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MPs are widely distributed in different aquatic ecosystems and raise concerns about their potential as contaminants (Battaglia et al., 2020). Comparison between the results of this research and other populations of S. guianensis was not possible because, as far as we know, this is the first study to document exposure to MP in the species. Additionally, there is no research on contamination by MPs in small cetaceans in coastal waters of South America that would allow extrapolations for comparisons and correlations in the region.

However, studies have reported contamination by microplastics in cetaceans from Europe, China, and North America, where the number of detected particles varied greatly. Findings ranged from 2 to 45 particles per individual (Zhu et al., 2019) and from 67 to 304 (Battaglia et al., 2020). These expressive differences in the number of detected particles are probably related to the different methods of isolation of MPs used associated with the type of sample used (stomach/intestine or both), in addition to the different feeding strategies of the target species of the study and the abundance of MPs in the middle of the study area (Di Beneditto & Ramos, 2014; Justino *et al.*, 2021).

However, the total number of 319 particles found here (1-30 MPs per individual) is similar to the study with common dolphins (Delphinus delphis) from Spain (Hernandez-Gonzalez *et al.*, 2018), which found 3 to 41 items per individual. Similar results may be related since both species are odontocetes, inhabit coastal and shallow waters, and have a similar pattern of a diverse diet composed of fish and cephalopods.

The absence of significant difference in the number of microplastics between the coastal sectors can be directly associated with three factors: (i) the carcass does not run aground in the same place where the animals lived and died; (ii) the size fraction of the microplastics detected here is not bioaccumulative in the stomach; (iii) the results are a snapshot of recently ingested prey, and S. guianensis frequently feeds to fulfill its high metabolic rate (Kastelein et al., 2010).

The positive correlation between the number of particles and the mass (g) of the food content associated with the absence of macroplastics in the food content of *S*. *guianensis* supports the hypothesis that individuals are not intentionally/actively ingesting plastic, which suggests that the plastic contamination may occur by trophic transfer (Farrel & Nelson, 2013) or by accidental ingestion of the contaminant during the foraging process.

Although the findings of this study have not been related to the cause of death of individuals, it is necessary to investigate the effect of microplastics as chemical pollutants since microplastic particles have a high adsorptive potential for persistent organic pollutants and heavy metals available in the environment (Van et al., 2012).

SENTINELAS DOS MARES

Dantas et al. (2020) documented contamination by microplastics in two species that make up the diet of *S.guianensis* in the state according to Campos (2012), which raises the alarm since the transfer of these contaminants through the food chain can seriously affect the Guiana dolphins, considering the biomagnification process (Clark, 2001).

Regarding the size of the extracted plastic particles, the differences may be associated with space-time variations. During the rainy season, there is a more significant contribution of microplastic particles from the continent to the sea that were retained along the banks of rivers, particles that once in the environment are exposed to weathering, which results in breaking into smaller pieces (Gewert et al. al., 2015) and are transported when runoff increases (Lima et al., 2014; Lebreton et al., 2017).

CONCLUSION

The study promoted unprecedented findings of contamination by MPs for the Guiana dolphin in South America. It opened up important space for undefined questions about the origin of the contamination of the animals, in addition to presenting itself as a reference in Brazil for the evaluation of contamination of Guiana dolphins and other MM, encouraging the objective of drawing up a panel about the contamination along the Brazilian coast. In addition, it reinforces the title and use of the species as a sentinel of the seas. Through the investigation of stomach contents of dead individuals, it was possible to verify the contamination along the entire coast of the state of Ceará. It is also essential to carry out more robust studies investigating the degree of contamination by plastic particles and the degree of contamination by chemical pollutants associated with plastics in the same specimen. Given the trophic route as the primary source of contamination of the species, it is important to alert human populations about the potential risk of human contamination by consuming fish from the region.





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