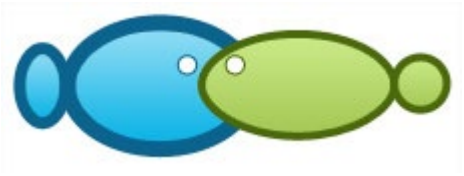

Analysis of microplastics in the intestines of stranded cetaceans

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Abstract. Microplastics derived from anthropogenic pollution have become a major issue today, as available data have shown they are present in all terrestrial and marine ecosystems. These pollutants originate from multiple sources, from trash dumped in rivers and lakes and travelling to the ocean, to fishing tools (such as nets, ropes, and baskets) abandoned at sea as well as various chemicals released in the water. As apex predators, cetaceans are critical in regulating the food web and maintaining ecosystem balance. They consume lower trophic-level organisms that have ingested microplastics, and, at present, the studies conducted on microplastics in cetaceans are insufficient. Further research on the prevalence and effects of microplastics in these animals is needed to support conservation efforts and shed light on the larger issue of plastic pollution in the oceans. Determining the concentration of microplastics in cetaceans can inform on the level of microplastic contamination in the entire food web, including seafood that humans consume. This research aimed to assess the presence and accumulation of microplastics in cetaceans to understand better their potential impacts on both these species and the marine environment. Microplastics were detected in 13 out of 17 samples of cetacean intestines; most of them were fibres and varied in length, size, and colour. Raman spectroscopy analysis showed that the microplastics were made of the polymer Nylon 6,6. The results of this study will contribute to the current knowledge of the impact of microplastics on marine life and ecosystems and can aid in developing strategies to mitigate and manage this urgent environmental issue.
Key Words: microplastics, plastic fibre, Raman spectroscopy analysis, stranding.

Introduction. Cetaceans are a key indicator of the health of marine ecosystems because they occupy the highest trophic level (Rogers & Greenaway 2005; Bossart 2006). The study of these animals can help to identify current or potential threats to marine ecosystems and define strategies to deal with them (Bossart 2006; Guzzetti et al 2018).

Most cetacean species have been shown to ingest a wide range of plastics (Ryan 2019), from microplastics, such as particles and fibres, to macroplastics, such as plastic sheets, fishing nets, fishing lines, household items, etc. (Fossi et al 2020). The International Whaling Commission considers plastic pollution an urgent issue since it may affect the mortality of cetaceans (IWC 2020).

Two extant cetacean suborders, i.e., baleen whales (Mysticeti) and toothed whales (Odontoceti), greatly differ in terms of their feeding ecology, which affects the evaluation of the impact of microplastics on them. Baleen whales can be exposed to plastic pollution through the direct uptake of contaminated water and prey as they filter large volumes of water while feeding (Bannister 2009; Desforges et al 2015; Germanov et al 2018; Burkhardt-Holm & N'Guyen 2019; Guerrini et al 2019). By contrast, toothed whales, which hunt and swallow the prey whole or torn into pieces, should be more prone to incorporate plastics through the ingestion of plastic-contaminated prey, i.e., through trophic transfer (Würsig et al 2009; do Sul & Costa 2014; Au et al 2017; Nelms et al 2018; Perez-Venegas et al 2018).

Both the direct and indirect ingestion of microplastics via filter feeding and prey consumption, respectively (Fossi et al 2020; Zantis et al 2021) may lead to bioaccumulation (Koelmans 2015; Miller et al 2020; Xu et al 2020). For example, a relatively high number of synthetic particles (mean value 0.057 particles g^{-1}) was found in krill ingested by fin whales on the western coast of Iceland (Garcia-Garin et al 2021). A study found about 45 plastic items in the body of a fin whale floated off conducted off Jeju Island, South Korea, including fishing lines, parts of fishing nets, plastic filaments, and styrofoam pieces (Im et al 2020). Several polymer types (polyethene, propylene, nylon, and polyethene terephthalate, among others) were found in humpback whales (*Megaptera novaeangliae*) in the eastern North Pacific (Besseling et al 2015; Alava 2020; Zantis et al 2021). In addition, another study found that blue whales (*Balaenoptera musculus*) feeding on krill in the Gulf of California are much more exposed to microplastic contamination through prey transfer than humpbacks, which feed predominantly on fish (Kahane-Rapport et al 2022).

Toothed whales are also commonly affected by microplastic pollution due to the consumption of contaminated prey. High amounts of microplastic particles were detected in the gastrointestinal tracts of beluga whales (*Delphinapterus leucas*) from Canada's Northwest Territories (Jacobsen et al 2010; Moore et al 2020). Similarly, a study of True's beaked whales (*Mesoplodon mirus*) stranded on the coasts of Ireland reported a large number of microplastics mixed with the remains of mesopelagic fish and cephalopods among their stomach contents (Lusher et al 2015).

It is crucial to improve our understanding of microplastic ingestion by cetaceans for various reasons. First, cetaceans are apex predators in the ocean and can accumulate high levels of microplastics that can potentially damage their health and threaten their survival (Harlacher 2020; Liu et al 2022). Furthermore, these species are valuable indicators of the health of marine ecosystems. Therefore, the analysis of microplastics present in their bodies can provide valuable insights into the extent and impact of marine plastic pollution (Wilcox et al 2016).

Hokkaido is located in the Northern part of Japan, surrounded by the Pacific Ocean, the Okhotsk Sea, and the Sea of Japan. The northwestern Pacific Ocean is known for its high levels of microplastic pollution (Pan et al 2019). On average, 70 cetacean strandings are observed annually, and the Stranding Network Hokkaido (SNH) collects specimens of stranded cetaceans and delivers them to various institutions for academic purposes.

The objective of the study was to evaluate the presence of microplastics in cetacean intestines collected in Hokkaido, Japan, in order to increase the available information for the investigation of the potential impacts of these pollutants on marine ecosystems.

Material and Method

Materials. Specimens collected by SNH were used for this research. The SNH is a nonprofit organisation that records information related to cetacean strandings, including beaching, drifting, and bycatch, and collects specimens of the stranded animals. Since its establishment in 2007, the network has called on the general public, administrative bodies, fishery officials, and others to report information on strandings to the dedicated receiving and reporting desk "IRUKA KUJIRA 110" and recorded an average of about 70 cases per year, with a cumulative total of over 1000 cases by 2022 (Stranding Network Hokkaido 2023). Upon receiving the information, the SNH investigates the approachability and condition of the animals and determines the feasibility of conducting a survey. If a survey is deemed feasible, permission is obtained from the relevant authorities, and the survey is carried out. Specimens are collected by searching for external morphological information and external injuries. The SNH obtains requests in advance from research institutions in regard to the samples needed for analysis. When the specimens are available, they are sent to the institutes free of charge and unconditionally. The use of stranded specimens allows research to be conducted without unnecessary collections of living specimens, thus contributing to conservation efforts.

The intestines of 11 cetacean specimens were obtained during 10 January 2019 and 14 April 2022 from different locations in Hokkaido. Table 1 shows the detailed information for each individual used in this study. A total of 17 samples were prepared and screened for the presence of microplastics. A 5-cm sample was taken from each received intestine. In three cases, multiple samples were prepared: five for individual SNH21007 and two each for individuals SNH20091 and SNH190032. To avoid contamination, samples were wrapped in aluminium foil and frozen before the analysis. The study of carcasses can be complicated due to uncertainties associated with the determination of the stage of decomposition. However, it is crucial to have a system in place to evaluate the quality of the material being studied. A code-based system was established by the Smithsonian Institution's Scientific Event Alert Network. In Table 1, CODE 2 defines a carcass in good condition, while CODE 3 means that the carcass is decomposed, but the organs are basically intact (Geraci & Lounsbury 2005). In the present study, the experimental intestines were considered adequate.

An experiment was conducted to ascertain if all plastics emitted fluorescent light and could thus be observed under a fluorescence microscope. Small pieces (less than 5 mm) were cut from several plastic items commonly used in households and industries, and they were marked and placed in one intestinal sample. This was then examined under a fluorescence microscope using the same conditions as those used for the other samples in this study. The materials used for this test were: polyurethane (PU), polyethylene terephthalate (PET), poly (methyl methacrylate), i.e., acrylic plastic and plexiglass (PMMA), and polystyrene, i.e., foams (PS). The observed microplastic fibers were classified based on their size, color, shapes, and possible origins. Furthermore, their chemical composition (polymer types) was identified using Raman analysis.

Methods. Raman spectroscopy is a useful tool for the analysis of microplastics. It can provide specific information about the chemical composition and structure of a sample without the need for sample preparation or destruction. This makes it a non-destructive and efficient method for identifying microplastics (Shim et al 2017). Another merit of Raman spectroscopy is its ability to differentiate between different types of polymers based on their unique Raman spectral signatures (Vankeirsbilck et al 2002). Based on their characteristic peaks, the spectra of polyethene, polypropylene, and polystyrene can be easily separated. Here, Raman spectra (RENISHAW inVia Raman Microscope) were used to identify the microplastics found in the samples. The measured spectra were uploaded to Open Specy and compared with those in the database library. Open Specy automatically displays the highest matching polymer spectra, thus allowing the identification of microplastics.

To detect microplastics more efficiently in the studied samples, the following three observation methods were adopted:

a). Digestion: microplastics were extracted from the intestines following Chen's procedure (Chen et al 2020). After lightly washing their surface with Mili-Q water, the samples were placed into a 10% KOH solution, and a water bath was used to accelerate the digestion process. With the help of a vacuum pump, the solution was filtered through filter paper (20-25 μm). Labelled plastic disks were placed into a labelled Petri dish and sealed with sticky tape. An optical microscope (Nikon ECLIPSE 50i) was used to preliminarily identify the particles and assess if they were made of plastic. Observations were made under 100x magnification. The obtained images were used to evaluate the shapes and sizes of the observed microplastics.

b). Direct observation: after the intestines were washed with Mili-Q ultrapure water, they were opened and flattened with the help of clips to expose the inner wall (Figure 1). A digital microscope was used to search for larger plastic fragments in the sample under a magnification of 200x.

c). Fluorescence microscopy: this method was used to reduce the likelihood of overlooking microplastics present in the studied samples. A cryostat (Leica CM 3050S) was used to slice the samples for further observation under a fluorescence microscope. Figure 2 shows the stages of this process.

Table 1

Stranding locations and species of sampled cetaceans

Sample ID	*Stranded location*	*Species name*	*Common name*	*Suborder*	*Body length*	*Sex*	*Decomposition stage*
SNH20091	41.986064N 140.906982E	*Balaenoptera acutorostrata*	Minke whale	Mysticeti	495.0 cm	Male	CODE 2
SNH19001	41.868298N 140.115493E	*Lagenorhynchus obliquidens*	Pacific white-sided dolphin	Odontoceti	219.9 cm	Male	CODE 3
SNH20032	41.940520N 143.242260E	*Stenella coeruleoalba*	Striped dolphin	Odontoceti	176.0 cm	Male	CODE 2
SNH21060	42.389176N 141.084797E	*Stenella coeruleoalba*	Striped dolphin	Odontoceti	191.1 cm	Female	CODE 2
SNH21052	42.600722N 141.488556E	*Stenella coeruleoalba*	Striped dolphin	Odontoceti	196.9 cm	Female	CODE 2
SNH22001	42.010660N 140.104330E	*Lagenorhynchus obliquidens*	Pacific white-sided dolphin	Odontoceti	202.4 cm	Male	CODE 3
SNH22006	41.468139N 140.029861E	*Phocoenoides dalli*	Dall's porpoise	Odontoceti	196.5 cm	Male	CODE 3
SNH21066	42.619847N 141.564035E	*Stenella coeruleoalba*	Striped dolphin	Odontoceti	215.7 cm	Male	CODE 2
SNH21007	42.697500N 140.051944E	*Phocoena phocoena*	Harbour porpoise	Odontoceti	178.6 cm	Female	CODE 3
SNH22016	42.331027N 141.022174E	*Phocoena phocoena*	Harbour porpoise	Odontoceti	136.1 cm	Female	CODE 2
SNH21036	42.393920N 140.906860E	*Stenella coeruleoalba*	Striped dolphin	Odontoceti	186.8 cm	Female	CODE 2

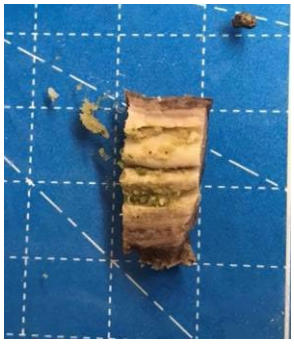

Figure 1. Dissection of the intestine to expose the inner wall.

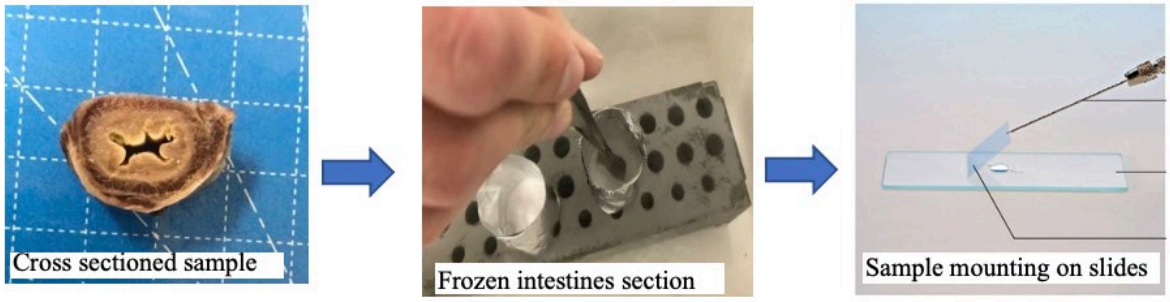

Figure 2. Images illustrating the preparation of an intestinal sample for observation under the fluorescence microscope.

Results. Microplastics were found in 13 out of the 17 samples of cetacean intestines examined (Table 2). The two samples without microplastics were from individuals SNH20032 and SNH22006 (Table 2). The observed particles showed differences in length, size, and colour (Figure 3), and most were fibres (Figure 4). Only Nylon 6,6 was identified by Raman spectroscopy (Figure 5). Figure 6 shows the test experiment results using common household items made of different plastic polymers.

Table 2

Presence of microplastics in stranded cetaceans from various locations in Japan (for species names and detailed location information, refer to Table 1)

Sample ID	*Number of samples taken*	*Observation of microplastics in the samples*
SNH20091	2	Presence
SNH19001	1	Presence
SNH20032	2	Absence
SNH21060	1	Presence
SNH21052	1	Presence
SNH22001	1	Presence
SNH22006	1	Absence
SNH21066	1	Presence
SNH21007	5	Presence
SNH22016	1	Presence
SNH21036	1	Absence

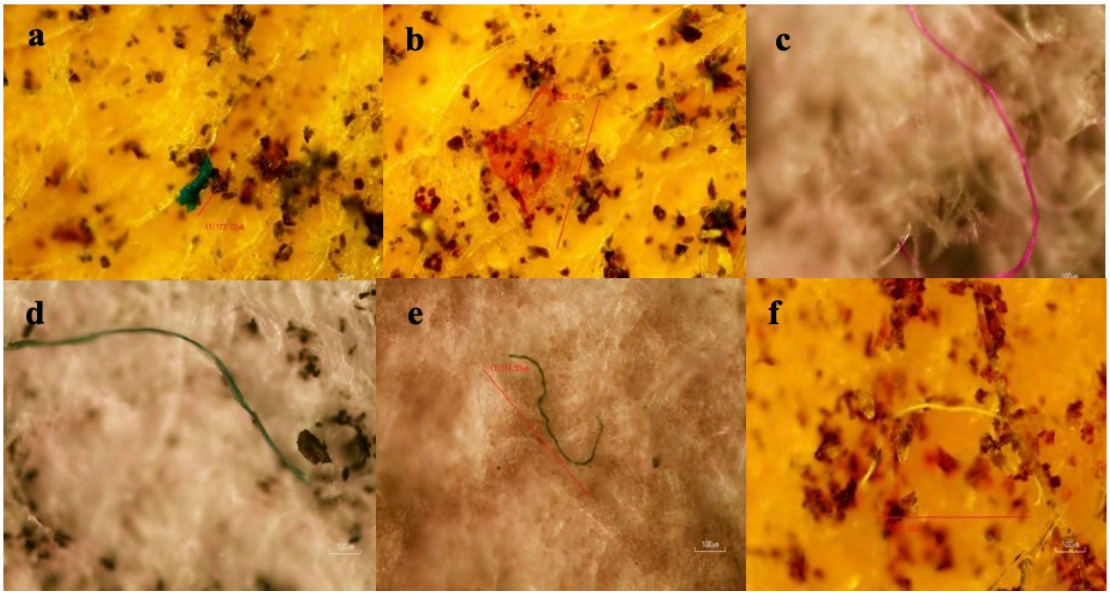

Figure 3. Typical microplastics found in cetacean intestines: a) green fragment; b) red fragment; c) pink fiber; d) green fiber; e) green fiber; f) yellow fiber.

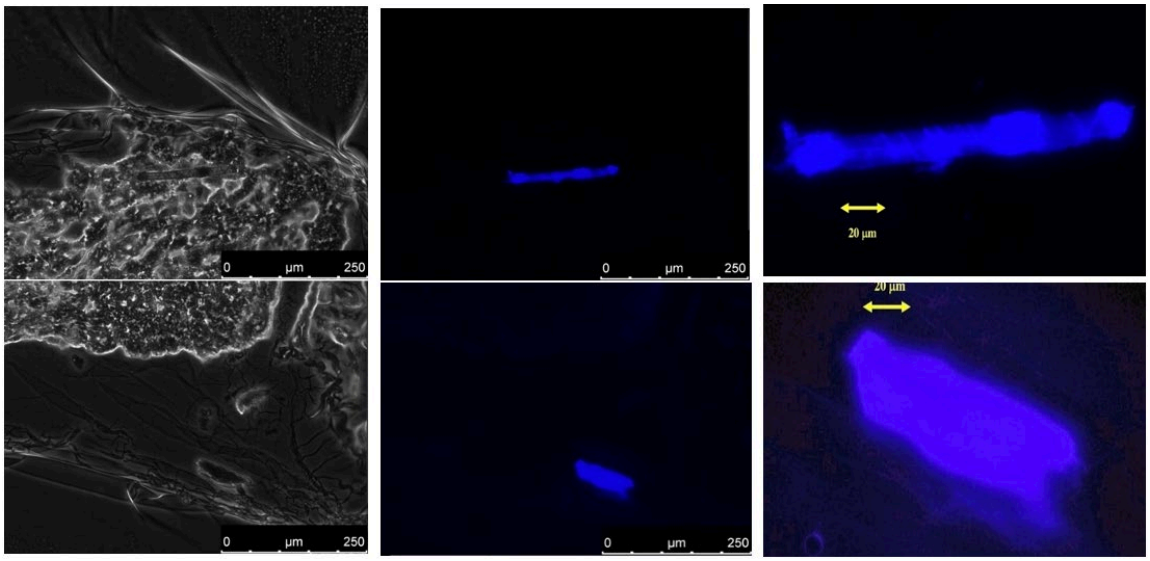

Figure 4. Detailed fluorescence microscope images of microplastic fragments and fibers found in cetacean intestines.

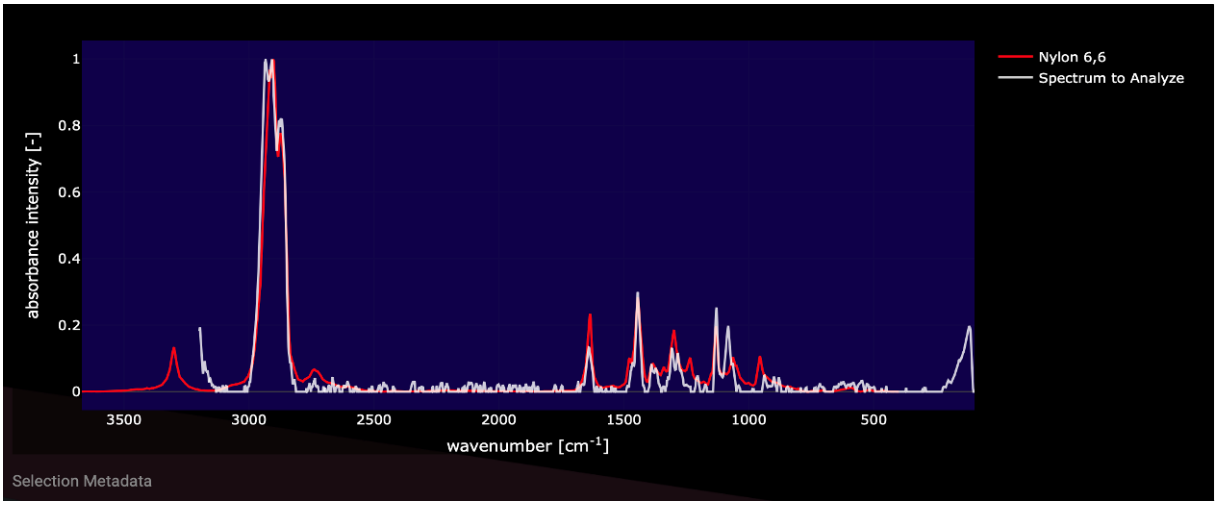

Figure 5. Raman spectra of Nylon 6,6.

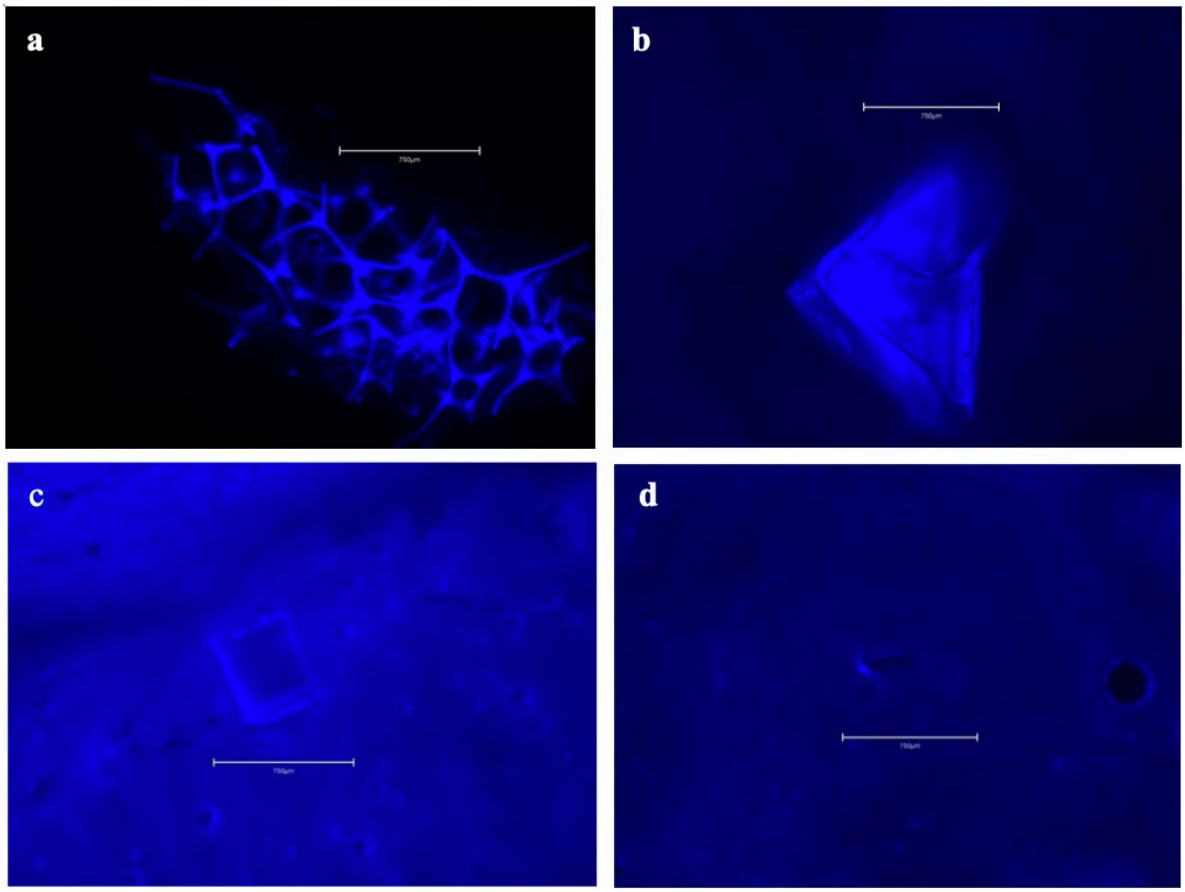

Figure 6. Images of the experiment testing different plastic materials. a) PU (polyurethane) and b) PET (polyethylene terephthalate) are clearly identified under the fluorescence microscope, while c) PS (polystyrene) and d) PMMA (poly (methyl methacrylate)) cannot be distinguished from the surrounding tissue under the fluorescence microscope.

Discussion. Our study aimed to ascertain the presence of microplastics in the intestines of stranded cetaceans in several areas of Japan and characterise the detected particles. Plastic production and its use are continuously increasing (Rhodes 2018) despite the alarming data reported in numerous studies (Thushari & Senevirathna 2020). Indeed, plastic pollution and its consequences for marine diversity have been widely documented (CBD 2012; IWC 2020; Zantis et al 2021). Although observations of microplastic ingestion in cetaceans have increased (Besseling et al 2015), they are still lagging behind those in other marine groups. Being top predators, cetaceans are key sentinel species to evaluate the health of the world's oceans (Rogers & Greenaway 2005; Bossart 2006; Guzzetti et al 2018). Thus, it is crucial to increase our knowledge of the consequences of plastic pollution to develop better strategies and guidelines for the protection of this important group of marine organisms.

As previously mentioned, toothed whales (Odontoceti) and baleen whales (Mysticeti) have different feeding habits, the former being top carnivore species while the latter obtaining nourishment through filter feeding (IWC 2020; Zantis et al 2021). Microplastic particles were found in most analysed samples through fluorescence and optical microscopy. The small amounts detected may be related to the fact that the intestines examined derived primarily from toothed cetacean species. Indeed, only one baleen species was examined in this study. Baleen whales are more exposed to macroplastics because of their feeding ecology (Jacobsen et al 2010; Unger et al 2016). Predictions based on observations and modeling have shown that toothed cetaceans feeding on fish and cephalopods would be less impacted by microplastics than krill-feeding baleen species (Kahane-Rapport et al 2022). The most numerous microplastics observed in this study were fibres, which is in line with the results of several previous

studies of cetaceans and other marine vertebrates (Lusher et al 2015; Lusher et al 2018; Nelms et al 2019).

In order to compare data, the same methodology should be followed, if at all possible. Unfortunately, it was not possible to access the instruments normally used for this type of study. Consequently, it was decided to use fluorescence microscopy. The use of this method to observe microplastics in cetacean intestines has not been reported before, so this analysis also has value as a methodological first trial. Even though fluorescence microscopy could clearly capture microplastic images, sample processing was long and inefficient. Therefore, only one sample was used for this trial to confirm that fluorescence microscopy produces clear images of microplastic particles. We would recommend this method for studies investigating smaller samples.

The small amounts of microplastics found in this study could be due to several reasons. First, fluorescence microscopy may not be the most efficient method to observe microplastics in the intestines of marine mammals, as it is normally used to detect microplastics in water (Qiu et al 2015; Dehghani et al 2017; Scircle & Cizdziel 2020). In addition, many microplastics have low intrinsic fluorescence signals or lack them together (Yan et al 2012; Spizzichino et al 2016; Karakolis et al 2019), making their observation quite challenging. Figure 6 shows that while PU and PET particles have strong fluorescence emission signals, the signals are very weak and cannot be observed under the fluorescence microscope. One way to improve the fluorescence emission signals is to stain the samples before observation using fluorescent dyes such as Nile Red (Maes et al 2017). It was our intention to check the reliability of this method in tissues. Unfortunately, our samples were very brittle after being processed for the microtome, so they could not be further used to separate the microplastics from the medium. Therefore, we relied on the digestion method to assess the presence of microplastics in the samples.

It is generally difficult to compare studies of microplastic contents in cetaceans due to methodological issues. As explained above, fluorescence microscopy was chosen because the instruments normally used in other studies were not available. Unfortunately, as our experiments with this method did not yield reliable results, we turned to less commonly used methods, such as the digestion of tissues. Furthermore, some previous studies examined both the stomach and intestines (Lusher et al 2015; Lusher et al 2018), while others used subsamples to extrapolate the total potential amounts of microplastics (Moore et al 2020). This study examined only the intestines, which was possibly not sufficient to detect the full extent of microplastic contamination. Several studies have reported blue and black as the most frequent microplastic colours (Nelms et al 2019). In contrast, this study found mostly green fibres and fragments (Figure 3), as well as a number of red and yellow fragments.

Conclusions. This study reports for the first time the presence of Nylon 6,6 in cetaceans. This robust plastic, often used to manufacture pelagic fishing nets, is considered a common source of microplastics in the ocean. More studies are necessary to assess its possible transfer to the animal food chain in the marine environment.

As plastic pollution increases in the oceans, more data from ecologically critical prey species are needed to accurately assess the risks posed by microplastics. New data obtained from different species at all trophic levels would allow to achieve a complete understanding of plastic distribution in marine food webs and its consequences for apex predators, including predatory fish, seabirds, and marine mammals.

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Conflict of interest. The authors declare that there is no conflict of interest.

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