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Data collection on marine litter ingestion in sea turtles

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COVER LETTER

Dear Editor,

This is a manuscript and video tutorial entitled "**Protocol for data collection on marine litter ingestion in sea turtles".**

It is produced to respond to the requirements set by the European Union's Marine Strategy Framework Directives (MSFD) for the D10C3 Criteria reported in the Commission Decision (EU), related to the amount of litter ingested by marine animals, and it could be easily applied for monitoring purposes along European coasts and Mediterranean countries, but also around the world for research activities.

Many authors worked on it and it has been tested in seven different Mediterranean and Atlantic countries in the frame of the European DG-ENV project GA No. 11.0661/2016/748064/SUB/ENV.C2

It is a deliverable of the project than, if It could be possible, we need a review asap.

We need to pay Jove journal by the 15Th January as the budget is linked to the project, that will finish at the end of January.

Here we suggest some possible reviewers but in general it could be better to invite someone more involved in marine litter monitoring, rather than researchers involved in sea turtles conservation.

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Best regards

Marco Matiddi et al.

TITLE:

Data Collection on Marine Litter Ingestion in Sea Turtles

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KEYWORDS:
Sea turtle, plastic ingestion, necropsy, marine litter, MSFD, EcAp process, good environmental status, thresholds

SUMMARY:
The protocol focuses on the collection of sea turtle samples, describing all the steps from the animal recovery and necropsy to the classification and quantification of ingested marine litter. Moreover, the representative results show how to use the collected data to elaborate the possible thresholds for Good Environmental Status.

ABSTRACT:
The following protocol is intended to respond to the requirements set by the European Union's Marine Strategy Framework Directives (MSFD) for the D10C3 Criteria reported in the Commission Decision (EU), related to the amount of litter ingested by marine animals. Standardized methodologies for extracting litter items ingested from dead sea turtles along with guidelines on data analysis are provided. The protocol starts with the collection of dead sea turtles and classification of samples according to the decomposition status. Turtle necropsy must be performed in authorized centers and the protocol described here explains the best procedure for gastrointestinal (GI) tract isolation. The three parts of the GI (esophagus, stomach, intestine) should be separated, opened lengthways and contents filtered using a 1 mm mesh sieve. The article describes the classification and quantification of ingested litter, classifying GI contents into seven different categories of marine litter and two categories of natural remains. The quantity of ingested litter should be reported as total dry mass (weight in grams, with two decimal places) and abundance (number of items). The protocol proposes two possible scenarios to achieve the good environmental status (GES). First: "There should be less than X% of sea turtles having Y g or more plastic in the GI in samples of 50–100 dead turtles from each sub-region", where Y is the average weight of plastic ingested and X% is the percentage of sea turtles with more weight (in grams) of plastic than Y. The second one, which considers the food remain versus plastic as a proxy of individual health, is: "There should be less than X% of sea turtles having more weight of plastic (in grams) than food remains in the GI in samples of 50–100 dead turtles from each sub-region".

INTRODUCTION:
Marine litter is a complex issue to address since it can enter the oceans via multiple sources and

forms. Over 80% of the litter that is encountered in marine environments is made up of plastic[1]. The role of this material from an economic perspective has been increasing in the last 50 years. As a consequence, its production has also increased twentyfold since 1960, reaching 335 million tons in 2016. This value is expected to double over the next 20 years[2]. Moreover, it has been estimated that around 5 to 13 million tons of plastic end up in the oceans every year (which is equal to 1.5 to 4% of global plastic production)[2,3]. Plastic movement is influenced by its physical properties (e.g., buoyancy) or environmental variables (e.g., tide and stream), and plastic can be accumulated in all marine compartments[4,5]. To face the plastic problem, it is important to bear in mind that, as many other environmental issues, it is transboundary and therefore governance solutions are complex to meet[6]. To better reach this goal we must take into consideration regional and international frameworks, so as to enhance or maintain marine environmental awareness and protection across the globe[7]. The final objective of the European Union's Marine Strategy Framework Directive (MSFD) is to achieve a good environmental status (GES) in European waters by 2020, to protect marine biodiversity, and to promote the sustainable use of marine environments. This will be done through 11 qualitative descriptors, of which Descriptor 10 focuses on marine litter and is defined as "Properties and quantities of marine litter do not cause harm to the coastal and marine environments". Within this descriptor, the New Commission Decision[8] decided to add criteria D10C3—"The amount of litter and micro-litter ingested by marine animals is at a level that does not adversely affect the health of the species concerned"—since it was considered to be a relevant criteria in the evaluation of GES. As a result, member states were requested to produce a list of species, to develop methodological standards and define threshold values through regional or sub-regional cooperation.

After the first scientific publication in 1838[9], on the Storm-Petrel with an ingested candle stick, over 500 marine species have been listed for ingesting marine litter[10,11,12,13,14], and sea turtles were among the first taxa recorded to ingest plastic debris[15]. Given their propensity for ingesting litter, their wide distribution and the large range of habitats used during their life, sea turtles, in particular the loggerhead species *Caretta caretta* (Linnaeus 1758), was chosen as a potential indicator for the Mediterranean basin[16], like the sea bird *Fulmarus glacialis* (Linnaeus, 1761) for Northern European waters[17]. Even after five decades of research, discussion of method standardization has been very limited[18] and a cohesive approach by the scientific community to quantify plastic ingestion by wildlife is lacking[19]. Standardized sampling protocols, and analytical detection methods and metrics to assess plastic ingestion by marine biota are needed; a recent paper showed the potential benefits and limitations of using marine species as bio-indicators on plastic pollution[20]. Following the Matiddi et al. proposal in 2011[21] to use the loggerhead as bio-indicator, and the Technical Group on Marine Litter Report[22], a specific protocol to consider marine litter ingested by sea turtle has been developed and tested by ten partners in seven countries in the Mediterranean Sea and Atlantic Ocean within the European Project INDICIT (GA n°11.0661/2016/748064/SUB/ENV.C2). This protocol provides standardized methodologies for the analysis of marine litter ingested by sea turtles in order to support the new Commission Decision (EU)[8], Criteria D10C3, where threshold values are requested. According to the definition provided by COM[8], the threshold value is a number or a range that allows to evaluate if the quality level criterion was achieved, therefore helping in assessing the GES. The proposed protocol for assessing the litter ingested by sea turtles, will be useful in gathering data on the

composition and abundance of litter and evaluate its impact on marine environments. Moreover, collecting this type of standardized data will help to define threshold values. Here we consider two types of scenarios. The first scenario takes into account the Fulmar litter EcoQO monitoring, which is implemented for the OSPAR area: "There should be less than X% of sea turtles having Y g or more plastic in the GI in samples of 50–100 dead turtles from each sub-region, where Y is the average weight of plastic ingested considering all the samples and X% is the percentage of sea turtles with more weight (in grams) of plastic than Y. The second one aims at considering a proxy of individual health level: "There should be less than X% of sea turtles having more weight of plastic (in grams) than food remains in the GI in samples of 50–100 dead turtles from each sub-region", where the weight of ingested plastic is compared with food remaining in each individual.

PROTOCOL:

A series of "basic" and "optional" parameters are proposed to be collected. The basic parameters correspond to the minimum parameters fundamental to accomplish the Criteria D10C3, while the optional parameters allow acquiring more knowledge on sea turtles' behavior/biology. An observation data sheet and a list of material necessary for sampling individuals in the field and analysis of litter in the laboratory are provided here in order to facilitate data recording and statistical analysis by following a standardized table. Marine litter subcategories are chosen according to the shape and type of items. Remains of sea turtle's food and anything natural that are non-food items (stone, wood, pumice, etc.) are requested for considering thresholds and the animal's diet. All the experimental activities of this protocol have been conducted on dead turtles according to the law of the involved countries and international rules. All the necropsies must be performed at the authorized centres.

1. Sampling from carcass: fill the observation sheet (Appendix 1 in Supplementary Files 1 and 2)

1.1. Fill in the contact details including name, contact (phone, mail) and institution of the observer(s) (data collector).

1.2. Identify the species as follows: **Cc** (*Caretta caretta,* Linnaeus 1758); **Dc** (*Dermochelys coriacea,* Vandelli 1761); **Cm** (*Chelonia mydas,* Linnaeus 1758); **Ei** (*Eretmochelys imbricata*, Linnaeus 1766); **Lo** (*Lepidochelys olivacea*, Eschscholtz 1829); **Lk** (*Lepidochelys Kempii,* Garman 1880); **Nd** (*Natator depressus*, Garman 1880).

1.3. Tags: If a tag already exists on the flipper, specify the number (N°. Indicate the presence and number of electronic chips. Otherwise, note NO.

1.4. Specify the animal identification code. For example: "two letters for the country"_"two letters for the location (e.g., region or institution)"_"YY" _ "MM"_"DD"_"chip number".

1.5. Note the date of discovery (yy/mm/dd).

1.6. Specify the location of discovery which is the recovery area or coordinates in decimal

degrees.

1.7. Report the specimen's body condition level: **1** (Alive), **2** (Fresh—dead recently), **3** (Partially decomposed—internal organs are still in good condition), **4** (Advanced decomposed—skin scales are raised or lost), **5** (Mummified—part of the skeleton or part of the body are missing). See **Figure 1**.

1.8. Discovery circumstances: Note the circumstances among the four categories: **Stranding** (animal found on the beach or on the shoreline); **By-catch/Fisheries** (animal captured actively by fishermen, e.g., ingestion of a hook, trapped in a net, brought back by fishermen, etc.); **Found at sea** (animal discovered on the sea surface); **Dead at the recovery centre** (the animal arrived alive, but died during its recovery).

[Place Figure 1 here]

2. Sea turtle necropsy: biometrics measurements and extraction of the contents of the gastro-intestinal tract

2.1. Arrange for the transport of the animal to the authorized center for necropsy. In case of an extremely decomposed animal, assess the integrity of the digestive tract before disposal at the authorized center. If the necropsy cannot be done immediately after the recovery, freeze the carcass at -20 °C.

2.2. Before the necropsy operation, record the biometric measurements in the specific section of the recovery file. The curved carapace length, notch to tip[23], is mandatory; the other measures are optional (e.g., curved carapace width, weight).

2.3. Conduct an external examination of the animal body and report the information in the specific section of the necropsy file. Also inspect the oral cavity for possible presence of foreign material.

2.4. Separate and remove the plastron from the carapace by making an incision along the edge as highlighted by the yellow line (**Figure 2a**).

2.5. Use a short blade or cut with a horizontal tilt avoiding damaging the interior parts (**Figure 2 b–c**). The ligament attachment to the pectoral and pelvic girdle must be cut when the plastron is detached from the carapace so that it is easy to access and handle it.

2.6. Expose the gastro-intestinal (GI) tract removing the pectoral muscles and the heart of the turtle (**Figure 2d**).

2.7. (Optional) Assess the trophic status qualitatively by evaluating the atrophy of pectoral muscles (none–moderate–severe) and fat thickness in the articular cavities and on the coelomic membrane (abundant–normal–low–none).

2.8. Extract the GI and place it on the examination table. Do this with two operators to make the actions easier. While one operator keeps the carcass laying on one side, the other separates the ligaments from the different organs and the membranes from the carapace using small blades or scissors and removes the GI from the animal (**Figure 2f**).

2.9. Isolate the esophagus, stomach and intestine using plastic clamps. Place these on the esophagus close to the mouth, at the esophageal valve, on the peg and at the cloaca, as close as possible to the anal orifice as indicated by yellow arrows (**Figure 2f**).

2.10. Record the sex of the animal when possible.

2.11. Separate the esophagus, stomach and intestine definitively by placing a second clamp (corresponding to the cutting point) to avoid spillage of the contents.

2.12. Open the GI section lengthwise using a scissor (or the fingers when possible), and then directly place the material contained into a 1 mm mesh sieve by cleaning the GI walls with running water.

2.13. Take note of each anomaly in the GI (e.g., ulcers, perforations, adhesions, inflammation).

2.14. Inspect the contents in the sieve to eventually detect any tar, oil, or fragile material that must be removed and treated separately.

2.15. Rinse the contents through the sieve in order to remove the liquid portion, mucus and unidentifiable digested matter.

2.16. Repeat the sequence for each GI portion separately.

2.17. Freeze all the material collected by the sieves or store it in jars containing 70% alcohol solution.

NOTE: For more details on the anatomy of the sea turtle see Wyneken[24].

[Place Figure 2 here]

3. Data collection and analysis: marine litter classification

3.1. Label the sample code and the respective GI section.

3.2. Empty the jars on a 1 mm mesh sieve, gathering all the material.

3.3. Rewash the collected material with water in order to eliminate alcohol and clean the litter.

3.4. Separate marine litter from the organic components or other materials, identifying the category of marine litter by visual analysis, sorting the material on a Petri dish, and sub-diving the collected items into the different categories.

3.5. Fill up the datasheet with collected information.

3.6. Use the stereomicroscope to take a closer look at any unidentifiable materials.

3.7. Dry the marine litter at room temperature or in an oven at 35 °C for 12 h.

3.8. Dry the organic fraction in an oven at 35 °C for 12 h or in a drier.

3.9. Report the number and the dry weight of the different categories of marine litter.

3.10. Report the dry weight of the organic fraction subdivided into food remain(s) and natural non-food remain(s). Total dry mass (weight in grams, accurate to the 2nd decimal place) is the main information used in the monitoring program, followed by the number of items (abundance).

3.11. Record other information such as the color of the items, volume of litter, different incidences of litter in the esophagus, stomach and intestine, and incidence per litter category as this is useful for research and impact analysis. The raw data will provide varied information for each single section of the GI; the total contents of the marine litter within the three parts will be accounted for in the final data.

REPRESENTATIVE RESULTS:

This protocol, derived from the MSFD guideline[22] and has been co-built and improved by more than 50 stakeholders (biologists from rescue centers, stranding networks, veterinarian and research laboratories) from 7 countries across the Mediterranean and the European Atlantic coasts, it proposes a homogenized, feasible and easy evaluation of litter ingestion by sea turtles. The protocol has been tested on loggerhead turtles, and most of the manipulations are also applicable to other sea turtle species. The first important result of this protocol is the description of marine litter items under seven categories according to their visual features (**Figure 3**). This classification has been derived by the Fulmar EcoQo[17,25], and modified as per the authors' experience in sea turtle ecology. The first category, and usually the least abundant one, is industrial plastic (**IND PLA**) comprised of plastic pellet and granules, usually cylindrical and round shape, but also oval or cubical shapes, rarely found to be ingested by the loggerhead turtle[16,26]. The second category comprises the remains of sheet-like (**USE SHE**) materials, such as plastic bags, agricultural sheets or plastic foil. They appear in irregular shapes but are always thin and flexible. The third category includes ropes, filaments, and other threadlike materials such as the remains of ghost fishing gear usually made of nylon (**USE THR**). The fourth category includes all foamed plastics (**USE FOA**) such as polystyrene foam or foamed soft rubber. The fifth category is includes fragments of hard plastic items (**USE FRA**). Fragments are highly abundant in the GI contents and they can be found in a variety of different colors. They are derived from broken larger pieces and are usually rigid, with an irregular shapes and sharp crooked edges. Any other

plastic items including elastics, dense rubber, balloon pieces, and soft air-gun bullets, are categorized as other user plastic (**USE POTH**). All the non-plastic marine litter such as cigarette butts, newspapers, rubbish and hard pollutant are included in the last category of litter other than plastic (**OTHER**) even if they are not easily found in sea turtles. The other two categories not classified as marine litter, are (i) remains of the turtle natural diet (**FOO**) and (ii) any natural item, not recognized as prey for the sea turtle such as stone, wood or pumice (**NFO**).

Figure 4 shows an example of representative results on the dry mass of marine litter categories, where sheet-like plastic (USE SHE) was the most abundant class, and plastic bags or parts of them, were the main ingested items. Similar results are shown in **Figure 5** in terms of the number of items (abundance). **Table 1** shows an example result of litter dry mass analysis in six different areas, which is useful for setting the threshold value according to the requirements from the European Union's MSFD. These areas should be represented, for example, by countries or sub-region of the Mediterranean basin. The reported average is calculated using all individuals examined, including samples without ingested marine litter. According to our example, area 5 represented the clearest zone of the Mediterranean basin and the data from this area could be used to set the threshold value to be reached. For this area the first scenario could be: "There should be less than **25%** of sea turtles having **0.5 g** or more plastic in the GI in samples of 50–100 sea turtles". The second scenario could be: "There should be less than **32%** of sea turtles having more plastic grams than food remains (**FOO**) in the GI in samples of 50–100 sea turtles".

[Place Figure 3 here]
[Place Figure 4 here]
[Place Figure 5 here]
[Place Table 1 here]

FIGURE AND TABLE LEGENDS:

Figure 1: Specimen's body condition level or decomposition status.

Figure 2: Sequence of turtle necropsy. (**a**) Ventral view of a dead turtle. The yellow line indicates the way to cut in order to separate the plastron from the rest of the turtle. (**b,c**) Horizontal cuts to prevent affecting the interior organs. (**d**) Ventral view of the opened turtle. (**e**) Extraction of the GI tract. (**f**) View of the entire GI, yellow arrows indicate where clamps must be attached in order to separate the three different GI sections.

Figure 3: Examples of marine litter categories established for marine turtle ingestion monitoring. (**a**) IND PLA, (**b**) USE SHE, (**c**) USE THR, (**d**) USE FOA, (**e**) USE FRA, (**f**) USE POTH, (**g**) OTHER, (**h**) FOO.

Figure 4: Example results of weights of marine litter ingested by sea turtles under the various categories. The average weight values are reported in grams of items per individual (± SE).

Figure 5: Example results for the number of marine litter categories ingested by sea turtles. The

353 average number of items per individual (± SE) are reported.
354
355 **Figure 6: Fragmentation of single items could occur before ingestion or during the feeding**
356 **process, producing bias in counting.**
357
358 **Table 1: Example of results from different areas (e.g., countries, sub-regions, etc.), using dry**
359 **mass of marine litter.**
360
361 **DISCUSSION:**
362 This protocol allows evaluation of the total abundance of marine litter, and identification of the
363 main litter categories ingested by sea turtles. It is less expensive compared to other monitoring
364 programs with sea activities because sea turtles could be collected after stranding on the beach
365 or be recovered by fishermen. The identification of marine litter categories is easy and rapid as
366 the lower limit on item size is 1 mm. A limitation of the protocol is the use of sea turtle considering
367 that all 7 species of marine turtles are listed on Appendix I of the Convention on International
368 Trade in Endangered Species of Wild Fauna and Flora[27]; therefore, only authorized personnel can
369 handle live and dead animals or parts of them. Turtle management and recovery should be
370 reported and coordinated with the corresponding authorities. Sanitary precautions must be
371 taken when handling dead or alive wild animals to minimize risks of zoonosis. This protocol has
372 been tested on loggerhead species but it is applicable to all the seven turtle species. Data analysis
373 should be carried out separately for each species. The specimen's body conditions as considered
374 under five levels from alive to mummified turtles. Level 1 (Alive) is considered for a more detailed
375 classification of the specimen's body condition in case the turtle died at the rescue center after
376 the recovery. The protocol is applicable to dead individuals from levels 2 to 4, but also on
377 individuals that died after recovery (circumstances: dead at the recovery center). Levels 2 and 3
378 are adequate for the protocol while level 4 allows to measure biometrics data and assessing the
379 presence/absence of ingested litter for the evaluation of frequency of occurrence (FO%), and the
380 percentage of turtles with ingested marine litter on the entire sample. Individuals of level 5,
381 where generally the gastrointestinal content has been lost cannot be considered for the
382 collection and quantification of litter ingestion. Taking photos of the animal before handling,
383 could provide additional information on the sample as the probable cause of death or main
384 injuries and entanglement. It is important to include a scale bar on the pictures. Even if often sea
385 turtles had fishing hooks in their GI, data do not have to be included in the analysis because
386 fishing hooks on which longline victims are actively caught are not considered as "marine litter".
387 Hook presence should be recorded in the notes. Collection of data should be performed
388 separately in each part of the GI (esophagus, stomach, intestine), in order to evaluate the degree
389 of tolerance to marine litter ingestion considering GI blockage or the capability to eliminate it
390 through defecation, as demonstrated in previous studies[16,28,29,30,31,32]. A critical step of the
391 protocol could be found in the collection of the number of items. Multiple pieces could be derived
392 from fragmentation of the same object inside the GI or as a consequence of separate ingestion.
393 Subjective interpretation of a single item or multiple separate pieces could correspond to a
394 potential bias in recording number (**Figure 6**). For this reason, threshold values have been
395 elaborated using only ingested marine litter mass data, like the Fulmar EcoQO[17,25].
396

[Place Figure 6 here]

The protocol requires the categorization of different plastic items according to their shapes (USE SHE, USE THR). This sub-division is useful to identify the source of marine litter with a list of items according to their abundance. It aides policy makers in their programs of measures, providing rapid evidence of their efficiency in targeting items by evaluating their strength. For example, the ban of plastic bags in the markets should correspond to a reduction of USE SHE category ingested (**Figure 4**, **Figure 5**) in sea turtle samples collected in the future. The application of this protocol will allow EU member states to answer to the MSFD requirements, evaluating their own baselines and defining the threshold values at which GES is achieved. Thresholds should be determined in pristine or next to pristine areas. Due to the ubiquity of plastic in the marine environment, a pristine area does not exist. According to the example data (**Table 1**), area 5, was the clearest zone and could represent the value (**Y**) to be reached for the Mediterranean basin. Member states should decide thresholds according to the significant reduction of their own distance from this value. According to a recent review[18], marine litter ingestion units should be normalized to the size of the turtle, especially if the goal is to compare different age classes. Nevertheless, a relationship between the mass of ingested litter and turtles size has been detected by different authors with positive, negative or zero values[16,26,32,33,34]. Our protocol does not include animal size in the first scenario, but it could be possible to estimate the body burden, evaluating mass of the turtle using curved carapace length (CCL)[35] and use the ratio of weight of plastic weight of turtle instead of only grams of ingested plastic (Y). In any case we suggest verifying any possible significant differences before merging oceanic stage turtles with neritic ones or early juveniles with adults, in order to better stratify the samples[16,26]. The second scenario is more related to the individual health status and could better answer to the Criteria D10C3: "The amount of litter and microlitter ingested by marine animals is at a level that does not adversely affect **the health of the species** concerned". In fact, impact of ingested plastic items consists most frequently in sub-lethal effects rather than lethal ones[28,36,37,38,39]. We also rarely found an occlusion or a perforation due to plastic ingestion, which could cause the death of the turtles. Sub-lethal effects are not easy to be detected and to be distinguished from impacts due to other pollutants[40]. Dietary dilution or assimilation of contaminants happens when marine litter is inside the GI of the turtle[41]. Thus the sample with more grams of plastic than food remains could indicate an animal in a very bad health condition. In order to remain in line with the Fulmar EcoQO[17,25] used by the northern European countries, both scenarios consider plastic weigth instead of marine litter weight.

Finally it is important to clarify the differences between (i) analyzing the ingestion of plastic in sea turtles as indicator of impact on population with consequences for population conservation and (ii) analyzing the ingestion of plastic in sea turtles as bio-indicator of impact on the coastal and marine environment[20,40]. To understand the implications of this impact on conservation of turtles population, more information is needed and better data stratification is necessary[42]. By confronting the opinion of 35 specialists from 13 nations, who are experts in sea turtle biology and conservation, it is clear that sea turtles have been widely studied across the years, although it is still necessary to investigate the interactions with human activities and therefore assess the population status and potential threats[43].

This means that a single protocol cannot be considered as exhaustive for all the thematic and more studies are necessary to understand the impact of plastic at population level.

Even tough plastic could be considered to cause a low level of damage to sea turtles, with respect to by-catch or habitat destruction, its reduction has been challenging in the last few years and quick methods of measurement must be elaborated. There is a controversy in the use of stranded turtles for monitoring purposes because, according to some authors they are not representative of the whole population[40], while others have declared that stranded turtles do not represent a bias of marine litter ingestion rates in the background population[44]. Moreover, in many countries there is not a well-organized stranding network or system linking rescue centers to fishermen and there is a lack of information on by-catch and post release mortality by fisheries. Hence, stranded samples cannot always be considered as sick turtles without normal feeding behavior for a time period before dying and reaching the beach; many of them are "death at sea" turtles washed ashore and are usually used as samples in monitoring activities[26,32,38,45]. We believe that stranded samples are useful in providing information on the level of marine litter abundance in the environment and we suggest excluding only turtles with completely empty gastrointestinal tract from this analysis as they could be sick from a long time before death. The use of this protocol would enable evaluation of environmental status and marine litter availability for marine organisms. It could also be helpful in improving our knowledge on turtle behavior. The significance of the method with respect to the MSFD TS-ML guidelines[22], is due to the harmonization in seven countries and the number of samples on which it has been tested (n = 700). Specimen's body condition level has been defined and marine litter ingested categories have been reduced according to the preliminary results. Moreover, this is the first time representative results have been shown and connected to the GES thresholds.

The protocol is an efficient tool for researchers to understand the impact of plastic on the marine environment, globally or at a local scale, and for comparing standardized data with neighboring countries. This result could not be reached before, due to the discrepancies in data among different countries, preventing any spatial comparison.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1: Specimen's body condition level or decomposition status.

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Figure 2: Sequence of turtle necropsy: a) Ventral view of a dead turtle. The yellow line indicates the way to cut in order to separate the plastron from the rest of the turtle; b-c) Horizontal cuts to prevent affecting the interior organs; d) ventral view

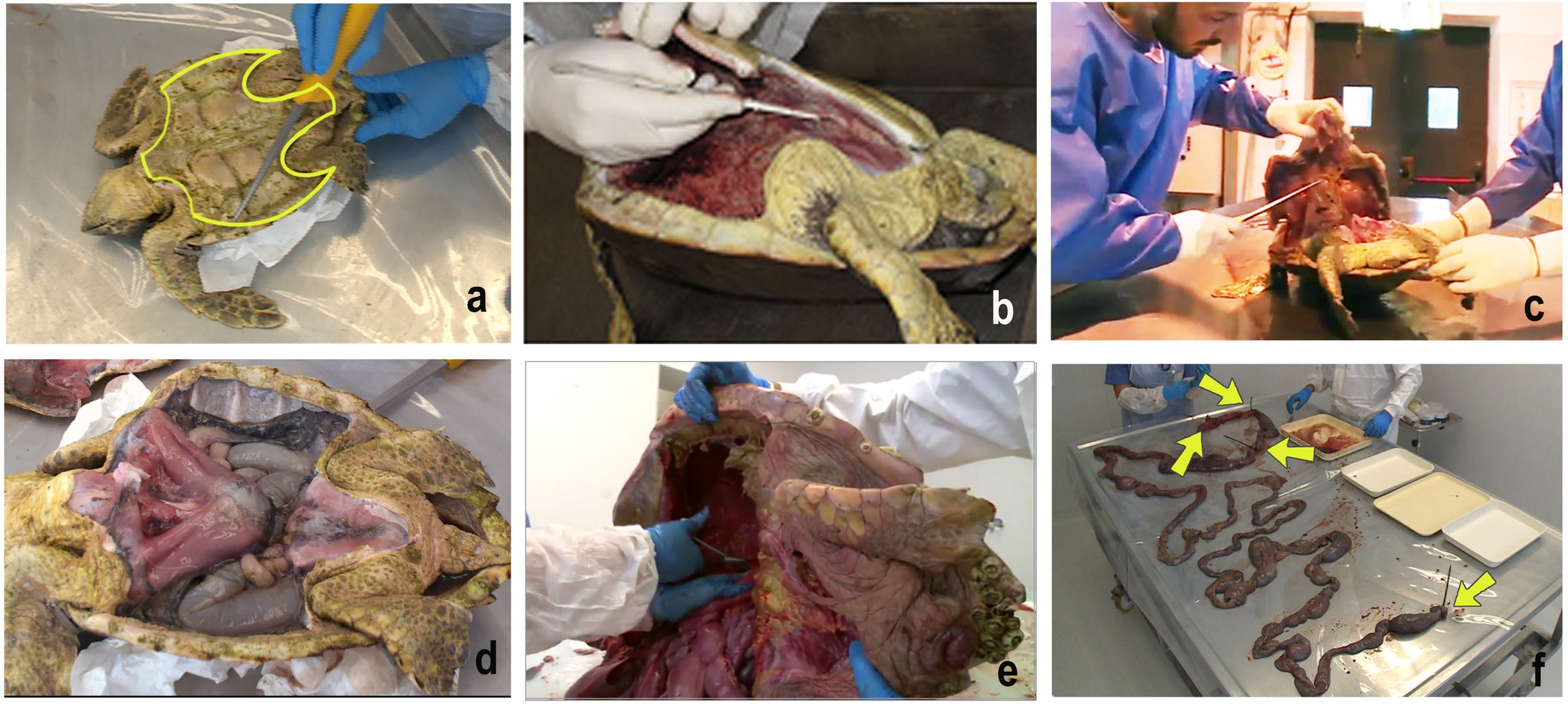

Figure 3: Examples of marine litter categories established for marine turtle ingestion monitoring: a) IND PLA b) USE SHE c) USE THR d) USE FOA e) USE FRA f) USE POTH g) OTHER h) FOO.

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Figure 4: Example of results for weight of marine litter categories

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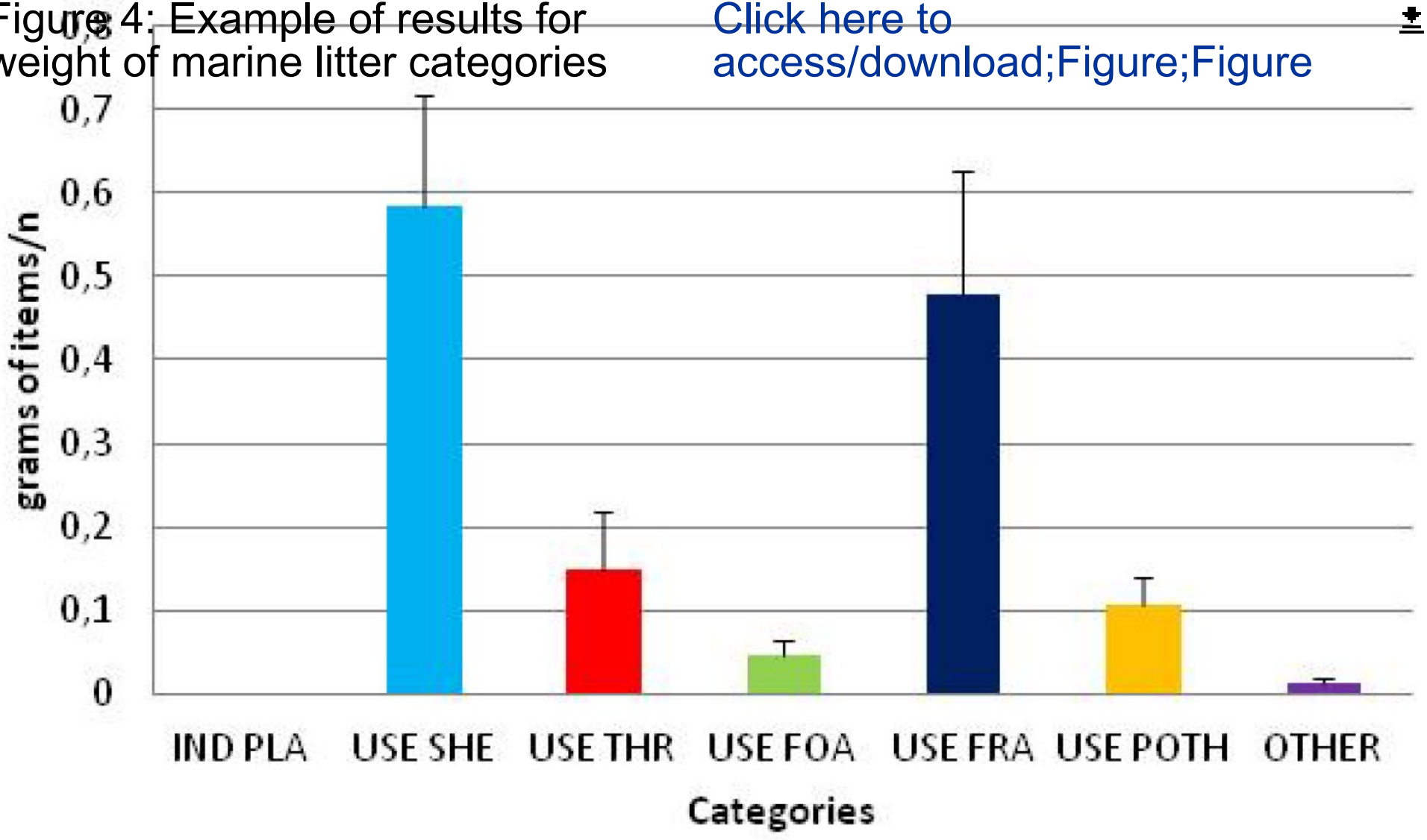

Figure 5: Example of results for number of marine litter categories

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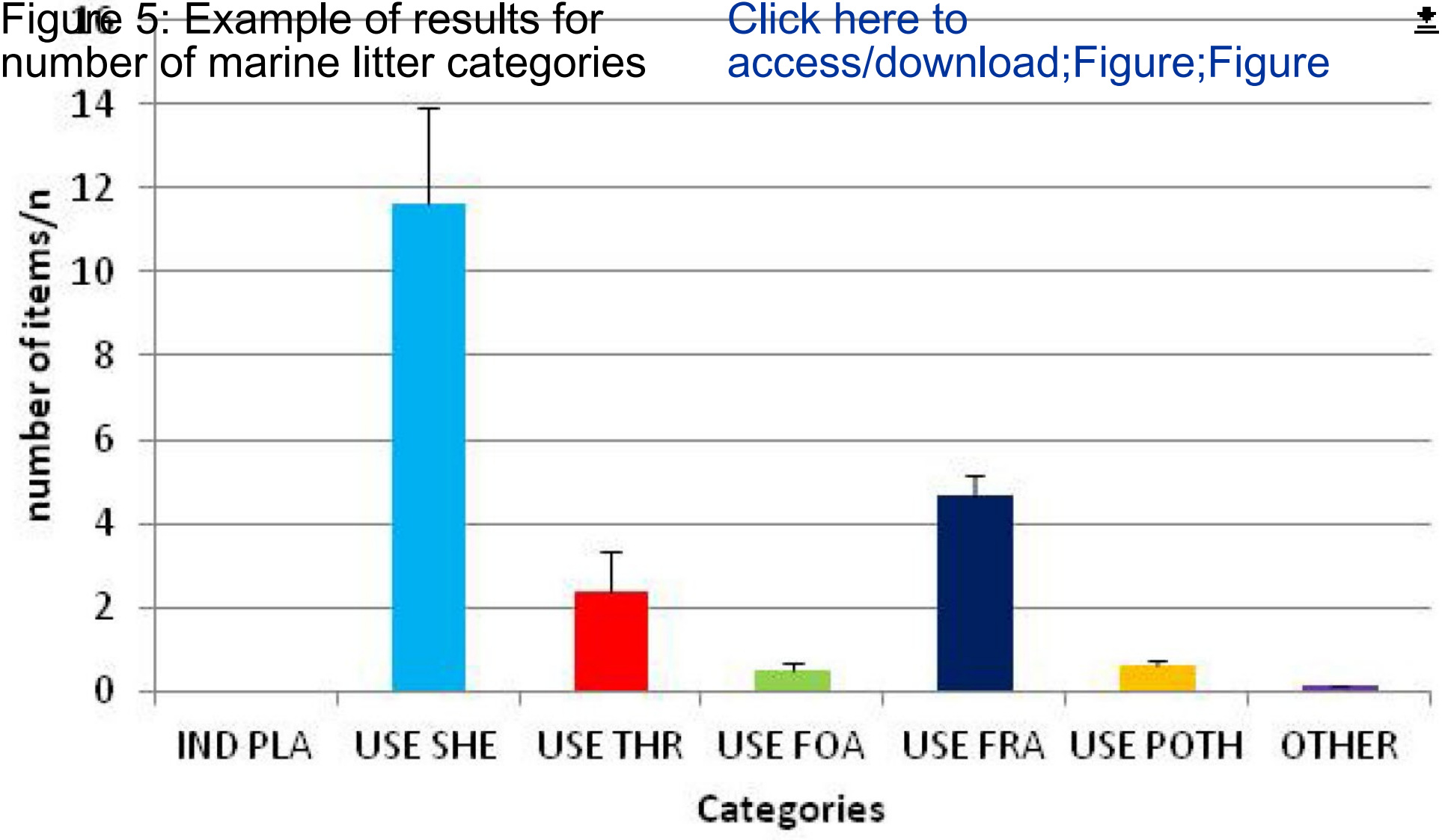

Figure 6: Fragmentation of single items could occur

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Table 1: Example of results from different AREAs (e.g. countries, sub-regions, etc.), using dry mass of marine litter.

AREA	Sample size (n)	Average value ±SE for dry weight of ingested plastic (g)	Percentage of turtles with more plastic than average value (%)
1	100	1.32±0.03	27
2	100	1.61±0.01	28
3	100	1.35±0.02	26
4	95	0.73±0.02	34
5	65	0.55±0.03	25
6	50	0.90±0.04	44

Percentage of turtles with more plastic than food remain (%)
64
67
62
40
32
54

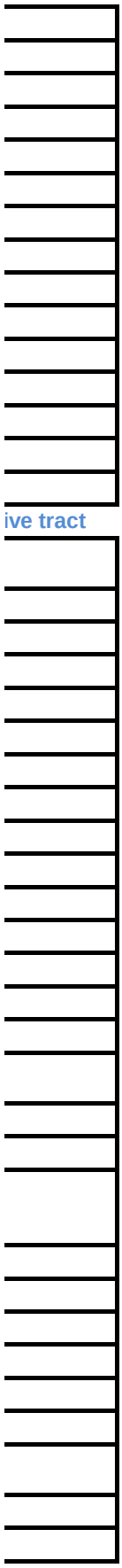
Table of Materials

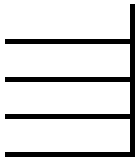
Click here to access/download;Table of Materials;Material Table.xlsx

For the recovery of the animal and the collection of samples at the discovery site
Boots
Bottle/ziploc bags
Camera
Cooler
Cut-resistant gloves
Garbage bag
Glasses and protective mask or shield
Gloves
Integral protective suit
Measuring tape
Observation sheet
Pen
Permanent marker
Rope (to marke-off the zone)
Transport bins or containers for the turtle

collection of samples on dead individuals in laboratory and the extraction of the ingested litter from the digesti
In the laboratory room
Cold chamber or chest freezers (-20°C) with large storage capacity
Garbage bags
Proofer (not mandatory)
For manipulators
Boots
Cut-resistant gloves
Glasses and protective mask or shield
Gloves
Integral protective suit
For notes and report
Camera
Observation sheet
Pen
Permanent marker
For biometric measurements
Measuring tape
Sliding calliper
For the necropsy and the collection of samples
Clamps (at least 6) and/or kistchen string or plastic cable clamps
Clips with claws
Containers for samples (Bottle/zipped bags)
Metal containers
Scalpel (possible with interchangeable blade)
Scissors
For the analysis of ingested litter
Binocular (optional)
Measuring cylinders (10 ml, 25 ml, 50 ml)

Measuring decimetre
Precision balance (0.01 g)
Sieve with 1 mm mesh
Sieve with 5 mm mesh (optional – for the study of the ingested micro-plastics (1-5 mm))


ive tract



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Rebuttal letter

Dear Editor,

We made all the changes you requested to the manuscript and to the video.

Please let us know your availability to review our work.

Marco Matiddi et al.

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TITLE:

~~Protocol for~~ Dd~~d~~ata collection on marine litter ingestion in sea turtles

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KEYWORDS:
Sea turtle, plastic ingestion, necropsy, marine litter, MSFD, EcAp process, Good Environmental Status, thresholds.

SUMMARY:

The protocol focuses on:

i. ~~The~~ the collection of samples describing ~~(individual to digestive tract)~~ all the steps from the animal recovery and necropsy~~.~~ to the classification and quantification of ingested marine litter. Moreover, the representative results show how to use the collected data to elaborate the possible thresholds for Good Environmental Status.

~~ii. The procedure for assessing the sea turtle body condition.~~
~~iii. The steps of the necropsy until the isolation of gastrointestinal tract.~~
~~iv. The classification and quantification of ingested marine litter.~~
~~v. Thresholds for Good Environmental Status.~~

ABSTRACT:

The following protocol is intended to respond to the requirements set by the European Union's Marine Strategy Framework Directives (MSFD) for the D10C3 Criteria reported in the Commission Decision (EU)[1], related to the amount of litter ingested by marine animals. Standardized methodologies for extracting litter items ingested from dead sea turtles along with guidelines on data analysis are provided. The protocol starts with the collection of dead sea turtles and classification of samples according to its decomposition status. Turtle necropsy must be performed in authorized centres and the protocol described here explains the best procedure for gastrointestinal (GI) tract isolation. The three parts of the GI (~~o~~esophagus, stomach, intestine) should be separated, opened lengthways and contents filtered in a 1 mm mesh sieve. The video describes the classification and quantification of ingested litter, classifying GI contents into seven different categories of marine litter and two categories of natural remains. The quantity of ingested litter should be reported as total dry mass (weight in grams, with two decimals) and abundance (number of items). The protocol proposes two possible scenarios to achieve the Good Environmental Status (GES). First: "There should be less than X% of sea turtles having Y g or more plastic in the GI in samples of 50-100 dead turtles

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from each sub-region", where Y is the average value of plastic ingested and X% the percentage of sea turtles with more weight (in grams) of plastic than Y. The second one, which considers the food remain *vs* plastic as a proxy of individual health, is: "There should be less than X% of sea turtles having more weight of plastic (in grams) than food remains in the GI in samples of 50-100 dead turtles from each sub-region".

INTRODUCTION:

Marine Litter is a complex issue to address since it can enter the oceans via multiple sources and forms. Over 80% of the Litter that is encountered in marine environments is made up of ~~plastic[2]~~plastic[1]. The role of this material from an economical perspective has been increasing in the last 50 years, as consequence also its production has increased twentyfold since 1960, reaching 335 million tonnes in 2016. This value is expected to double over the next 20 ~~years[3]~~years[2]. Moreover, it has been estimated that around 5 to 13 million tonnes of plastic end up in the oceans every year (which is equal to 1.5 to 4% of global plastic production)~~.[4]~~[2,3]. ~~Due to the dynamics and environmental conditions of the oceans combined with the physical properties of plastic, the latter can be found in all the five environmental marine compartments: coastline, water surface, water column, seafloor and biota.~~ Plastic movement ~~can be~~is influenced by its physical properties (e.g. buoyancy)~~and~~ or environmental ~~variables[5]~~variables (e.g. tide and stream), and can be accumulated in all the environmental marine compartments[4,5] To face the plastic problem, it is important to bare in mind that, as many other environmental issues, it is transboundary and therefore governance solutions are complex to meet[6]. To better reach this goal we must take into consideration regional and international frameworks, so to enhance or maintain marine environmental awareness and protection across the globe[7]. The final objective of the European Union's Marine Strategy Framework Directive (MSFD) is to achieve a good environmental status (GES) in European waters by 2020 and to protect marine biodiversity and to promote the sustainable use of marine environments. This will be done through 11 qualitative descriptors, of which Descriptor 10 focuses on marine litter and is defined as "*Properties and quantities of marine litter do not cause harm to the coastal and marine environments*". Within this descriptor, the New Commission ~~Decision[1]~~ Decision[8] decided to add Criteria D10C3 "*The* amount of litter and microlitter ingested by marine animals is at a level that does not adversely affect the health of the species concerned" since it was considered to be a relevant criteria in the evaluation of GES. As a result, Member States were requested to produce a list of species, to develop methodological standards and define threshold values through regional or sub-regional cooperation.

After the first scientific publication in ~~1838[8]~~1838[9], on the Storm-Petrel with an ingested candle stick, over 500 marine species have been listed for ingesting marine ~~litter[9]~~litter[10,11,12,13,14], and sea turtles were among the first taxa recorded to ingest plastic ~~debris[11]~~debris[15]. Given their propensity to ingest litter, their wide distribution and the large range of habitats used during their life, sea turtles, in particular the loggerhead species *Caretta caretta (*Linnaeus 1758*)*, was chosen as a potential indicator for the Mediterranean ~~basin[12]~~basin[16,3], alike the sea bird *Fulmarus glacialis* (Linnaeus, 1761) for the Northern European ~~waters[13]~~waters[17]. Even after five decades of research, discussion of method standardization has been very ~~limited[14]~~limited[18] and a cohesive approach by the scientific community to quantify plastic ingestion by wildlife is

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~~lacking~~[15]lacking[19]. Standardized sampling protocol and analytical detection methods and metrics to assess plastic ingestion by marine biota are needed, a recent paper show the potential and limits of using marine species as bio-indicators on plastic pollution[3420]. Following the Matiddi *et al.*, proposal in ~~2011~~[16] 2011[21] to use the loggerhead as bio-indicator, and the Technical Group on Marine Litter ~~report~~[17] ~~r~~Report[22], a specific protocol to consider marine litter ingested by sea turtle has been developed and tested by ten partners in seven countries in the Mediterranean Sea and Atlantic Ocean within the European Project INDICIT (GA n°11.0661/2016/748064/SUB/ENV.C2). This protocol provides standardized methodologies for the analysis of marine litter ingested by sea turtles in order to support the new Commission Decision (EU)[1188], Criteria D10C3, where threshold values are requested. According to the definition provided by ~~COM~~[1] COM[188] the threshold value is a number or a range that allows to evaluate if the quality level criterion was achieved, therefore helping in assessing the Good Environmental Status. The proposed protocol, in assessing the litter ingested by sea turtles, will be useful in gathering data on the composition and abundance of Litter and evaluate its impact on marine environments. Moreover, by collecting this type of ~~standardised~~standardized data will help to define threshold values. Here we consider two types of scenarios. The first scenario takes into account the Fulmar litter EcoQO monitoring, which is implemented for the OSPAR area: "There should be less than X% of sea turtles having Y g or more plastic in the GI in samples of 50-100 dead turtles from each sub-region, where Y is the average value of plastic ingested considering all the sample and X% the percentage of sea turtles with more weight (in grams) of plastic than Y. The second one aims at considering a proxy of individual health level: "There should be less than X% of sea turtles having more weight of plastic (in grams) than food remains in the GI in samples of 50-100 dead turtles from each sub-region", where the weight of ingested plastic is compared with food remain in each individual.

PROTOCOL:

A series of "basic" and "optional" parameters are proposed to be collected. The basic parameters correspond to the minimum parameters fundamental to accomplish the Criteria D10C3, while the optional parameters allow acquiring more knowledge on sea turtles' behaviour/biology. An observation data sheet and a list of material necessary for sampling individuals in the field and analysis litter in laboratory are provided here in order to facilitate banking and statistical analysis, following a standardized table. Marine litter subcategories are chosen according to the shape and type of items. Remains of sea turtle's food and anything natural that are no food items (stone, wood, pumice etc.) are requested for threshold considerations and animal's diet.

All the experimental activities of this protocol have been conducted on dead turtles according to the law of the involved countries and international rules. All the necropsies must be performed at the authorized centres.

1. **Sampling from carcass: fill the observation sheet (Appendix 1)**

1. Contact: Name, contact (phone, mail) and institution of the observer(s) (data collector).
2. Species identification: **Cc** (*Caretta caretta,* Linnaeus 1758); **Dc** (*Dermochelys coriacea,* Vandelli 1761); **Cm** (*Chelonia mydas,* Linnaeus 1758); **Ei** (*Eretmochelys imbricata,*

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Linnaeus 1766); **Lo** (*Lepidochelys olivacea*, Eschscholtz 1829); **Lk** (*Lepidochelys Kempii*, Garman 1880); **Nd**(*Natator depressus*, Garman 1880). ~~etc.~~

3. Tags: If a tag already exists on the flipper, specify the number. Indicate the presence and number of electronic chip. Otherwise, note NO.
4. Animal Identification Code: Example: 2 letters for the country _ 2 letters for the location (e.g. region or institution) _ Year _ Month _ Day_N° turtle.
5. Date of discovery: (dd/mm/yyyy).
6. Location of discovery: Recovery area or coordinates in decimal degrees.
7. Report the specimen's body condition level: 1 (A~~a~~**live**), 2 (F~~f~~**resh**), 3 (P~~p~~**artially decomposed**), 4 (A~~a~~**dvanced decomposed**), 5 (M~~m~~**ummified**), (Fig.1).
8. Discovery circumstances: Note the circumstances among the 4 categories: **Stranding:** animal found on the beach or on the shoreline; **By-catch/Fisheries**: animal captured actively by fishermen (e.g. ingestion of a hook, trapped in a net, brought back by fishermen, etc.); **Found at sea**: animal discovered on sea surface; **Dead at the recovery centre**: the animal arrived alive, but died during his recovery.

[Place Figure 1 here]

2. Sea turtle necropsy: biometrics measurements and ~~gut content extraction~~Gastro Intestinal tracts content extraction

1. Organize the transport of the animal to the authorized centre for necropsy. In case of an extremely decomposed animal, assess the integrity of the digestive tract ~~should be assessed~~ before disposal at the authorized centre. If the necropsy cannot be done immediately after the recovery, freeze the carcass at -20°C.
2. Before the necropsy operation~~,~~ record the biometrics measurements ~~should be recorded~~ in the specific section of the recovery file. The curved carapace length, notch to tip[23], is mandatory; the other measures are optional (e.g. curved carapace width, weight).
3. Conduct ~~An~~ an external examination of the animal body ~~should be conducted~~ and report the information ~~must be reported~~ in the specific section of the necropsy file. Also the oral cavity must be inspected, for possible presence of foreign material.
4. Separate and remove the p~~P~~lastron ~~must be separated and removed~~ from the carapace by performing an incision on the edge as highlighted by the yellow line (~~in figure~~ Fig. 2~~.~~a)
5. ~~It is important to~~ U~~u~~se a short blade or ~~to~~ cut with a horizontal tilt avoiding ~~to~~ damagin~~g~~e the interior parts (Fig. 2 b-c). The ligament attachment to the pectoral and pelvic girdle ~~should have to~~must be cut when plastron is detached from the carapace and it is easy to access and handle it.
6. Expose ~~The~~ the Gastro Intestinal tracts (GI) ~~should be exposed by~~ removing the pectoral muscles and the heart of the turtle (Fig. 2d).
7. Optional: the trophic status ~~should~~ have to be qualitatively assessed by evaluating the atrophy of pectoral muscles (none – moderate – severe) and fat thickness in the articular cavities and on the coelomic membrane (abundant – normal – low – none).
8. Extract t~~T~~he GI and place ~~must be extracted and entirely placed~~ it on the examination

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surface. , ~~possibly by~~Do this with two operators to make the actions easier. While one of the operators keep the carcass laying on one side, the other separates the ligaments from the different organs and the membranes from the carapace using small blades or ~~fingers~~ scissors and removing the GI from the animal (Fig. 2f).

9. E~~O~~oesophagus, stomach and intestine must be isolated using plastic clamps, these are placed on the ~~o~~esophagus close to the mouth, at the ~~o~~esophageal valve, on the peg and at the cloaca, as close as possible to the anal orifice as indicated by yellow arrows (Fig. 2f).
10. Record the sex of the animal when possible.
11. E~~O~~oesophagus, stomach and intestine must be definitively separated placing a second clamp (in correspondence of the cutting point) to avoid spillage of the contents.
12. Open ~~The~~ the GI section ~~should be opened~~ lengthways using a scissor (or the fingers when possible), the material contained must be placed directly into a 1mm mesh sieve by cleaning the GI walls with running water.
13. Take note of each anomaly in the GI (e.g. ulcers, perforations, adhesions, inflammation).
14. Inspect the contents in the sieve to eventually detect any tar, oil, or particular fragile material that must be removed and treated separately.
15. Rinse t~~T~~he contents ~~must be rinsed~~ through the sieve in order to remove the liquid portion, mucus and digested unidentifiable matter.
16. Repeat the sequence for each GI portion separately.
17. Freeze a~~A~~ll the collected material by the sieves ~~should be freezed~~ or store it~~d~~ in jars with 70% alcohol solution.

Note: For more ~~detailed~~details on the anatomy of sea turtle see also Wyneken, (2001)[24].

[Place Figure 2 here]

3 Data collection and analysis: marine litter classification

1. Label the sample code and the respective GI section.
~~1.~~2. Empty the jars on a 1mm mesh sieve, gathering all the material.
~~2.~~3. Rewash the collected material with water in order to eliminate alcohol and clean litter.
~~3.~~4. Separate m~~M~~arine litter ~~should be separated~~ from the organic components or other materials, identifying the category of marine litter by visual analysis, ~~following the analytical protocol.~~sorting the material on a Petri dish subdiving the collected items in the different categories.
~~4.~~5. Fill the data sheet with collected information.
~~5.~~6. Use the stereomicroscope for any uncertain materials.
~~6.~~7. Dry out the marine litter at room temperature or in a stove at 35°C for 12 hours.
~~7.~~8. Dry the organic fraction in a stove at 35°C for 12 hours or in a drier.
~~8.~~9. Report the number and the dry weight of the different categories of marine litter.
~~9.~~10. Report the dry weight of the organic fraction subdivided in Food remain(s) and Natural Non Food remain(s).

~~10.~~11. Total dry mass (weight in grams, accurate to 2nd decimal) is the main information useful for monitoring program, followed by number of items (abundance).

~~11.~~12. Other information as colour of items, volume of litter, different incidence of litter in ~~o~~esophagus, stomach and intestine, incidence per litter category, are useful for research and impact analysis.

~~12.~~13. The raw data will provide different information for each single section of the GI; the total content of the marine litter within the three parts will be accounted in the final data.

REPRESENTATIVE RESULTS:

This protocol, derived from the MSFD ~~guideline[17]~~ guideline[22] and has been co-built and improved by more than 50 stakeholders (biologists from rescue centers, stranding networks, veterinarian and research laboratories) from 7 countries across the Mediterranean and the European Atlantic coasts, it proposes a homogenized, feasible and easy evaluation of litter ingestion by sea turtles. The protocol has been tested on loggerhead turtles, and most of the manipulations are also applicable to other sea turtle species.The first important result of this protocol is the description of marine litter items into seven categories according to their visual features (Fig. 3). This classification has been derived by the Fulmar EcoQo[17,25], modified according to the authors experience on sea turtles ecology. The first category, and usually the less abundant one, is Industrial plastic (**IND PLA**) composed by plastic pellet and granules, usually cylindrical and round shape, but also oval or cubical shapes, rare to find ingested by ~~turtles~~loggerhead turtle[16,26]. The second category concerns remains of sheetlike (**USE SHE**), as plastic bags, agricultural sheets or plastic foil. They appear in irregular shapes but always thin and flexible. ~~White and transparent are the main abundant colors found in sea turtles GI.~~

The third category includes ropes, filaments, other threadlike materials and remains of ghost fishing gears usually made of nylon (**USE THR**). The fourth category includes all foamed plastics (**USE FOA**) as polystyrene foam or foamed soft rubber. The fifth category is represented by fragments of hard plastic items (**USE FRA**). Fragments are highly abundant in the GI contents and they can be found in a variety of different colors. They are derived from broken larger pieces usually rigid, with an irregular shape and sharp crooked edges. Any other plastic items including elastics, dense rubber, balloon pieces, soft air-gun bullets, ~~etc.~~ are categorized as other user plastic (**USE POTH**). All the non plastic marine litter as, cigarette buts, newspapers, rubbish and hard pollutant are included in the last category litter other than plastic (**OTHER**) even if they are not easy to be found in sea turtles. Other two categories, not classified as marine litter, are 1) remains of the turtle natural diet (**FOO**) and 2) any natural item, not recognized as prey for the sea turtle as stone, wood or pumice (**NFO**).

Figure 4, shows an example of representative results on the dry mass of marine litter categories, where sheetlike plastic (Use SHE) is the most abundant class, being plastic bags or part of them, the main ingested items. Similar results are showed in figure 5 considering number of items (abundance). Table 1, shows an example of results of litter dry mass analysis in six different AREAs, useful for setting of threshold value according to the requirements by the European Union's MSFD. These AREAs should be represented, for example, by countries or sub-region of the Mediterranean basin. The average reported is calculated using all individuals examined, including samples without ingested marine litter. According to our example, AREA 5

represents the clearest zone of the Mediterranean basin and data from this area could be used to set the threshold value to be reached.
For this AREA the first scenario could be: There should be less than **25%** of sea turtles having **0.5 g** or more plastic in the GI in samples of 50-100 sea turtles".

Second scenario could be: "There should be less than **32%** of sea turtles having more plastic grams than food remains (**FOO**) in the GI in samples of 50-100 sea turtles".

[Place Figure 3 here]

[Place Figure 4 here]

[Place Figure 5 here]

[Place Table 1here]

FIGURE AND TABLE LEGENDS:

Figure 1: Specimen's body condition level or decomposition status.
Figure 2: Sequence of turtle necropsy: a) Ventral view of a dead turtle. The yellow line indicates the way to cut in order to separate the plastron from the rest of the turtle; b-c) Horizontal cuts to prevent affecting the interior organs; d) ventral view of the opened turtle; e) extraction of the GI tract; f) view of the entire GI, yellow ~~narrows~~ harrows mark where clamps must be attached in order to separate the 3 different GI sections.
Figure 3: Examples of marine litter categories established for marine turtle ingestion monitoring: a) IND PLA b) USE SHE c) USE THR d) USE FOA e) USE FRA f) USE POTH g) OTHER h) FOO.
Figure 4: Example of results for weight of marine litter categories ingested by sea turtles. It is reported the average weight value in grams of items per individual (± SE)~~n=510).~~
Figure 5: Example of results for number of marine litter categories ingested by sea turtles. It is reported the average number of items per individual (± SE).~~(n=510).~~
Table 1: Example of results from different AREAs (e.g. countries, sub-regions, etc.), using dry mass of marine litter.
Figure 6: Fragmentation of single items could occur before ingestion or during the feeding process, producing bias in count**.**

TABLE OF MATERIALS:
Appendix 1
Appendix2

DISCUSSION:
This protocol allows ~~to have rapid information on~~to evaluate the total abundance of marine litter ~~in the marine environment and more important~~, and identify the ~~different~~ main ~~availability of specific items~~ categories ingested by sea turtles. ~~for sea turtles.~~
It is ~~easy, rapid and intuitive and~~ less expensive compared to other monitoring programs with

sea activities because sea turtles could be collected stranded on the beach or recovered by fishermen. The identification of marine litter categories it is easy and rapid as the lower items limit is 1 mm. A limitation of the~~–~~ protocol ~~–It~~is the use of sea turtle considering ~~must be considered~~ that all 7 species of marine turtles are listed on Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora[27], therefore only authorized people can handle live and dead animals or parts of them. Turtle management and recovery should be reported and coordinated with the corresponding Authorities. Sanitary precautions must be taken when handling dead or alive wild animals to minimize risks of zoonosis. This protocol has been tested on loggerhead specie~~s~~ but it is applicable to all the seven species. Data analysis should be carried out separately for each species. The specimen's body conditions consider 5 levels from alive to mummified turtles~~.~~. The Level 1 (Alive) has been considered for a more detailed classification of specimen's body condition in case the turtle dead at the rescue centre after the recovery. The protocol ~~being~~ is applicable to dead individuals from levels 2 to 4, but also on individuals died after recovery (Circumstances: Dead at the recovery centre). Level 2 and 3 are adequate for the protocol while level 4 allows to measure biometrics data and assessing the presence/absence of ingested litter for the evaluation of Frequency of occurrence (FO%), the percentage of turtles with ingested marine litter on the entire sample. Individuals of level 5, where generally the gastrointestinal content~~s~~ has been lost, cannot be considered for the collection and quantification of litter ingestion. Taking photos of the animal before handling, could give additional information on the sample, as probable cause of death or main injuries and entanglement type. It is important to include into the picture ~~something as~~ a scale bar. Even if often sea turtles had fishing hooks in their GI, data do not have to be included in the analysis because, fishing hooks on which longline victims are actively caught are not considered as "marine litter". Hook presence should be recorded in the notes. Collection of data should be performed separately in each part of the GI (~~o~~esophagus, stomach, intestine), in order to evaluate degree of tolerance to marine litter ingestion considering GI blockage or the capability to eliminate it trough defecation, as demonstrated in previous studies[16~~,~~,28,29,30,31,32]. A critical step of the protocol could be found in the collection of number of items. ~~Usually in the scientific papers the word item is commonly used to identify part of an object.~~ Multiple pieces could be derived from a fragmentation of the same object inside the GI or as a consequence of separately ingestion. Subjective interpretation of single item or multiple separate pieces could correspond to a potential bias in recording number (Fig.6). For this reason, ~~, in this manuscript,~~ threshold values have been elaborated ~~giving emphasis mainly~~ using only ~~on~~ ingested marine litter ~~dry~~ mass data, ~~the same happens for~~ alike the Fulmar EcoQO[17,25].

[Place Figure 6 here]

The protocol requires the categorization of different plastic items according to their shape (USE SHE, USE THR, ~~etc.~~).This sub-division is useful to identify source of marine litter with a top list of items, according to their abundance. It supports policy makers for their programs of measures, giving a rapid evidence of their efficiency for target items by evaluating their strength. For example, the ban of plastic bags in the markets, should correspond to a reduction, of USE SHE category ingested (Fig.4-5) in sea turtle samples collected in the future.The application of this protocol will allow~~s~~ Member States to answer to the MSFD requirements, evaluating own

baseline and defining the thresholds value to which GES is achieved. Thresholds should be determined in the pristine or near to pristine area. Due to the ubiquity of plastic in the marine environment, a pristine area does not exist. According to the example data (Tab.1), AREA 5, as the clearest zone, could represent the value (**Y**) to be reached for the Mediterranean basin. Member States should decide threshold according to the significant reduction of their own distance from this value. According to a recent review[18], marine litter ingestion units should be normalized to the size of the turtle, especially if the goal is to compare different age classes. Nevertheless, a relation between mass of ~~litter~~ ingested litter and turtles size ~~of the turtles have~~ has been detected by different authors with positive, negative or zero value [16,26,32,33,34].Our protocol does not include animal size in first scenario, but it could be possible to estimate the body burden, evaluating mass of turtle using CCL[35] and use the ration grams of plastic on grams of turtle instead of only grams of ingested plastic (Y). In any case we suggested verifying any possible significant differences before merging oceanic stage ~~samples~~ turtles with neritic ones or early juveniles with adults ~~turtles~~, in order to better stratify the samples[16,26]. ~~etc~~. The second scenario is more related to the individual health status and could better answer to the Criteria D10C3: "The amount of litter and microlitter ingested by marine animals is at a level that does not adversely affect **the health of the species** concerned". In fact, impact of ingested plastic items consists most frequently in sub-lethal effects rather than lethal ones[28,36,37,38,39]. We also rarely found an occlusion or a perforation due to plastic ingestion, which could cause the death of the turtles. Sub-lethal effects are not easy to be detected and to be distinguished from impacts due to other pollutants[40]. ~~It is obvious that dietary~~ Dietary dilution or assimilation of contaminants happens when marine litter is inside the GI of the turtle[41]. Thus the sample with more grams of plastic than food ~~remains,~~remains could indicate an animal in a very bad health condition. In order to remain in line with the Fulmar EcoQO[17,25] used by the northern European countries, both scenarios consider plastic grams instead of marine litter grams.

Finally it is important to clarify the differences between:

- analyzing the ingestion of plastic in sea turtles as indicator of impact on population with consequences for population conservation;
- analyzing the ingestion of plastic in sea turtles as bio-indicator of impact on the coastal and marine environment[20,40].

To understand implication of this impact on conservation of turtles population, more information is needed and a better data stratification is necessary[42]. By confronting the opinion of 35 ~~specialist~~specialists from 13 nations, which are experts in sea turtle biology and conservation, it is clear that sea turtles have been widely studied across the years, although it is still necessary to investigate the interactions with human activities and therefore assess the population status and potential threats.[43]~~.~~

This means that a single protocol cannot be considered as exhaustive for all the thematic and more studies are necessary to understand the impact of plastic at population level.

Even if plastic could be considered at a lower level of damage for sea turtles, in respect to by-catch or habitat destruction, its reduction is the challenge of the last few years and quick methods of measurement ~~had~~ have to be elaborated. There is a controversy in the use of stranded turtles for monitoring purposes because, according to some authors they are not

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representative of the whole population[40], while others declared that stranded turtles do not represent a bias of marine litter ingestion rates in the background population[44]. Moreover it should be considered that, in many countries, there is not a well organized stranding network or system linking Rescue Centers to fishermen and there is a lack of information on by-catch and post release mortality by fisheries. Hence stranded samples cannot be considered always as sick turtles without normal feeding behavior a time before dying and reaching to the beach, but many of them are "death at sea" turtles washed ashore and they are usually used as samples in monitoring activities[26,32,38,45]. We believe that stranded samples are useful in providing information on the level of marine litter abundance in the environment and we suggest excluding from this analysis only turtles with completely empty gastrointestinal tract, that could be sick from a long time before death. The use of this protocol is addressed to evaluate environmental status and marine litter availability for marine organism, nevertheless it could helpful improving knowledge on turtle behavior. The significance of the method respect to the MSFD TS-ML guidelines[22], is due to the harmonization in seven countries and the number of samples on which it has been tested (n=700). Specimen's body condition level has been defined and mMarine litter ingested categoriesy haves been reduced according to the preliminary results. Moreover, here it is the first time where representative results have been showed and connected to the GES thresholds.

The protocol is an efficient tool for researchers to understand the pressure of plastic on marine environment, globally or at local scale, comparing standardized data with neighboring countries. This result could not be reached before, due to the discrepancy of data among different countries, preventing any spatial comparison.

ACKNOWLEDGMENTS:

Authors are grateful to the French Rescue Centres (Jean-Batiste Senegas), stranding networks (Jacques Sacchi) and veterinarian laboratories (Joanne Belfort) and Jessiaca Martin and Marie Sabatte.

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The members of the INDICIT Advisory Board and the PO for their suggestion.

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DISCLOSURES:

The Authors Have Nothing To Disclose.

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Editor

1 Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
Verified.

2 Please use American English.
Done.

3. Please sort the Materials Table alphabetically by the name of the material.
Done.

4. Please shorten the title to remove extraneous words: Data collection on marine litter ingestion in sea turtles.
Done: "Data collection on marine litter ingestion in sea turtles".

5 Please revise the Summary to be complete sentences and not a list
Done: The protocol focuses on the collection of samples, describing all the steps from the animal recovery and necropsy to the classification and quantification of ingested marine litter. Moreover, the representative results show how to use the collected data to elaborate the possible thresholds for Good Environmental Status.

6. Abstract: Please avoid quotations in the abstract.
Done.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.
We changed all the sentences in imperative tense.

8. Please provide a legend for each supplemental file.
Done.

9. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file.
Done.

Reviewer 1

Major suggestions:
This is a good protocol, well thought through and written. This protocol is a good contribution to the standardization of the study of plastic ingestion by sea turtles. However, before its acceptance, I have two major suggestions. First, although, the title of this manuscript is "Protocol for data collection on marine litter ingestion in sea turtles", the authors put a big emphasis on the "Thresholds for Good Environmental

Status" which is an objective of the European Union's Marine Strategy Framework Directive. I don't think that this manuscript should put emphasis on this topic, mainly because, as a protocol, it doesn't contribute to a better understanding on how to define threshold values to GES. By removing the emphasis on GES, this protocol will become more comprehensible. Define threshold to GES requires a different approach, that is way beyond the scope of this manuscript.

This protocol has been produced thanks to the European DG Environment Agreement N: 11.0661/2016/748064/SUB/ENV.C2. The main goal of the project was to give an useful tool for European Member State to propose thresholds on GES for the Criteria D10C3 "*The amount of litter and microlitter ingested by marine animals is at a level that does not adversely affect the health of the species concerned*". How to establish the threshold values is the aim of this protocol and the significance of the method respect to other alternative methods.

Second, authors should provide detailed information on the "3. Marine litter should be separated from the organic components or other materials, identifying the category of marine litter by visual analysis, following the analytical protocol." (line 235). Providing detailed information about how to perform the separation between organic components and marine litter is an important part of this protocol. For example, in my experience, work on small samples of the total GI's content per time in a recipient with water enhance the ability to detect plastic fragments.

We changed line 235 providing detailed information as you requested.

We agree with you to use water on small samples but due to the fact that the lower limit of detection in this protocol is 1mm, we think it is not necessary and too time consuming. In any case, we suggest the use of stereomicroscope for any uncertain material.

Minor suggestions:

1 Line 119: "...over 500 marine species have been listed for ingesting marine litter." Please, verify this information, I believe that this number includes entanglement and ingestion, not only ingestion.

Kuhn *et al*., 2015, considered also entanglement but the exact number of species with documented record of ingestion of marine litter is 536.

2 Line 162 or Line 183: I recommend adding more detailed information on the health classification. Although it is a subjective analysis, authors may consider describing the protocol suggested by Walsh, 1999. Walsh, M., 1999. Rehabilitation of sea turtles. In: Eckert, K.L., Bjorndal, K.A., Abreu-Grobois, F.A., Donnelly, M. (Eds.). Research and Management Techniques for the Conservation of Sea Turtles. IUCN/SSC Marine Turtle Specialist Group. pp. 202-207.

Regarding the health classification more detailed information are requested in the Appendix 1 but as optional parameter. As the management and conservation of sea turtle is really important this protocol could improve some biological information, but the real focus is on marine litter ingestion using sea turtle as target specie, as described in discussion. The suggested protocol (Walsh *et al*., 1999) focus on rehabilitation and conservation of sea turtle and it is too difficult to merge both protocols considering the Jove journal rules for editing.

3 The authors should revise their reference to ensure that key studies are cited, mainly those that have applied a similar protocol of marine litter quantification and classification. I also believe that Wyneken (2001) (The Anatomy of Sea turtle) must be cited as a reference for the necropsy process.

We add Wyneken as reference as you suggested, other key studies on this topic were already cited (van Franeker[17,25], Matiddi[16], MSFD-TSGML[22]).

4. Line 351 - "Subjective interpretation of single item or multiple separate pieces could correspond to a potential bias in recording number (Fig.6). For this reason, in this manuscript, threshold values have been elaborated giving emphasis mainly on ingested dry mass, the same happens for the Fulmar EcoQO."
I suggest that the authors define 5 mm as the minimum size of plastic fragment that can be considered a single item (this is also the limit between macro and microplastic). It will help to standardize the quantification process. When this threshold is used, some studies have found a high correlation among number of items, volume and weight. The only exception will be plastic pellets, because they are obviously not a result of fragmentation. However, I do agree that weight is the best measure to quantify plastic ingestion.
There is a growing discussion on this point among the authors, with many different ideas. Micro litter found in the sea turtle could be a result of direct ingestion or fragmentation of larger items during the feeding activities and inside the GI.
Your definition of items is good and personally I agree with you, but an agreement on this point has not been reached by all the authors. For this reason we proposed the weight, we cannot delete number of items because it is the second measure requested by the EC to quantify plastic.

Reviewer 2

This manuscript MS was interesting to read and certainly warrants publication as it provides a well-defined protocol to collect and analyse data on marine litter ingestion by sea turtles. This protocol description and data analysis on marine litter ingestion by sea turtles also bring new insights as there is to my knowledge no such standardized existing protocol.
The authors have examined their samples using different approaches and proposed two possible scenarios to achieve the Good Environmental Status. This result is relevant for future works as it provides significant quantitative arguments for the use of standardized and easy methods to assess for debris ingestion on sea turtles. Although discussion deserve a more critical point of view and perspectives of the protocol, the MS covers a large panel of research on the ingestion of plastic debris on sea turtles. The MS is in overall well written and clear although few sentences may be confusing or written in a more concise manner.

Major Concern:
In the protocol it is not described neither discussed on the procedures to follow in the case where the animal is alive - I would like more information on this - Feces collection?
This protocol takes into consideration only analysis performed trough necropsy operation.
The Level 1 ALIVE, has been considered only for a more detailed classification of specimen's body condition, in case the turtle dead at the rescue centre or after the recovery (more than fresh).
The Jove journal rules for editing cannot allow us to enlarge too much the protocol, including all the procedures on alive turtle (number of words, time of the video etc.), that should follow a new protocol.

Follow your suggestion we better specified this point in the discussion (Line337-338: *The Level 1 (Alive) has been considered for a more detailed classification of specimen's body condition in case the turtle dead at the rescue centre after the recovery*).
For Alive turtle you are right, the analysis should be performed on the fecal pellet and even if the marine litter classification could be the same, the sampling procedure are completely different.

Minor Concerns:
SUMMARY
Ok

ABSTRACT
Clear and synthetic

Line 73: Do not include references
Deleted.

INTRODUCTION
Line 99 & 100: Please provide more details and references for your statement "Due to the dynamics and environmental conditions of the oceans combined with the physical properties of plastic". I would like to know in particular what are the environmental conditions that drive plastic to oceans and seas. Line 103: Give some examples of physical properties of plastics and environmental variables that influence plastic movement.
Now Line 98: We better explained the sentence and added examples
Plastic movement is influenced by its physical properties (e.g. buoyancy) or environmental variables (e.g. tide and stream), and can be accumulated in all the environmental marine compartments[4,5].

Line 119: Gall and Thompson (2015) reported encounters between organisms and marine debris in 693 species. ; Please include the following references : Derraik, J. G. The pollution of the marine environment by plastic debris: a review. Mar. Pollut. Bull. 44, 842–852 (2002). Gall, S. C. & Thompson, R. C. The impact of debris on marine life. Mar. Pollut. Bull. 10.1016/j.marpolbul.2014.12.041 (2015).
Suggested References included[12,13].

Line 127-128: Recently, Bonanno and Orlando-Bonaca (2018) have also discussed on using loggerheads as biodindicators of plastic pollution. Bonanno, G. and Orlando-Bonaca, M. Perspectives on using marine species as bioindicators of plastic pollution. Marine Pollution Bulletin 137, 209-221,(2018). Please provide main advantages and drawbacks of using loggerheads as an bio-indicator of this pollution.
The main advantage and drawbacks of using sea turtle as bio-indicator has been already higlight by Bonanno and Orlando-Bonaca. We have included their reference for details, but it is not possible here to go so in deep. The protocol starts from the statement that the sea turtle is already approved as bio-indicator of plastic pollution. These authors have been also cited in the discussion for their point of view on this topic.

PROTOCOL

In the protocol it is not described what are the procedures in the case where the animal still alive (Level 1) - I would like more information on this - Feces collection ?

Feces collection should be described in a different protocol (see replay to major concern).

Line 166: Please avoid "etc" - As there are 7 marine species (all documented to have ingested marine plastic debris), list them all.

Now Line 164 Done all the seven species have been included: Species identification: **Cc** (*Caretta caretta,* Linnaeus 1758); **Dc** (*Dermochelys coriacea,* Vandelli 1761); **Cm** (*Chelonia mydas,* Linnaeus 1758); **Ei** (*Eretmochelys imbricata*, Linnaeus 1766); **Lo** (*Lepidochelys olivacea*, Eschscholtz 1829); **Lk** (*Lepidochelys Kempii,* Garman 1880); **Nd**(*Natator depressus*, Garman 1880).

Line 173-174: Please use the same words for the specimen's body condition level as in the Figure 1

We changed figure 1 and the sentence.

Line 178-179: What if the individual is dead after a surgical intervention: perhaps make a distinction between Dead at the recovery center before any medical care and Dead at the recovery after medical care or surgical operations?

There is the possibility to insert “Note and Remarks” and/or including this information in Appendix in “cause of death”.

Line 183: GI track rather than "gut content" because it appears the entire GI content is extracted, isn't it ?

Yes, changed.

Line 191: What are the other optional measures? Please be as much precise as possible.

Now Line 193: We added weight and CCW.

Line 213: Add yellow before "arrows".

Added yellow.

Line 220: Please provide some examples of anomalies that can found in sea turtles with references that described these anomalies.

We add ulcers, perforations, adhesions, inflammation.

REPRESENTATIVE RESULTS

In the protocol it is not described what are the procedures in the case where the animal still alive (Level 1) - I would like more information on this - Feces collection?

As feces collection is not the focus of this protocol, but we are working on this topic, if you are interested we can discuss this procedure via private e-mail.

Line 258: Start with a new sentence with: The protocol proposes a homogenized. Don't forget the "s" at "proposes".

Now Line 261: corrected

It proposes a homogenized, feasible and easy evaluation of litter ingestion by sea turtles.

Line 260: "applicable to other sea turtle species" rather than "applicable to othe species"Now line 262 Corrected *The protocol has been tested on loggerhead turtles, and most of the manipulations are also applicable to other sea turtle species.*

Line 263: Please provide reference(s) stating that "usually the less abundant one"
Now Line 268References included and focus on sea turtle *rare to find ingested by loggerhead turtle*[16,26]

Line 267-268: Please provide reference(s) stating that "White and transparent are the main abundant colors in sea turtles GI". Also this may be not true for all turtle species.
As you suggest this cannot be true for all the turtle, we decide to delete the Line

Line 275: Avoid "Etc."
Deleted

Line 291: insert "the" before "first".
Inserted .

FIGURE AND TABLE LEGENDS
Line 304: I think the authors meant "yellow arrows" rather than "Yellow narrows".
Yes corrected.

Line 309-310: Please indicate if the average is reported with the SE, SD %95CI ?
Added ±SE to the average.

Line 311-312: Please indicate if the average is reported with the SE, SD %95CI ?
Added ±SE to the average.

In Table 1: Please report the SD of "average value for dry weight of ingested plastic"
Table 1: We added ±SE of average for dry mass.

DISCUSSION
In the discussion it is not discussed the procedures in the case where the animal still alive (Level 1) - I would like more information on this - Feces collection?
Explained before.

This section should further discuss the following with citations: *Critical steps in the protocol.
Now Line 354-359 We add references in this part and delete the sentence "Usually in the scientific papers the word item is commonly used to identify part of an object" that cannot be verified for all the papers.
A critical step of the protocol could be found in the collection of number of items. Multiple pieces could be derived from a fragmentation of the same object inside the GI or as a consequence of separately ingestion. Subjective interpretation of single item or multiple separate pieces could correspond to a potential bias in recording number (Fig.6). For this reason, threshold values have been elaborated using only ingested marine litter mass data, alike the Fulmar EcoQO[17,25].
Marine litter ingestion should be related to the animal size, but up to now no homogenous data have been collected by different studies as explained on Line 375-376 *According to a recent review*[18], *marine litter ingestion units should be normalized to the size of the turtle,*

especially if the goal is to compare different age classes. Nevertheless, a relation between mass of ingested litter and turtles size has been detected by different authors with positive, negative or zero value [16,26,32,33,34].

***Modifications and troubleshooting of the method.**
This method has been tested on over 700 loggerhead turtles and any kind of trouble have been solved during the processing in order to have a complete harmonization.

***Limitations of the method.**
We underlined the limitation on the use of the protocol LINE 329-335 :
A limitation of the protocol is the use of sea turtle considering that all 7 species of marine turtles are listed on Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora[27], therefore only authorized people can handle live and dead animals or parts of them. Turtle management and recovery should be reported and coordinated with the corresponding Authorities. Sanitary precautions must be taken when handling dead or alive wild animals to minimize risks of zoonosis.
This protocol target on marine litter and it is not exhaustive for all the thematic as explained on line 404-405 *This means that a single protocol cannot be considered as exhaustive for all the thematic and more studies are necessary to understand the impact of plastic at population level.*

***The significance of the method with respect to existing/alternative methods.**
Line 422-431
The significance of the method respect to the MSFD TS-ML guidelines[22], is due to the harmonization in seven countries and the number of samples on which it has been tested (n=700). Specimen's body condition level has been defined and marine litter ingested categories have been reduced according to the preliminary results. Moreover, here it is the first time where representative results have been showed and connected to the GES thresholds.
The protocol is an efficient tool for researchers to understand the pressure of plastic on marine environment, globally or at local scale, comparing standardized data with neighboring countries. This result could not be reached before, due to the discrepancy of data among different countries, preventing any spatial comparison.

Future applications or direction of the methods.

On Line 363 we already described future application: *It supports policy makers for their programs of measures, giving a rapid evidence of their efficiency for target items by evaluating their strength. For example, the ban of plastic bags in the markets should correspond to a reduction, of USE SHE category ingested (Fig.4-5) in sea turtle samples collected in the future.*
On Line 366 it has been expressed using the future verb in order to underline the future application:
The application of this protocol will allow Member States to answer to the MSFD requirements, evaluating own baseline and defining the thresholds value to which GES is achieved.

Line 323-324: Please reformulate this sentence.
Reformulated: "*This protocol allows to evaluate the total abundance of marine litter and identify the main categories ingested by sea turtles*."

Line 325-326: What is easy, rapid and intuitive? Be specific.
Now Line 327-330:

It is less expensive compared to other monitoring programs with sea activities because sea turtles could be collected stranded on the beach or recovered by fishermen. The identification of marine litter categories it is easy and rapid as the lower items limit is 1 mm.

Line 337: Give a definition of FO% (Frequency of occurrence?).
We added Frequency of occurrence.

Line 342: Avoid "something".
Deleted something.

Line 344-345: Hook presence should be recorded and also what kind of hook (e.g., J, circle ...).
Hook are already requested to be recorded in the note, where you can also write the kind or other.

Line 348: Maybe a short discussion may be done on plastic retention time in GI track ?
Few studies have measured transit time of ingested plastic in sea turtle. Retention time in GI track is under analysis in some rescue centre with hospitalized alive turtle, using plastic balls as marker. The results are not still homogenous among different centre and are un-published data. We considered retention time for alive protocol, not include here.

Line 349: Discuss also on the measured parameters on plastics: for instance Clukey et al. (2016) found that "Six approaches for quantifying amounts of ingested plastic strongly correlated with one another and included: number of pieces, mass, volume and surface area of plastics, ratio of plastic mass to body mass, and percentage ofthe mass of gut contents consisting of plastic"Clukey, K. E., Lepczyk, C. A., Balazs, G. H., Work, T. M., & Lynch, J. M. (2017). Investigation of plastic debris ingestion by four species of sea turtles collected as bycatch in pelagic Pacific longline fisheries. Marine pollution bulletin, 120(1-2), 117-125.
We added Clukey to the literature.
It should be considered that this protocol focus on the MSFD requirements (COM Dec. 2017) that are targeted only on number of items and mass of marine litter.
We agree with you on the importance of the other measures, for this reason we included optional parameters in the protocol, (Data collection paragraph point 11; appendix 1).
There is only a lack in the litter surface measure in respect to Clukey.
As we pointed on Line 375-376 a relation between mass of ingested litter and animal size showed a different results ([16,26,32,33,34])

Line 353-354: Ok but must be re-phrased
Now Line 358Re-phrased: *For this reason, threshold values have been elaborated using only ingested marine litter mass data, alike the Fulmar EcoQO[17,25]*.

Line 357: Delete "etc." and list all categories.
Delete. etc.

Line 371: Delete "value". Also provide more information on the relationship between biometric variables of sea turtles and amount of ingested debris.
Deleted value and see previous comment Line 375-376

Line 373 : Please discuss more on other parameters such as volume and mean length of plastics that may have an influence on sea turtle survival (e.g., Hoarau et al. 2014) Hoarau, L., Ainley, L., Jean, C., Ciccione, S. Ingestion and defecation of marine debris by loggerhead sea turtles, Caretta caretta, from by-catches in the South-West Indian Ocean Marine Pollution Bulletin. 84, 90-96, (2014).
See previous comment .

Line 375 : Avoid "etc.">- Develop this point then. Deleted etc. and developed this point.

Line 379 "effects"
Corrected

Line 382-383: The authors must provide references comforting this statement. I don't think "it is" so "obvious" We added references for sub-lethal effect and for dietary dilution. We deleted obviously.
Now Line 386-388 *Sub-lethal effects are not easy to be detected and to be distinguished from impacts due to other pollutants[40]. Dietary dilution or assimilation of contaminants happens when marine litter is inside the GI of the turtle[41].*

Line 402: "had" or "have"?
Corrected "Have".

References :
Corrected as you suggested.

appendix 1

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APPENDIX 1 – OBSERVATION SHEET 1/2

OBSERVATION SHEET - Litter ingestion by sea turtles	
COLLECTOR:	**LOCAL CODE:**
INSTITUTION:	
CONTACT:	

Discovery circumstances:

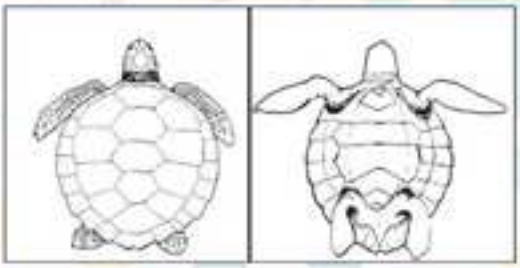
SPECIES	☐ Caretta caretta ☐ Dermochelys coriace ☐ Chelonia mydas ☐ Other
INDIVIDUAL TAG	Tag number: Electronic chip N°:
INDIVIDUAL CODE:	- - - - - -
DATE OF DISCOVERY (yyyy/mm/dd):	
LOCATION	X CORD : Y CORD :
CIRCUMSTANCES	☐ By-catch/Fishery ☐ Stranding ☐ Dead at rescue centre ☐ Found at sea ☐ Other ☐ NR
BY-CATCH ENGINE CAUSE	☐ Longline ☐ Trawl ☐ Drift net ☐ Fishing rod ☐ Other ☐ NR
CAUSE OF DEATH/ STRANDING	☐ Bycatch/Fisheries ☐ Entanglement in debris ☐ Ingestion of litter ☐ Anthropogenic trauma ☐ Natural trauma ☐ Natural disease ☐ Oils ☐ Healthy ☐ Other ☐ NR
ENTANGLEMENT TYPE	☐ Active ☐ Passive ☐ NR
LITTER CAUSING ENTANGLEMENT	☐ Net pieces ☐ Monofilament lines ☐ Rope/s ☐ Plastic bags ☐ Raffia ☐ Other ☐ NR
PICTURES ☐	Picture names :

Animal body condition:

CONSERVATION STATUS	☐ 1 - Alive ☐ 2 - Fresh ☐ 3 - Partialy ☐ 4 - Advanced ☐ 5 - Mummified ☐ NR
HEALTH STATUS (Plastron shape)	☐ Poor (concave) ☐ Fair (plane) ☐ Good (convex) ☐ NR
MAIN INJURIES	☐ No injuries ☐ Fracture ☐ Amputation ☐ Sectionning ☐ Abrasion ☐ Other
AFFECTED PARTS	☐ Flipper ( ) ☐ Carapace ☐ Neck ☐ Head ☐ Plastron ☐ Other
FAT RESERVES	☐ Thin ☐ Fat ☐ Normal ☐ NR

Biometric measurements:

Curved measurements (0,01cm)		Straight measurements (0,01cm)	
CCLst	cm	SCLst	cm
CCLmax	cm	SCLmax	cm
CCLmin	cm	SCLmin	cm
CCW	cm	SCW	cm
CPL	cm	SPL	cm
CPW	cm	SPW	cm



WEIGHT (0,01kg)	
NOTES AND REMARKS (Discovery and Animal conditions):	

appendix 2

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APPENDIX 1 – OBSERVATION SHEET 2/2

INDIVIDUAL CODE: - - - - - -

CC (Region) YY MM DD n°

Extraction of ingested litter

PROTOCOL	☐ Necropsy
ARRIVAL DATE / / **DEAD DATE** / /	
FAT RESERVES	☐ Thin ☐ Fat ☐ Normal
Please describe :	
VISCERAS STATUS *(note the presence of any infection, suspect colour, fluid effusion, perforation, presence of oil, etc.):*	
DIGESTIVE TRACT *(note the presence of any infection, suspect colour, fluid effusion, perforation, presence of oil, etc.):*	
TURTLE BEHAVIOUR AND TREATMENTS:	

Capacities of digestive tract section and gut content

	FULL				EMPTY			
	mass	Vol (V1)	vol (V0)	V1-V0	mass	Vol (V1)	vol (V0)	V1-V0
ESOPHAGUS								
STOMACH								
INTESTINES								

Marine debris measurements

	ESOPHAGUS		STOMACH		INTESTINE	
	DRY MASS	NUMBER	DRY MASS	NUMBER	DRY MASS	NUMBER
IND. PLA						
USE SHE						
USE THR						
USE FOA						
USE FRAG						
USE POTH						
Other non plastic						
FOO (nat. Food)						
NFO (nat. no food)						
TOTAL						

TOTAL DEBRIS		NUMBER OF ITEMS		NUMBER OF ITEMS	
dry mass		micro (1-5mm)		white transparent	
number of items		meso (5-25mm)		dark coloured	
volume		macro (>25mm)		light coloured	