Impacts of plastic ingestion on green sea turtles (*Chelonia mydas*) in Uruguayan waters

Thesis submitted by

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Acknowledgements

The initial ideas for this PhD research emerged in 2016, prompted by the alarming levels of plastic ingestion by green turtles in Uruguay. Over the past decade, through the efforts of the Karumbé NGO, we have been actively involved in grassroots conservation to tackle this issue, from rehabilitating dozens of turtles impacted by this hazard to working with local communities to raise awareness of the plastic pollution threat and empower them to act for the preservation of the Uruguayan marine environment. However, we felt the need to support and complete these efforts by assessing such critical situation for the green turtle population in Uruguayan waters from a scientific perspective. Hence, this was the main reason that led me to pursue a PhD at the renowned institution, James Cook University.

This endeavour has not been easy and has been filled with challenges. The main one was the global COVID-19 pandemic, which affected thousands of people worldwide and rattled the foundations of the present society. That situation caused me to be stuck outside Australia for over a year while conducting my fieldwork in Uruguay. It was a tumultuous and confusing time, as little was known about what the pandemic would bring. Despite this, and always with the invaluable support of my supervisors, I persevered through all the difficulties to complete this PhD project successfully.

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I am immensely satisfied to have completed this PhD project and contributed my bit to the conservation of the wonderful creatures that are marine turtles. They are the true protagonists of this story, beyond the achievement of a personal goal or an academic milestone. The purpose and reason for this endeavour have always been the same: working for a universal value such as nature conservation. Perhaps it has been a long and arduous path, but as one of Uruguay's most renowned writers, Eduardo Galeano, once said, "*Many small people, in small places, doing small things, can change the world*."

Muchas gracias,

Daniel González-Paredes
22 December 2023
Townsville, QLD, Australia

Statement of the Contribution of Others

Chapter	Publication on which based	Role of each author
1	**González-Paredes, D.,** & Estrades, A. (2021). Plastics Versus Turtles: An Overview of the Uruguayan Case. In: B. Nahill (Ed.), *Sea Turtle Research and Conservation*, (pp 83-92). Elsevier Inc. Editorial.	DGP and AE compiled all the information regarding the threat of plastic pollution to green turtles in Uruguayan waters in the published manuscript. The thesis chapter was based on this publication and the assistance of JPL, MH and HM in editing.
2	**González-Paredes, D.**, Ariel, E., David, M. F., Ferrando, V., Marsh, H., & Hamann, M. (2021). Gastrointestinal transit times in juvenile green turtles: An approach for assessing digestive motility disorders. *Journal of Experimental Marine Biology and Ecology*, 544, 151616.	DGP and EA co-designed the experiments. EA, MFD and VF, all of them Doctors of Veterinary Medicine, contributed to assesing the health status of turtles. Volunteers of Karumbé NGO and JCU Turtle Health Research, under supervision of DGP, helped to monitor turtle faecal matter and collect plastic ingested. MH and HM assisted DGP in analysing and interpreting data. DGP wrote the thesis chapter with the assistance of all the authors in editing.
3	**González-Paredes, D.,** Vélez-Rubio, G.M., Marsh, H., & Hamann, M. (*in prep.*). Plastic ingestion by green turtles (*Chelonia mydas*) in Uruguayan waters; Insights from different studies approaches. I plan to submit a revised version of this chapter to *Endangered Species Research*, as the target journal.	DGP and GMVR co-designed the research and DGP secured the funding for the fieldwork. DGP and GMVR co-led the fieldwork with the assistance of volunteers of Karumbé NGO. Volunteers of Karumbé NGO and JCU, under supervision of DGP, helped to analyse the plastic samples. MH and HM assisted DGP in analysing and interpreting data. DGP wrote the thesis chapter with the assistance of all the authors in editing.
4	**González-Paredes, D.,** Jones R., De la Fuente, A., Ferrando, V., Vélez-Rubio, G.M., Hamann, M., & Marsh, H. (*in prep.*). Impact severity associated with plastic ingestion in juvenile green turtles in relation to the volumes and characteristics of ingested plastics.	DGP and VF co-designed the research and DGP secured the funding for the fieldwork. DGP and GMVR co-led the fieldwork with the assistance of volunteers of Karumbé NGO. VF, who is a Doctor of Veterinary Medicine, contributed to assesing the health status of turtles and conducting the necropsies. Volunteers of Karumbé NGO and JCU, under supervision of DGP, helped

	I plan to submit a revised version of this chapter to *Marine Pollution Bulletin*, as the target journal.	to analyse the plastic samples. RJ and ADF assisted DGP in analysing data. MH and HM contributed to interpreting data. DGP wrote the thesis chapter with the assistance of HM and MH in editing.
5	**González-Paredes, D.,** Duncan, E., Godley B. J., Marsh, H., & Hamann, M. (*in prep.*). A best practice framework for assessing plastic ingestion in marine turtles. I plan to submit a revised version of this chapter to *Conservation Biology*, as the target journal.	The conceptualisation and structure of this chapter was conceived by DGP, HM and MH, in collaboration with ED and BJG. DGP wrote the thesis chapter with the assistance of all the authors in editing.
6	**González-Paredes, D.,** Fallabrino, A., Godley B. J., Marsh, H., & Hamann, M. (*in prep.*). Impacts of plastic ingestion on green turtles (*Chelonia mydas*) in Uruguayan waters. I plan to submit a revised version of this chapter as a report to the IUCN-SSC Marine Turtle Specialist Group.	DGP, HM and MH, conceptualised this chapter in collaboration with AF and BJG. DGP wrote the chapter with the editorial assistance of HM and MH.

List of authors acronym:

DGP	Daniel Gonzaléz-Paredes	EA	Ellen Ariel
AE	Andrés Estardes	MFD	Maria Florencia David
JPL	Juan Pablo Lozoya	VF	Virginia Ferrando
HM	Helen Marsh	GMVR	Gabriela M. Veléz-Rubio
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ED	Emily Duncan	ADF	Alejandro de la Fuente
BJG	Brendan J. Godley	AF	Alejandro Fallabrino

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Ethics Approvals

This research was conducted under the following permits:

- **James Cook University Animal Ethics Permit A2309**
- **Research permit WITK 15765815 issued by the Australian Government**
- **Research license (No. 4/2018) issued by the Ministry of Environment, Uruguay (MVOTMA)**
- **Experimentation permit (No. 0024/12) issued by the National Committee for Animal Experimentation, Uruguay (CNEA)**

Associated Outputs

Publications

González-Paredes, D., & Estrades, A. (2021a). *Plastics Versus Turtles: An Overview of the Uruguayan Case.* In: B. Nahill (Ed.), *Sea Turtle Research and Conservation*, (pp 83-92). Elsevier Inc. Editorial (related to Chapter 1) https://doi.org/10.1016/B978-0-12-821029-1.00009-X

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Technical reports

Brad Nahill, B., Kakai, T., Matilde, E., Berendse, S., & **González-Paredes, D.** & (2018). More Turtles, Less Plastic. In: R. B. Mast, L. M. Bailey, B. J. Hutchinson, A. Hutchinson, K. Koenig & M. S. Rowe (Eds.), *SWOT Report—The State of the World's Sea Turtles, 19,* 40-41. (related to Chapter 1)

González-Paredes, D. & Estrades, A. (2018). Addressing the plastic pollution challenge in Uruguay. In: R. B. Mast, L. M. Bailey, B. J. Hutchinson, A. Hutchinson, K. Koenig & M. S. Rowe (Eds.), *SWOT Report—The State of the World's Sea Turtles, 13,* 42-43. (related to Chapter 1)

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Conference presentations

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González-Paredes, D. (2021). *Current threats: bycatch and plastic pollution*. Keynotes speak at the 6th Mexican National Meeting on Sea Turtles, Quintana Roo, MEXICO 2021.

González-Paredes, D. (2018) *Plastics vs. Turtles and how to deal with this issue. An overview of the Uruguayan case.* Oral presentation at the 4th Australian Marine Turtle Symposium. Bundaberg, QLD, Australia.

González-Paredes, D., Estrades, A. (2019). *Plastics vs. Turtles and how to deal with this issue. An overview of the Uruguayan case.* Poster presentation at the 39th Annual Symposium on Sea Turtle Biology and Conservation. Charleston, SC, USA.

González-Paredes, D., Vélez-Rubio, G.M., Teryda, N.S., Estrades, A., Fallabrino, A. (2018) *Efectos de la contaminación plástica sobre la salud de tortugas verdes (Chelonia mydas) en el Uruguay.* Oral presentation at the 5th Uruguayan Zoology Congress. Montevideo, Uruguay.

Workshops participation

González-Paredes, D. (2023). *IV Workshop on Marine Debris & Sea Turtles*. Chair and organiser of the workshop held at the 41st Annual Symposium on Sea Turtle Biology and Conservation. Cartagena de Indias, Colombia.

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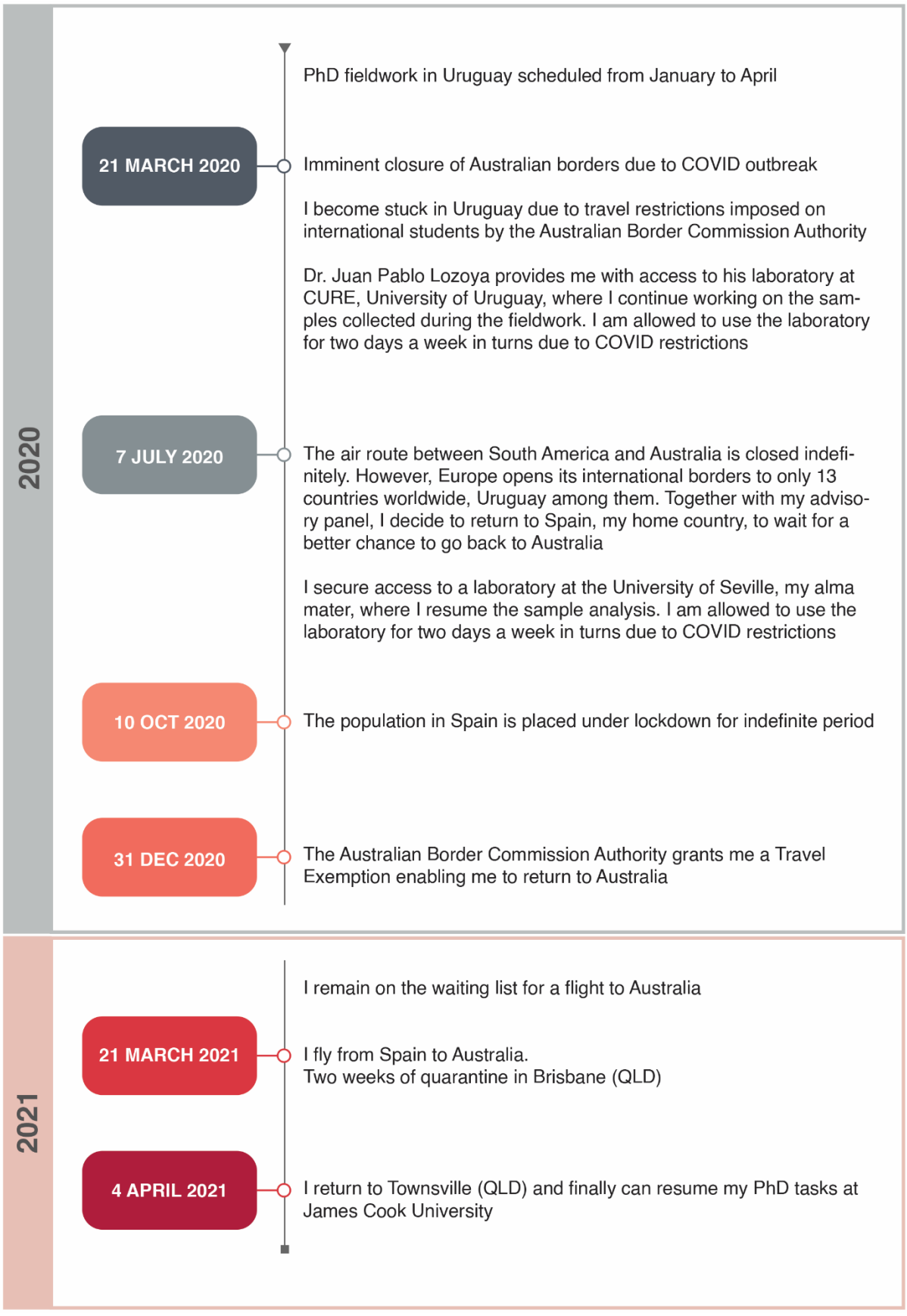
González-Paredes, D. (2018). *II Workshop on Marine Debris & Sea Turtles*. Chair and organiser of the workshop held at the 38th Annual Symposium on Sea Turtle Biology and Conservation. Kobe, Japan.

González-Paredes, D. (2019). *Workshop on Marine Debris*. Organiser and speaker of the workshop held at the 4th Australian Marine Turtle Symposium. Bundaberg, QLD, Australia.

González-Paredes, D. (2017). *I Workshop on Marine Debris & Sea Turtles.* Chair and organiser of the workshop held at the 37th Annual Symposium on Sea Turtle Biology and Conservation. Las Vegas, NV, USA.

Annex

Timeline of the disruptions to my PhD research due to COVID-19.



Thesis Abstract

Plastic ingestion is recognised as an emerging threat and a priority conservation concern for marine turtles. Understanding the impacts caused by plastic ingestion is crucial to assessing the vulnerability of marine turtles to this threat. Research and monitoring efforts have increased in recent years, enhancing our knowledge of the physical impacts and adverse effects caused by plastic ingestion at the individual level. However, significant knowledge gaps persist in comprehending the magnitude of this threat at a broader scale or the population level. Evaluating the impacts of plastic ingestion on marine turtle populations is particularly challenging due to their complex life history and extensive distributions. One of the challenges in this research field involves assessing plastic ingestion in early life stages, which are considered the most affected but also the hardest to survey technically and logistically in the wild. In addition, there is still limited consensus on adopting consistent research protocols and methodologies in these studies, which hampers comparing of their results for obtaining broader impact assessments.

My PhD research analyses the impact of plastic ingestion on the stock of juvenile green turtles (*Chelonia mydas*) in Uruguayan waters from 2014 to 2020, discussing the conservation implications that such impacts could have on the green turtle subpopulation of the South Atlantic - Regional Management Unit (RMU). To address this concern, I proposed measures for a more comprehensive examination of this threat to inform future listing assessments of this subpopulation, along with recommendations for conservation plans and mitigation actions, and future investigations in this field. Additionally, I provided a best practice framework for designing and implementing research to assess plastic ingestion in marine turtles, based on common research protocols and standardised methods.

My study includes samples collected from a representative subset of animals, enabling a comprehensive assessment of plastic ingestion. These turtles are related to different sources of specimens, including stranded, bycaught, captured (for scientific purposes) and rescue turtles.

Plastic samples were collected by necropsy of the dead turtles, gastric lavage or monitoring faecal matter through routine examinations of the live turtles. Faecal matter monitoring is a reliable approach for assessing plastic ingestion in live animals, provided adequate time is allocated to collect all ingested plastic. Therefore, in Chapter 2, I conducted an experiment using inert plastic markers to estimate the gastrointestinal transit times of juvenile green turtles in Uruguay, which lasted 22 days. Establishing monitoring periods exceeding the upper limit of ingesta time maximises the likelihood of collecting all plastic pre-ingested in the natural environment. In addition, I assessed different experimental conditions by simulating the same experiment on juvenile green turtles from Heron Island, Australia. As conclusions, the husbandry conditions, such as water temperature and diet administered, ideally should mimic natural conditions for a more accurate estimation of gastrointestinal transit times. I also observed that inert plastic markers are more efficient than organic markers (corn kernels in my second experiment) as they do not degrade or discolour during the digestive process, ensuring a higher recovery success. This baseline data on gastrointestinal transit times is central to assess digestive motility disorders in juvenile green turtles, and also has the potential to contribute to toxicology studies related to exposure to toxic substances leached from ingested debris.

In Chapter 3, I analysed plastic ingestion on juvenile green turtles (N = 294) present in Uruguayan waters between 2014 and 2020, assessing incidences and patterns of ingestion. For this purpose, I quantified and characterised ingested plastic collected through three sampling techniques (necropsy, faecal matter monitoring, and gastric lavage) across different sources of specimens (stranded and bycaught dead turtles, and live wild-captured and rescued turtles). This allowed me to provide additional information about the strengths and limitations of these approaches. The overall incidence of plastic ingestion was 76% among the examined turtles, with quantities of ingested plastic recorded higher than those found in green turtles in the rest of the Southwestern Atlantic region. Uruguayan waters exhibit high levels of plastic pollution, representing a significant hazard to marine turtles. No identifiable annual trend was detected in the incidence of plastic ingestion or in

the quantities of plastic ingested across the subgroups of turtles examined. Laminar soft plastics in white and clear/transparent colours constituted the most consumed plastic type, accounting for over 40% of the plastic retrieved from each subgroup. This potential selectivity behaviour could be driven by the resemblance of these plastics to the most common dietary items for green turtles in Uruguayan waters, such as macroalgae and gelatinous macrozooplankton.

Regarding the different sources of specimens, bycaught and wild-captured turtles are more reliable indicators of a population's overall exposure to plastic ingestion if sampling is systematic. Conversely, stranded dead and rescued turtles can provide valuable insights into the severity of the impact caused by plastic ingestion at an individual level. Necropsy examination remains the most reliable sampling technique for assessing plastic ingestion, enabling retrieval of all the digestive contents. While faecal monitoring enables live animals to be sampled, it provides adequate monitoring time for collecting all the ingested plastic. Gastric lavage proved inefficient for studying plastic ingestion in marine turtles.

In Chapter 4, I explored the factors influencing the severity of the impact caused by plastic ingestion by assessing the cumulative volumes and characteristics of ingested plastic in a subsample of the examined turtles (n = 150), for which complete necropsy reports or health assessments were available. The analysis detected a positive relationship between the ingested volume of plastic and the severity of the impact, with a highly significant difference in volumes between non-affected and impacted turtles. Furthermore, the accumulation of plastic followed an increasing pattern along the digestive tract: oesophagus < stomach < intestines. Consistent with my previous observations, laminar soft plastics were the most consumed plastic-type in terms of volume. These plastics pose a particular risk for turtles due to their pliability characteristics. Turtles can ingest large pieces of soft plastics without restricting their mouth-gape. Once in the intestines, these large and malleable pieces can act as a mesh, entangling other plastic items and part of the solid fraction of the digestive contents, which results in fecaloma compaction and gut obstruction. This condition was determined

by veterinarians as the cause of death in 10 turtles and led to emaciation and chronic debilitation syndrome in another 14 animals. I also found that juveniles with a curved carapace length (CCL) below 40 cm were more susceptible to the impacts of plastic ingestion. This could be associated with their opportunistic feeding behaviour related to their previous oceanic stage. Due to their low discrimination in selecting dietary items, these smaller turtles are potentially more exposed to ingesting a wider range of plastic and higher volumes.

The 'best practice framework for designing and implementing research to assess plastic ingestion in marine turtles' presented in Chapter 5 aims to assist researchers in articulating the optimal available strategies and standard methods suitable for fulfilling research objectives, considering the accessibility of resources and capabilities. This framework compiles the knowledge I gained from conducting my PhD research, combined with the collective experience of my collaborators. It has also been enriched by discussions with other experts during the four editions of the Workshop on Plastic Pollution & Sea Turtles, which I led at the International Sea Turtle Symposiums.

The findings of my PhD thesis provide evidence that Uruguayan waters, considered a critical development and feeding area for juvenile green sea turtles, are a hotspot for plastic ingestion by the species in the South Atlantic Ocean. Since plastic ingestion mainly affects the early life stages of green turtles in the region, the future viability of their subpopulation in the South Atlantic - Regional Management Unit (RMU) may be compromised. In Chapter 6, I finished recommending several approaches based on the current criteria of the IUCN Red List and a Population Viability Analysis (PVA) for a more comprehensive examination of this concern, aiming to inform future listing assessments of this subpopulation. Urgent measures to reduce plastic pollution in the area and mitigate its impact on the marine environment must also be considered.

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PhD Chapter 1

General introduction

Chapter objective

To provide a general introduction to the threat of plastic ingestion to marine turtles as a rationale for the thesis, articulate the objectives of my PhD research and outline the structure of my thesis.

Methodology

I conducted a comprehensive literature review to synthesise existing knowledge on the impact of plastic ingestion on marine turtles as the basis for the objectives and structure of my PhD research.

Related publication

González-Paredes, D., & Estrades, A. (2021a). *Plastics Versus Turtles: An Overview of the Uruguayan Case.* In: B. Nahill (Ed.), Sea Turtle Research and Conservation, (pp 83-92). Elsevier Inc. Editorial. https://doi.org/10.1016/B978-0-12-821029-1.00009-X

1.1 A history of plastic pollution

The term “plastic” stems from the Greek *plasticos* and the Latin *plasticus,* meaning ‘something able to be shaped or moulded’. Nowadays, plastic refers to a manufactured sub-product composed of large chains of polymers, which is used for a wide variety of applications due to its malleable properties. Its low production cost and great versatility have resulted in a worldwide increase in production by around 9% each year since 1950 (Ryberg et al., 2018), reaching 390.7 million tonnes in 2021 (PlasticEurope, 2022). Such amount is predicted to double by 2050 if production continues at present rates (Geyer et al., 2017). This massive production generates large volumes of waste of which only 9% of plastic is recycled, 12% is incinerated, and 79% is deposited in landfills (Geyer et al., 2017). Ultimately, a significant portion of this plastic waste enters the environment due to inappropriate waste management and accidental loss (Beaumont et al., 2019).

1.2 Marine plastic pollution

Conservative estimates predict an annual increase of 20 to 53 million tonnes of plastic emissions to aquatic ecosystems by 2030 if current rates of primary production and deficient disposal methods persist (Borrelle et al., 2020). Plastic debris that originates from land- and marine-based sources now occurs as persistent and pervasive contaminants throughout nearly every aquatic ecosystem (Cózar et al., 2014; Galgani et al., 2015; Hardesty et al., 2021; Ryan et al., 2009). Consequently, plastic pollution is recognized as one of the major threats to the marine environment (Gall & Thompson, 2015; Rochman et al., 2013; Senko et al., 2020; Vegter et al., 2014). Plastic debris results in ubiquitous pollutants that tend to be concentrated in oceanic gyres and coastal fronts by the combined actions of winds and currents (Cózar et al., 2014; Kershaw & Rochman, 2015). These vast quantities of waste, therefore, represent a potential long-term hazard through habitat degradation, entanglement, ingestion, and bioaccumulation of toxic substances for marine wildlife and vectors of dispersion for non-native species (Lavers et al., 2021; Miller et al., 2020; Rech et al., 2018; Santos et

al., 2021; Stelfox et al., 2016). In addition, plastics are exposed to UV light and the physical pressure of waves in the marine environment, and thus, their polymer bonds weaken, leading to fragmentation into increasingly smaller pieces by mechanical and photochemical forces (Andrady, 2015; Galloway et al., 2017). Apart from the toxic chemicals content in these plastics, the smaller pieces can also accumulate contaminants already present in the environment (e.g., heavy metals and polychlorinated biphenyls (PCBs) and persistent, bio accumulative, and toxic chemicals (PBTC)) due to their hydrophobic properties and the large surface area to volume ratio of microplastics (Engler, 2012; Fazey & Ryan, 2016; Ziccardi et al., 2016). At least 5.25 trillion plastic particles are believed to be in the marine environment (Eriksen et al., 2014).

1.3 Plastic ingestion by marine turtles

Plastic ingestion has been recognized as an emerging threat and a priority conservation concern for marine turtles (Fuentes et al., 2023; Hamann et al., 2010; Nelms et al., 2016; Senko et al., 2020). The ingestion of plastic debris has been reported in all seven marine turtle species (Duncan et al., 2019b; Lynch, 2018; Schuyler et al., 2014a), affecting vital processes across their stre (Do Sul et al., 2011; Duncan et al., 2021; Schuyler et al., 2014a). Marine turtles are believed to be particularly vulnerable to the impacts of plastic ingestion due to their long-life spans, complex life history and migratory behaviour (Duncan et al., 2021; Lynch, 2018; Santos et al., 2015). Indeed, these species have been recognized by organisations such as the European Marine Strategy Framework Directive (MSFD) through the INDICIT project as reliable bioindicator taxa for evaluating Good Environmental Status (GES) and assessing plastic pollution levels (Darmon et al., 2022; Fossi et al., 2017; Galgani et al., 2014; Matiddi et al., 2019).

Ingestion may occur directly when plastic is mixed with natural food or selectively due to similarities in shape and appearance with dietary items, such as leatherback turtles (*Dermochelys coriacea*) ingesting semi-buoyant soft plastics resembling jellyfish, their prey (Duncan et al., 2019a; Nelms et

al., 2016; Schuyler et al., 2014b). Turtles can also be attracted to plastic debris due to biofilm adhering to its surface, resembling food taste or smell; Pfaller et al. (2020) experimentally demonstrated that oceanic-stage loggerheads (*Caretta caretta*) respond to airborne odorants emitted by biofouled plastic in the same way they respond to food odorants. In addition, indirect ingestion can occur by consuming prey such as filter feeders, which have previously ingested microplastic, for example, loggerheads feeding on clams or mussels (Di Beneditto & Awabdi, 2014; Miller et al., 2020).

The likelihood of plastic ingestion is closely linked to turtle feeding behaviour, which differs between species and life stages. Opportunistic foraging species, for instance loggerheads, are potentially exposed to consuming a wider variety of plastics because of their low discrimination in selecting dietary items (Lynch, 2018; Schuyler et al., 2014a). Specialist animals, such as leatherback turtles feeding mainly on gelatinous organisms, have a greater likelihood of ingesting particular types of debris similar to their prey, such as soft plastics resembling jellyfish, , as outline above (Constantino & Salmon, 2003; Mrosovsky et al., 2009; Schuyler et al., 2014b). Feeding strategies are also subject to adaptive changes across different life stages and food accessibility. For example, post-hatchling and early juvenile green turtles exhibit an opportunistic feeding behaviour during their oceanic stage, before undergoing an ontogenetic shift to herbivory once they move to neritic habitats (Arthur et al., 2008; Bolten, 2003; Reich et al., 2007). This opportunistic feeding behaviour makes these smaller potentially more exposed to the risk of plastic ingestion due to their low discrimination in consuming dietary items. The exposure to the risk of plastic ingestion can also vary geographically, because plastic debris tends to concentrate differently across the marine environment (Schuyler et al., 2014a). Some authors consider that the ecological behaviours guiding early life stage turtles to specific areas with high levels of plastic pollution for feeding and development could pose an evolutionary trap for the species (Duncan et al., 2021; Santos et al., 2021).

The impacts of plastic ingestion on marine turtles' health are diverse, ranging from negligible to deleterious and lethal injuries. The severity of impact depends mainly on the quantities and

characteristics of ingested plastics. Specific amounts of plastic can be retained for long periods within the gut with low or no effect on turtle health (Hoarau et al., 2014). However, in the long term, the displacement of dietary items by ingested plastic might reduce stomach capacity and the stimulus to feed, leading to dietary dilution and malnutrition (McCauley & Bjorndal, 1999; Santos et al., 2020; Tourinho et al., 2010). In other cases, veterinarians have reported blockages of the digestive tract caused by large pieces or significant quantities of plastic (Rizzi et al., 2019; Vélez-Rubio et al., 2018a), which resulted eventually in ischemic necrosis and septicaemia with lethal consequences (Mashkour et al., 2020; Tagliolatto et al., 2020). In addition, the bioaccumulation of toxic substances leached out from ingested plastic into blood and tissues can cause sub-lethal effects. These additives or derivatives of plastics, commonly known as plasticisers, are speculated to lead to malfunctions in metabolic and endocrine systems, as well as disorders in somatic growth rates and reproductive outputs (Anderson et al., 2016; Clukey et al., 2018; Marn et al., 2020; Nelms et al., 2016; Ryan et al., 2016). Diverse studies have detected the presence of phthalates (SanJuan et al., 2023; Savoca et al., 2018) and organophosphate esters (OPEs) (Sala et al., 2021) in the tissues, organs, and fluids of turtles. However, the cryptic effects derived from the accumulation of these toxins are still poorly understood in marine turtles.

Understanding the impacts of plastic ingestion on marine turtles is central to assessing their vulnerability to this emerging threat. Nevertheless, Key knowledge gaps remain in understanding the magnitude of this threat at a population level and/or over long-time scales, as well as for specific unrepresented geographical areas, species, and life stages (Casale et al., 2016; Fuentes et al., 2023; Lynch, 2018; Nelms et al. 2016; Senko et al., 2020). Despite several regional efforts (e.g., the Marine Strategy Framework Directive (MSFD) and the INDICIT Initiative in Europe), there is also limited global consensus on the use of consistent research protocols. This lack of standardisation hinders the comparison of results across studies and reduces the applicability of data for long-term monitoring and assessments of plastic pollution as a population or species-level threat (Casale et al., 2016; Hamann et al., 2010; Nelms et al. 2016; Provencher et al., 2017).

1.4 Plastic ingestion by green turtles in Uruguay

In this study, I analyse the impact of plastic ingestion on juvenile green turtles in Uruguay between 2014 and 2020. Uruguayan waters are a key feeding and development area for a mixed stock of juvenile green turtles within the Southwestern Atlantic region (López-Mendilaharsu et al., 2006, 2016; Vélez-Rubio et al., 2013, 2016, 2018b). These waters host a mixed stock of juveniles, which distribution extends from Bahia Blanca, Argentina (-38.7819, -62.3418), to the north coast of Sao Paulo, Brazil (-23.4523, -44.9917) (González-Carman et al., 2012; Vélez-Rubio et al., 2018b). These juveniles are recruited to coastal/neritic habitats from the main breeding site in the South Atlantic Ocean, Ascension Island (UK), and other rookeries across the region: Trindade Island (Brazil), Aves Island (Venezuela), Surinam and Guinea Bissau (Africa) (Almeida et al., 2021; Caraccio 2008; Patricio et al., 2017; Proietti et al., 2012; Prosdocimi et al., 2012). The green turtle exhibits a seasonal occurrence in Uruguayan waters (González-Carman et al., 2012; Vélez-Rubio et al., 2018b). Its presence is driven by changes in the sea surface temperature (SST). The higher occurrences of green turtles occur during the austral summer, coinciding with SST above 20° C due to the influence of the Brazil Current, a warm water current flowing southward (Figs. 1.1 and 1.2). Inversely, during the austral winter, the influence of the Malvinas Current, a cold water current flowing northward, cools the SST to below 15° C when turtles tend to migrate north following the retreat of warm waters (Figs. 1.1 and 1.2) (Franco-Fraguas et al., 2014; González-Carman et al., 2012; Piola et al., 2008; Palma et al., 2008; Vélez-Rubio et al., 2018b). Although this is the primary migratory pattern of juvenile green turtles at these latitudes, it is estimated that some individuals may remain in Uruguayan waters for extended periods, exposing them to low temperatures, or even throughout the winter, potentially undergoing brumation (Vélez-Rubio et al., 2017).

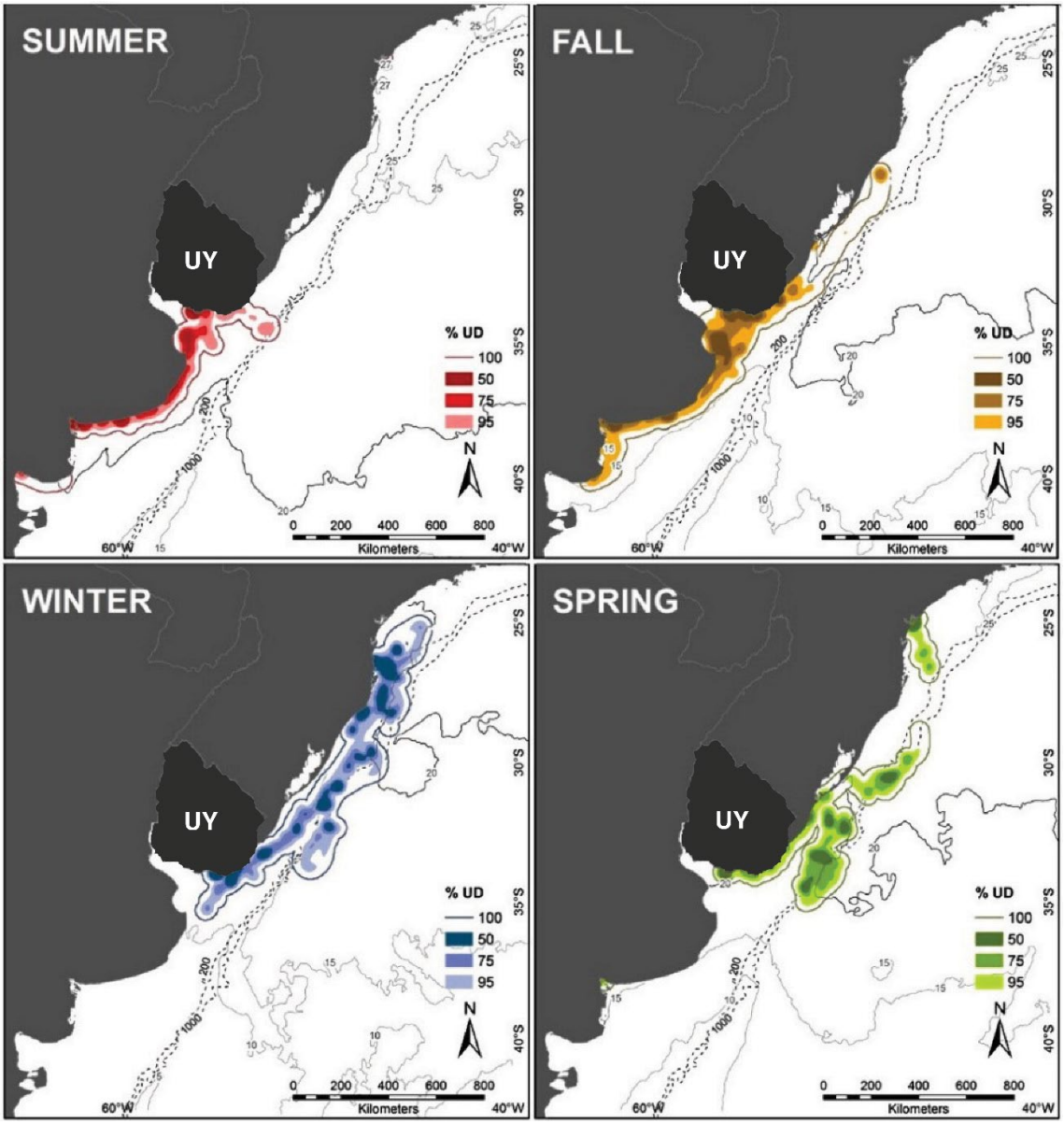

Figure 1.1. Seasonal habitat use of green turtles, *Chelonia mydas*, in the Southwestern Atlantic Ocean (adapted from González-Carman et al., 2012). The 100% and 50% UD represent the overall distribution range of the turtle and the core activity areas, respectively. Grey full lines (20 °C isotherm highlighted) represent monthly isotherms for February, May, August and November of 2009, respectively.

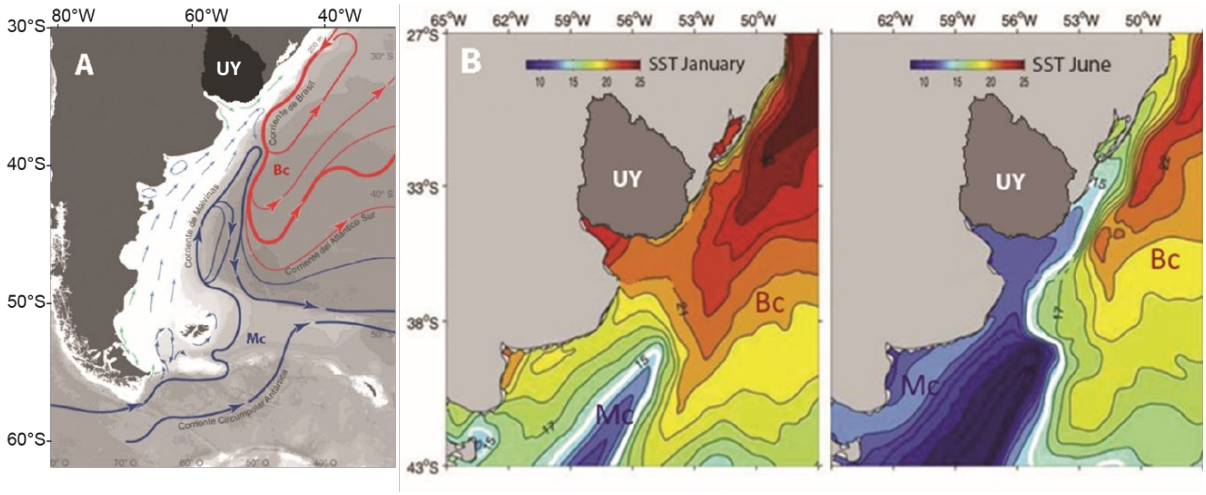

Figure 1.2. (A) Subtropical convergence of the Brazil Current (Bc; arrows in red), and the Malvinas Current (Mc; arrows in blue) offshore of Uruguay (UY). (B) Oceanographic and sea surface temperatures (SST) dynamics in the Southwestern Atlantic Ocean during summer (January, left isotherms map) and winter (June, right isotherms map) (adapted from Palma et al., 2008).

This stock of juvenile green turtles is relatively highly exposed to the risk of plastic ingestion due to the high levels of plastic pollution in these waters (González-Carman et al., 2014; Vélez-Rubio et al., 2018a). This significant accumulation of plastic originates from the sub-tropical convergence of the Brazil and Malvinas currents (Fig. 1.2), transporting debris from other latitudes and creating an aggregation zone offshore Uruguay (Franco-Fraguas et al., 2014; Manta et al., 2022; Ortega & Martínez 2007; Piola et al., 2008; Simionato et al., 2006). This situation is enhanced by the accumulation of debris arising from the Paraná River and its main tributaries coupled with the action of a benthic salinity front within the Rio de la Plata (Fig. 1.3), which drags debris toward the oceanic zone (Acha et al., 2003; González-Carman et al., 2014; Lebreton et al., 2017; Lozoya et al., 2015, 2016; Moreira et al., 2013; Rodríguez et al., 2020).

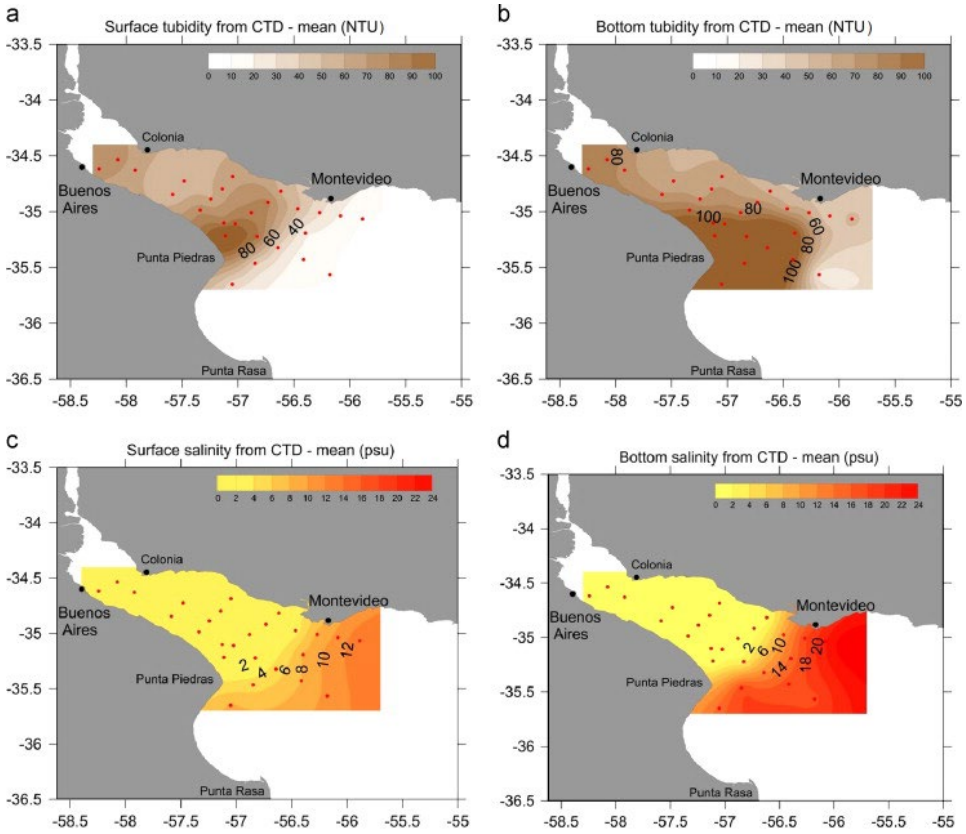

Figure 1.3. Surface and bottom mean turbidity (a,b); and surface and bottom salinity (c,d) computed from CTD/OBS observations into the Rio de la Plata estuary (adapted from Moreira et al., 2013).

Previous studies reported between 70% to 90% of the green turtles examined from Rio de la Plata and adjacent waters of Uruguay had ingested plastic (González-Carman et al., 2014; Vélez-Rubio et al., 2018a). Nonetheless, the magnitude of the plastic ingestion impacts on this stock of green turtles remains unclear since this data are limited to necropsy-based analysis of bycaught turtles (González-Carman et al., 2014) and stranded dead turtles (Vélez-Rubio et al., 2018a) collected opportunistically. Samples from necropsy examinations are subject to biases when establishing whether animals were in a healthy condition before death is not possible. Unhealthy animals might exhibit abnormal feeding behaviour or habitat use. In addition, the report by González-Carman et al. (2014) refers to the years 2008 – 2011, and Vélez-Rubio et al. (2018a) report covers the periods 2005 - 2007 and 2009 - 2013. Hence, an updated impact assessment is crucial since plastic ingestion is considered an ever-growing threat affecting the stock population of green turtles in these latitudes.

1.5 PhD thesis objectives

My PhD thesis aims to analyse the impacts of plastic ingestion on the stock of juvenile green turtles present in Uruguayan waters between 2014 and 2020. To achieve this goal, I identified the following objectives:

1. Generate baseline data on gastrointestinal transit times in juvenile green turtles in Uruguayan waters to determine the monitoring period required for detecting ingested plastic in the faecal matter of live animals.
2. Analyse the incidence and temporal trends of plastic ingestion by green turtles in Uruguayan waters and quantify and characterize the ingested plastic to assess ingestion patterns.
3. Assess the severity of the impacts caused by plastic ingestion on green turtles in Uruguayan waters in relation to the volumes and characteristics of ingested plastics.
4. Develop a best practice framework of strategies and standard methods for assessing plastic ingestion in marine turtles, considering the accessibility of resources and capabilities required.
5. Inform an assessment of the vulnerability of the green turtle to plastic pollution and implications for its conservation status at the scale of the South Atlantic - Regional Management Unit (RMU).

Objective 1: Generate baseline data on gastrointestinal transit times in juvenile green turtles in Uruguayan waters to determine the monitoring period required for detecting ingested plastic in the faecal matter of live animals.

To conduct a comprehensive assessment of plastic ingestion on the stock of green turtles in Uruguayan waters, I included a subset of dead and live animals to ensure a representative sample for analysis. While necropsy allows the retrieval of all the digestive contents for examination of ingested plastics in dead turtles; faecal matter examinations enable assessing plastic ingestion in live animals, provided adequate monitoring time is allocated to collect all potential plastic pre-ingested

in the environment. In Chapter 2, I conduct an experiment using inert plastic markers to estimate gastrointestinal transit times in juvenile green turtles. Knowing the upper limit of ingesta transit time enables me to establish the appropriate monitoring period for live turtles in my study. Additionally, I evaluate different experimental conditions replicating the experiment on juvenile green turtles from Heron Island, Australia.

Objective 2: Analyse the incidence and trends of plastic ingestion on green turtles in Uruguayan waters and quantify and characterize the ingested plastic to assess ingestion patterns.

Assessing the impact of plastic ingestion on green turtles in Uruguay is crucial to understanding the vulnerability of this stock population to plastic pollution. In Chapter 3, I analyse plastic ingestion of plastic in juvenile green turtles (N = 294) present in Uruguayan waters between 2014 and 2020 to assess the incidence and patterns of plastic ingestion over the study period. For this purpose, I quantified and characterised ingested plastic collected through three sampling techniques (necropsy, faecal matter monitoring, and gastric lavage) across different sources of specimens (stranded and bycaught dead turtles, and live wild-captured and rescued turtles), allowing me to provide additional information about the strengths and limitations of these approaches.

Objective 3: Assess the severity of the impacts caused by plastic ingestion on green turtles in Uruguayan waters in relation to the volumes and characteristics of ingested plastics.

The severity of the impact caused by plastic ingestion depends largely on the quantities and characteristics of ingested plastics. In Chapter 4, I analyse the factors influencing the severity of the impact caused by plastic ingestion in relation to the cumulative volumes and characteristics of plastic ingested plastic by a subsample of turtles examined in Chapter 3 (n = 150).

Objective 4: Develop a best practice framework of strategies and standard methods suitable for fulfilling research objectives, considering the accessibility of resources and capabilities required.

Plastic ingestion is recognised as an emerging threat and a priority conservation concern for marine turtles. Nevertheless, there is still limited consensus on adopting consistent research protocols and

methodologies for assessing this threat in marine turtles. This situation hampers the comparison of reports and results across studies, as well as the applicability of data for longer-term monitoring and assessments of plastic pollution as a population or species-level threat. In Chapter 5, I develop a best practice framework for designing and implementing research to assess plastic ingestion in marine turtles, discuss key aspects for establishing and fulfilling research objectives, and outline strategies for best practices to strengthen monitoring and research initiatives.

Objective 5: Inform assessment of the vulnerability of green turtles to plastic pollution and implications for its conservation status at the scale of the South Atlantic Regional Management Unit (RMU).

In 2019, the conservation status of the green turtle subpopulation within the South Atlantic Regional Management Unit (RMU) was down-listed from 'Endangered' to 'Least Concern' on the IUCN Red List based on an increase in nesting and hatching trends (Broderick & Patricio, 2019). However, the future viability of this subpopulation could be compromised, considering the current levels and future projections of plastic pollution that affect the early life stages of the species in the region. In Chapter 6, I discuss the conservation implications of my PhD findings, proposing approaches to address this concern, aiming for a more comprehensive examination of the vulnerability of green turtles to the impact of plastic pollution, ultimately informing future conservation status assessments of this subpopulation. I also emphasize the urgent need to take measures for reducing plastic pollution in the area and mitigate its impact on the marine environment.

1.6 PhD thesis outline

This PhD thesis is organised as a series of chapters (Fig. 1.4), each of which has been written in a format to facilitate publication in peer-reviewed journals. The associated publications (or intended publication) and co-authors are indicated in each chapter.

Chapter 1 (present chapter) provides a general introduction to the threat of plastic ingestion to marine turtles, as well as an overview of the conceptualization and structure of my PhD thesis, '*Impacts of plastic ingestion on green turtles (Chelonia mydas) in Uruguayan waters*.' The research strategy outlined in this thesis and the conservation efforts to address the plastic pollution issue in Uruguay have been published as a peer-reviewed book chapter. The reference for the publication is González-Paredes, D., & Estrades, A. (2021a). Plastics Versus Turtles: An Overview of the Uruguayan Case. In: B. Nahill (Ed.), *Sea Turtle Research and Conservation*, (pp 83-92). Elsevier Inc. Editorial. https://doi.org/10.1016/B978-0-12-821029-1.00009-X

Chapter 2 outlines the experimental design for estimating gastrointestinal transit times in juvenile green turtles. This baseline data is central for establishing appropriate monitoring periods to assess plastic ingestion by examining faecal matter in live turtles. A version of this chapter has been published: González-Paredes, D., Ariel, E., David, M. F., Ferrando, V., Marsh, H., & Hamann, M. (2021b). Gastrointestinal transit times in juvenile green turtles: An approach for assessing digestive motility disorders. *Journal of Experimental Marine Biology and Ecology*, 544, 151616. https://doi.org/10.1016/j.jembe.2021.151616

Chapter 3 assesses the incidence and patterns of plastic ingestion by green turtles in Uruguayan waters between 2014 and 2020. Different sources of specimens and sampling methods were used in the analysis, allowing for comparisons and providing insights into the advantages and disadvantages of these approaches. This chapter will be submitted to *Endangered Species Research* (target journal) as 'Plastic ingestion by green turtles (Chelonia mydas) in Uruguayan waters: insights from different study approaches' with authorship of Daniel González-Paredes, Gabriela Maria Vélez-Rubio, Helene Marsh, and Mark Hamann.

Chapter 4 evaluates the severity of the impact caused by plastic ingestion on juvenile green turtles in Uruguay through a dose-response analysis and assesses the risks of ingesting different plastic types. This chapter will be submitted to *Marine Pollution Bulletin* (target journal) as ' Impact severity

associated with plastic ingestion in juvenile green turtles in relation to the volumes and characteristics of ingested plastics' with authorship of Daniel González-Paredes, Rhondda Jones, Alejandro de la Fuente, Virginia Ferrando, Gabriela Maria Vélez-Rubio, Mark Hamann, and Helene Marsh.

Chapter 5 proposes a globally applicable best practice framework for designing and implementing research to assess plastic ingestion in marine turtles. The chapter discusses key aspects for establishing and fulfilling research objectives and outlining strategies for best practices to strengthen monitoring and research initiatives. This chapter will be submitted to *Conservation Biology* (target journal) as 'A best practice framework for assessing plastic ingestion in marine turtles', with authorship by Daniel González-Paredes, Emily Duncan, Brendan Godley, Helene Marsh, and Mark Hamann.

Chapter 6 summarises of the previous chapters' outcomes and outlines a general discussion on the vulnerability to plastic ingestion of green turtles in Uruguayan waters and its conservation implications for the conservation of the species in the South Atlantic Ocean – Regional Management Unit (RMU). A version of this chapter will be submitted to the IUCN-SSC Marine Turtle Specialist Group as '*Impacts of plastic ingestion on green turtles (Chelonia mydas) in Uruguayan waters'* with authorship by Daniel González-Paredes, Alejandro Fallabrino, Brendan J. Godley, Helene Marsh, and Mark Hamann, to emphasise the threat of plastic ingestion in future conservation strategies for marine turtles.

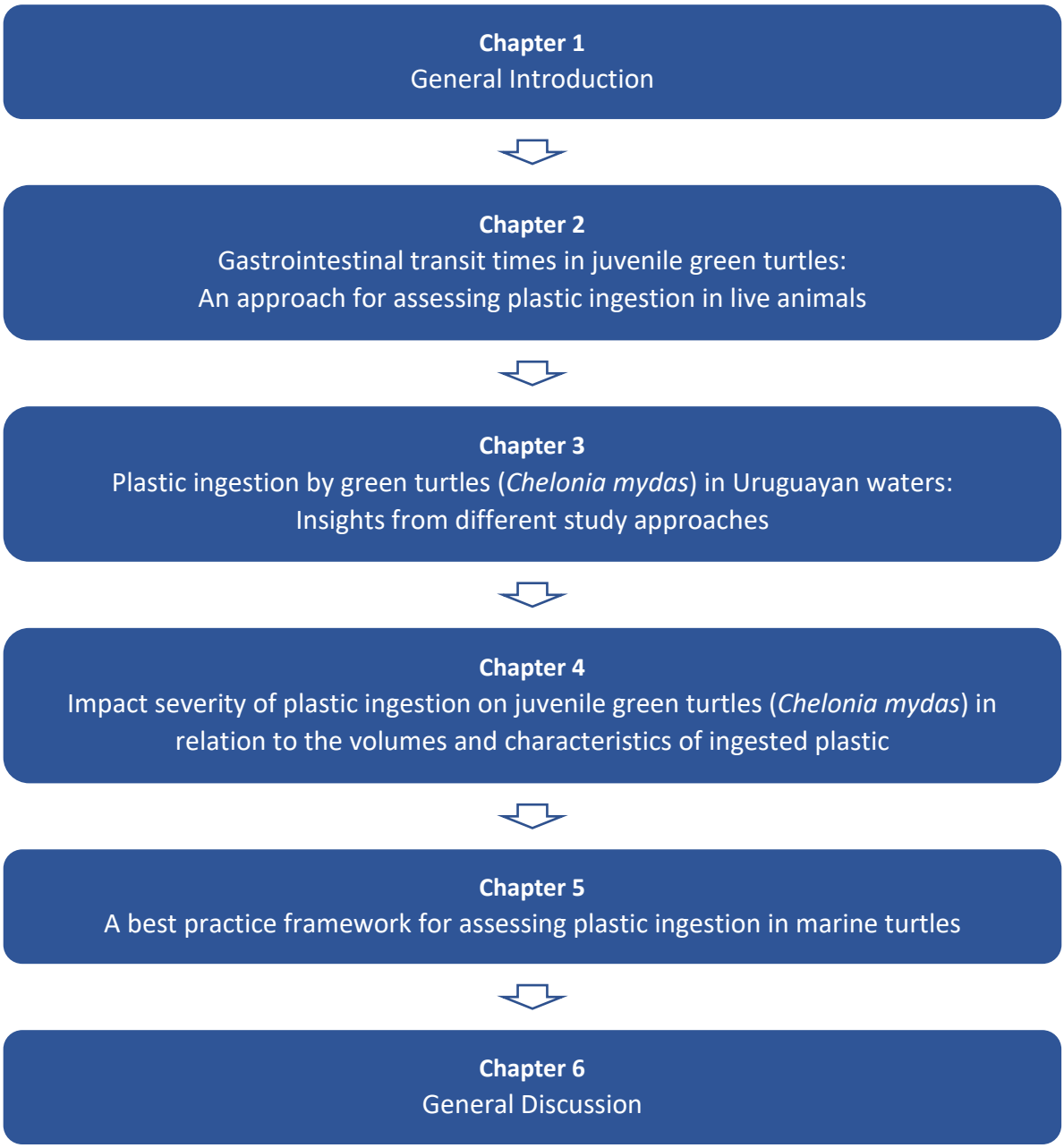

Figure 1.4. Chapter structure of this PhD.

PhD Chapter 2

Gastrointestinal transit times in juvenile green turtles: An approach for assessing digestive motility disorders

Chapter objective

To determine the appropriate monitoring period required for collecting of all ingested plastic from the faecal matter of live turtles in my study.

Methodology

To establish adequate monitoring periods, I conducted an experiment using inorganic markers (inert plastic discs) to estimate the gastrointestinal transit time of juvenile green turtles in Uruguayan waters. I replicated the experiment with juvenile green turtles from Heron Island in Australia, employing organic markers (corn kernels), to compare the effectiveness of the two marker types and other experimental conditions.

Key Findings

- Gastrointestinal transit times of juvenile green turtles examined in Uruguayan waters lasted up to 22 days. Establishing monitoring periods over the upper limit of ingesta time maximises the likelihood of collecting all plastic pre-ingested from the faeces of the live turtles examined in my study (see Chapter 3).
- Inert plastic discs (inorganic markers) are more efficient markers than corn kernels (organic markers). They are not degraded or discoloured by the digestive process, enabling high recovery success from faeces.
- Husbandry conditions, such as water temperature and diet administered, influence gastrointestinal transit times. Hence, these should ideally reflect natural conditions.

Conclusions

This study provides novel information on the gastrointestinal transit time of juvenile green turtles, particularly in a class size for which there is no previous data.
This baseline data on gastrointestinal transit times can contribute toward assessing digestive motility disorders and toxicology studies related to exposure to toxic substances leached from ingested debris.

Related publication

González-Paredes, D., Ariel, E., David, M. F., Ferrando, V., Marsh, H., & Hamann, M. (2021b). *Gastrointestinal transit times in juvenile green turtles: An approach for assessing digestive motility disorders.* Journal of Experimental Marine Biology and Ecology, 544, 151616. https://doi.org/10.1016/j.jembe.2021.151616

2.1 INTRODUCTION

Plastic ingestion is considered an emerging threat to marine turtles, affecting vital processes across their entire life cycle and key habitats (Duncan et al., 2021; Lynch, 2018; Schuyler et al., 2014a). Recognized as a priority conservation concern, research and monitoring efforts have intensified in recent years to assess this threat to marine turtle populations (Hamann et al., 2010; Nelms et al., 2016; Schuyler et al., 2014a). These assessments are generally based on necropsied animals, either stranded or bycaught turtles, collected opportunistically (Casale et al., 2016; Lynch 2018). Necropsy examination enables the retrieval of all digestive contents for the detection and analysis of ingested plastics. However, establishing whether these turtles were in a healthy condition before death or encounter is challenging unless the animal has recently died and a reliable post-mortem health assessment can be conducted (Casale et al., 2016; Lynch 2018; Nelms et al., 2016). Unhealthy turtles might exhibit abnormal feeding behaviour and/or altered habitat use, which can result in a higher consumption of plastic. For example, turtles exhibiting positive buoyancy associated with pneumonia face difficulties diving, forcing them to feed at the sea surface, where plastic concentrations are higher. Consequently, the ingestion rate for these unhealthy turtles may be higher, introducing biases in the analysis of the overall ingestion rate of a stock or population (Casale et al., 2016; Domènech et al., 2019; Lynch 2018).

Studies on plastic ingestion should incorporate representative samples, including turtles in healthy condition. Faecal matter monitoring represents a reliable approach for assessing plastic ingestion in live animals, provided adequate monitoring time is allocated to collect all ingested plastic. Hence, monitoring periods should extend beyond the upper limit of ingesta time to maximise the likelihood of collecting all plastic pre-ingested in the environment. In this respect, assessing gastrointestinal transit times represents a non-intrusive and low-cost approach for determining specific species and life stage monitoring periods.

The gastrointestinal transit time is defined as the time taken by an ingested item to pass through the entire digestive tract. Studies on gastrointestinal transit times have been conducted on several testudine species (Barboza, 1995; Hatt et al., 2002; Meyer, 1998; Spencer et al. 1998; Taylor et al., 1996) including marine turtles: loggerhead turtles, *Caretta caretta* (Di Bello et al. 2006; Valente et al. 2008); and green turtles, *Chelonia mydas* (Amorocho & Reina 2008; Brand et al. 1999; Hadjichristophorou & Grove 1983; McDermott et al. 2006). In reptiles generally, gastrointestinal transit times vary according to digestive efficiency associated with specific feeding strategies (Diaz-Figueroa & Mitchell, 2006). For instance, Valente et al. (2008) recorded mean transit times of 13.2 ± SD 4.6 days in juvenile loggerheads, a carnivorous species. While Amorocho and Reina (2008) recorded average transit times of 23.3 ± SD 6.6 days in juvenile green turtles, a predominately herbivorous species. The loggerhead turtle shows higher digestive efficiency than the green turtle (Di Bello et al. 2006; Valente et al. 2008), which results in shorter gastrointestinal transit times. In contrast, the green turtle uses a hindgut-fermentation strategy to digest the structural carbohydrates in plant cell walls, which requires longer gastrointestinal transit times (Brand et al., 1999; Bjorndal, 1980; Mackie, 2002).

Diet composition is one the factors influencing gastrointestinal transit times within a species. This is particularly interesting in green turtles due to the changes in their feeding behaviour and diet composition across different life stages. Juvenile green turtles usually undergo an ontogenetic shift in diet once they move to neritic habitats at the completion of their pelagic life-stage. At that time, their feeding behaviour changes from opportunistic and omnivorous to primarily herbivorous, and from a pelagic to a benthic-based diet (Arthur et al., 2008; Bolten, 2003; Reich et al., 2007). However, some juvenile green turtles in temperate and subtropical waters forage omnivorously even at neritic habitats, indicating an adaptive capacity according to food availability (Arthur et al. 2008; Cardona et al., 2009, 2010; Gama et al., 2016; González-Carman et al., 2012; Vélez-Rubio et al., 2015, 2016, 2018b).

Water temperature also influences gastrointestinal transit times in reptiles. The metabolism of poikilothermic reptiles, such as marine turtles, is regulated by the ambient temperature (Skoczylas, 1978; Williard, 2013). The optimal metabolic rate and highest digestive efficiency of green turtles occur within a specific range of temperatures, which Southwood (2003) experimentally estimated to be between 17 and 26°C. At lower temperatures, turtles can remain active, but their metabolic rate decreases to a thermal threshold inducing dormancy or hibernation. This thermal limit varies geographically among different green turtle aggregations, for example, 18°C in Florida (Mendonça, 1983), 15°C in the north-eastern Pacific Ocean (Seminoff, 2000), and 14°C in south-eastern Australia (Read et al., 1996). The effects of higher temperatures on the metabolic rates of green turtles have been less studied. However, high temperatures are likely to lead to greater food intake and faster digestive rates as observed by Bjorndal (1980) in green turtles exposed to temperatures above 34 °C for long periods in the Bahamas during atypical years of El Niño - Southern Oscillation (ENSO) (Ortega & Martínez 2007).

In this chapter, I aim to generate baseline data by estimating the gastrointestinal transit times in green turtles to determine the monitoring period required for detecting ingested plastic in faecal matter. I conducted an experiment using inert plastic markers to estimate gastrointestinal transit times in juvenile green turtles in Uruguay, enabling me to establish the appropriate monitoring period for live turtles in my study. Additionally, I evaluated different experimental conditions replicating the experiment on juvenile green turtles from Heron Island, Australia, using organic markers.

I also intend to validate the assessment of transit times as a non-intrusive complementary approach that could be used as an early warning sign of digestive motility disorders as well as highlighting its utility in furthering the study of sub-lethal impacts caused by bioaccumulation of toxins leached out from anthropogenic debris ingested, as an index of the time that these substances remain within the organism.

2.2 METHODS

I conducted a trial using inorganic markers to estimate gastrointestinal transit times in juvenile green turtles in Uruguay. I replicated this experiment on juvenile green turtles from Heron Island, Australia, using organic markers. Results of the trials will allow to compare the efficiency of both markers and evaluate other experimental conditions, such as administrated diet and water temperatures.

2.2.1 Inorganic marker trial

Six green turtles were intentionally caught in the wild from Uruguayan waters using scientific capture techniques (see methods in Vélez-Rubio et al., 2016). All turtles were assessed by a veterinarian as being in healthy condition after capture, following standard procedures described in Eckert et al. (1999). These animals ranged in size from 33.7 to 47.0 cm of curved carapace length (CCL) (mean = 40.6 ± SD 4.5 cm); and weighed between 4.4 – 10.9 Kg (mean = 7.5 ± SD 2.3 Kg) (biometrics collected following methods described in Eckert et al. 1999).

The turtles were transferred to the Karumbé NGO rehabilitation facilities in La Coronilla (Rocha, Uruguay), and placed individually into 1.5 m in diameter / 500 L tanks in a semi-shaded outdoor area. Husbandry followed the protocols approved by Uruguayan National Commission for Animal Experimentation (0024/12). During the trial, the tanks were cleaned daily, and salt water was exchanged every three days. Water temperature and salinity were controlled to reflect natural conditions. Turtles were fed daily up to 10% of their body weight on a macroalgae *Ulva* sp., the main dietary item of green turtles in Uruguayan waters (Vélez-Rubio et al., 2016).

Prior to the trials, turtles were allowed an adaptation period (6-8 days) under veterinary observation to detect any behaviour anomalies, and ensuring turtles exhibit normal digestive motility, meaning they feed and defecate regularly. On the first day of the trial, each turtle was given five purple

markers made from 7 mm diameter discs of polypropylene (Alfepa Ltd., Uruguay). The markers were administrated by intubating the turtles and introducing the markers one by one into the oesophagus with freshwater.

2.2.2 Organic marker trial

Eight green turtles, 32-months post-hatching, originating from Heron Island (Queensland, Australia) were held in captivity for research purposes in the Turtle Health Research Facility at James Cook University (Queensland, Australia), under research permit WITK 15765815 issued by the Australian Government. All turtles were under regular veterinary observation prior and during the trial period (following standard procedures described in Eckert et al., 1999). Additionally, blood analyses conducted along the experiment reflected regular haematological values (see values in Bolten & Bjorndal, 1992; Whiting et al., 2007; Flint et al., 2010). The turtles were assessed as clinically healthy with normal activity levels and behaviour. The animals ranged in size from 33.9 to 37.0 cm in CCL (mean = 35.9 ± SD 1.1 cm) and weighed between 4.4 – 5.3 kg (mean = 4.7 ± SD 0.3 kg) (biometrics collected following methods described in Eckert et al. 1999).

Husbandry followed the protocols established by the JCU Turtle Health Research Facility, which is approved by the JCU Animal Ethics Committee (A 2309). Animals were placed individually into 1.5 x 4 m / 500 L and 1.5 x 8 m / 1000 L tanks in a semi-shaded outdoor area. The seawater was sterilized by UV light and re-circulated through micro filters and fractionators for removing solids and oils. Water temperature and salinity were monitored during the trial period.

Coprophagia was observed prior to the experiment and was considered part of the continuous foraging behaviour of the study turtles (Lance & Morafka 2001). Therefore, I installed a mesh layer at the bottom of the tanks, facilitating faeces collection and creating a barrier to prevent turtles from re-ingesting their own faeces and markers.

Turtles were fed daily at a rate of 4% body weight with a blended diet of vegetables, fish pellets, tinned sardines, and vitamins (Sea Tabs®), compacted into gelatine cubes. On the first day of the trial, each turtle was fed 15 pre-cooked corn kernels (Coles Group Ltd. Australia), as organic markers, in batches of five with other food. According to prior observations when testing diet composition at JCU Turtle Health Research Facility (unpublished data), green turtles can ingest corn but do not easily digest it, resulting in whole corn kernels within their faeces.

2.2.3 Gastrointestinal transit time estimation

Turtles were monitored by veterinarians during the trial period at both locations to detect any anomalies in activity and feeding behaviour. The monitoring tanks were checked several times each day for faeces collection. Faeces were then examined for the presence and quantity of markers (Fig. 2.1). Gastrointestinal transit times were recorded as the number of days between marker administration and its expelling.

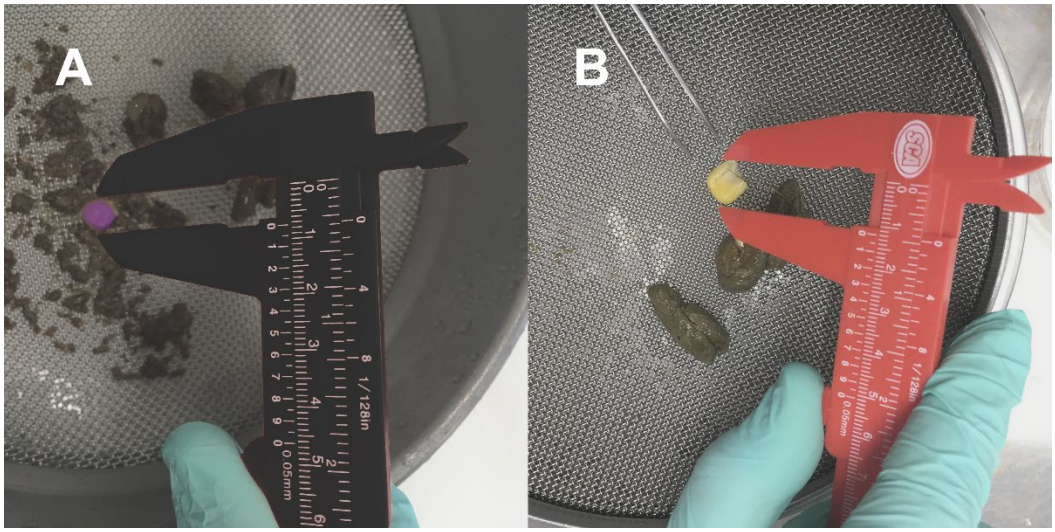

Figure 2.1. Markers recovered in the experiments: (A) inert plastic disc, inorganic marker; and (B) corn kernel, organic marker. Pictures copyright © Karumbé NGO.

2.3 RESULTS

2.3.1 Inorganic marker trial

The gastrointestinal transit time for the expulsion of the first marker averaged 14.6 ± SD 3.6 days, and the transit time for the expulsion of the last marker averaged 22.5 ± SD 4.2 days in the inorganic marker trial (Table 2.1). Markers were easily detected and recovered from faeces, recovery success was 96%; all the markers were recovered, excepting the last one from turtle UY03 (Table 2.1).

The experimental conditions reflected natural conditions in Uruguayan waters during the austral summer when the trial was conducted. The mean water temperature in the tanks was 23.4 ± SD 3.1°C (ranged 16 – 32 °C); while the salinity averaged 30.5 ± SD 0.5. The changes in weight (gain or loss) of individual turtles during the trial period averaged 6%, ranging from 5% weight gain to 9% weight loss.

It should be mentioned that turtle UY06 was excluded from the trial. Its ingestion rate decreased until stopped defecating on day six after starting the trial, when the animal was immediately transferred to the Karumbé rehabilitation area for appropriate treatment by veterinarians. A large number of plastic pieces was found in its faeces when defecation resumed, after turtle recovery (24 plastics fragments of different types totally 0.66 g). Subsequently, veterinarians diagnosed the individual with a partial obstruction caused by plastic ingested pre-capture while it was in its natural environment. I concluded that this obstruction was likely the main reason for the failed recovery of the markers during the trial period.

Table 2.1. Summary of the results obtained from the trial conducted using inorganic markers to estimate the gastrointestinal transit time of juvenile green turtles (*Chelonia mydas*). The trial took place at the rehabilitation facilities of Karumbé NGO (Rocha, Uruguay). The table includes biometric data, husbandry conditions, and gastrointestinal transit times corresponding to each marker expelled in the inorganic marker trial.

Turtle code	CCL (cm)	Weight (kg)	Water temperature, mean ± SD (°C)	Salinity, mean (ppt)	Gastrointestinal transit time (days)				
					1st marker	2sd marker	3rd marker	4th marker	5th marker
UY01	38.4	5.52	24.3 ± 4.2	30.0	19	19	20	22	23
UY02	47.0	10.90	25.6 ± 3.3	30.0	12	13	16	17	18
UY03	33.7	4.40	22.2 ± 3.6	31.0	11	11	11	13	-
UY04	40.8	7.40	21.7 ± 3.6	30.0	13	15	16	17	21
UY05	43.4	9.00	21.1 ± 2.9	31.0	18	21	21	25	28
UY06*	40.2	7.73	20.3 ± 3.0	31.0	-	-	-	-	-

*Turtle excluded from the trial due to health issues

2.3.2 Organic marker trial

The gastrointestinal transit time registered for the expulsion of the first marker averaged 6.63 ± SD 1.6 days in the organic marker trial. Markers were expelled in several defecations, reaching an overall recovery success of 72.5%. However, it was not possible to recover the last markers in any of the experimental animals (Table 2.2). The total markers recovery per turtle averaged 10.9 ± SD 1.46 corn kernels of the 15 administered initially, ranging from 8 - 13 corn kernels per turtle. I observed that markers expelled within the first 18 days were easily detected and recovered; in contrast to those expelled after > 18 days, which were markedly degraded by the digestion process and were more challenging to detect and collect from the faeces.

The experimental conditions remained within the parameters established by the JCU Turtle Health Research Facility. The mean water temperature in the tanks was 27.7 ± SD 1.2 °C (range 24.1 – 30.0 °C); while salinity averaged 28.4 ± SD 0.2. The changes in weight (gain or loss) of individual turtles during the trial period averaged 2%, ranging from 3% weight gain to 3% weight loss.

Table 2.2. Summary of the results obtained from the trial conducted using organic markers to estimate the gastrointestinal transit time of juvenile green turtles (*Chelonia mydas*). The trial took place at the Turtle Health Research Facility, James Cook University (Queensland, Australia). The table includes biometric data, husbandry conditions, and gastrointestinal transit times corresponding to each marker expelled in the organic marker trial.

					Gastrointestinal transit time (days)														
Turtle code	CCL (cm)	Weight (kg)	Water temperature, mean ± SD (°C)	Salinity, mean (ppt)	1st mrk	2sd mrk	3rd mrk	4th mrk	5th mrk	6th mrk	7th mrk	8th mrk	9th mrk	10th mrk	11th mrk	12th mrk	13th mrk	14th mrk	15th mrk
JCU01	36.3	4.63	27.3 ± 1.1	28.6	6	6	10	11	17	17	17	17	18	19	19	-	-	-	-
JCU02	33.9	4.36	27.3 ± 1.1	28.6	5	6	6	8	9	10	10	10	17	20	20	-	-	-	-
JCU03	35.7	4.62	27.5 ± 1.0	28.3	6	6	6	6	6	6	7	8	8	8	8	11	14	-	-
JCU04	36.2	4.77	27.4 ± 1.0	28.3	6	9	9	10	10	10	10	11	11	11	21	-	-	-	-
JCU05	34.9	4.35	27.8 ± 1.0	28.4	9	9	11	11	16	16	16	17	17	17	-	-	-	-	-
JCU06	37.0	5.15	27.8 ± 1.2	28.4	9	9	9	10	10	10	10	10	11	16	16	20	-	-	-
JCU07	36.4	4.54	28.2 ± 1.4	28.3	7	14	14	14	14	14	14	15	-	-	-	-	-	-	-
JCU08	37.0	5.28	28.2 ± 1.4	28.3	5	6	6	11	11	12	12	12	12	12	12	-	-	-	-

Transit times recorded were expressed as the percentages of markers recovered in order to compare the results of both trials (Fig. 2.2). I defined T1 as the time between the ingestion of the markers and the first defecation containing at least one of the markers; and subsequently T40, T60, T80 and T100 as the times required to expel 40, 60, 80 and 100 percent of the markers respectively. Turtles in the inorganic marker trial showed overall gastrointestinal transit times longer than turtles in the organic marker trial. I tested for potential correlations between the gastrointestinal transit times and data on temperature water, CCL and body mass, calculating Pearson's correlation coefficient in both experiments. Low or no significant correlation was observed between variables tested ($p > 0.05$). However, these results must be treated with caution due to the small sample sizes.

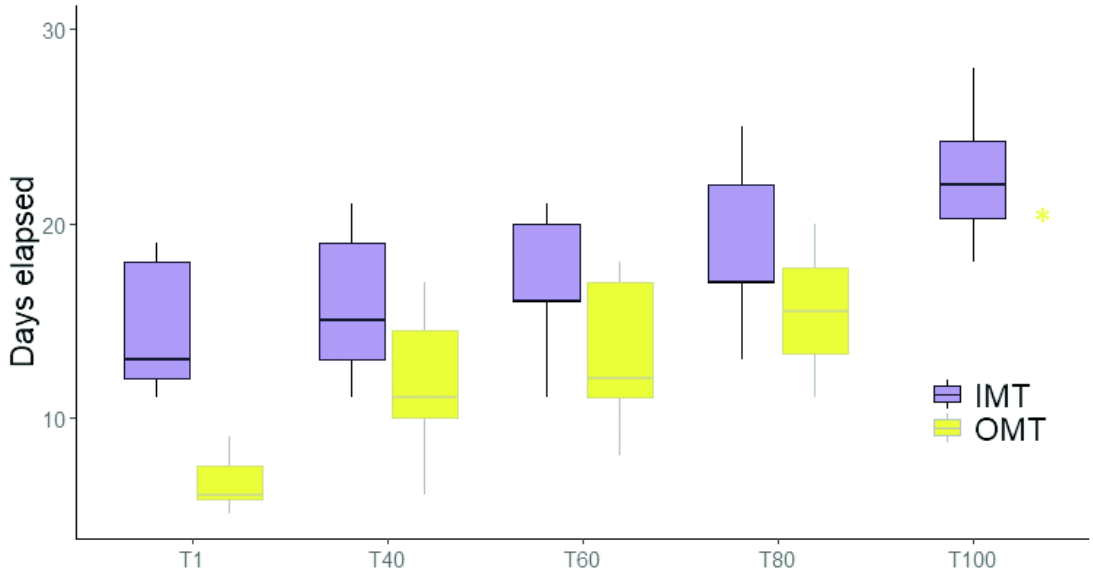

Figure 2.2. Gastrointestinal transit times registered in the inorganic marker trial, IMT (purple boxes) and organic marker trial, OMT (yellow boxes). Values are expressed as the percentage of markers recovered. The intervals are defined as: T1, time for the expelling the first marker; T40, T60, T80 and T100 for times required to expel 40, 60, 80 and 100 percent of the markers respectively.
(*) No values registered for T100 in the organic markers trial.

2.4 DISCUSSION

The mean length registered for the recovery of the first marker were 15 days for the inert plastic discs (inorganic marker) and to 7 days for corn kernels (organic marker). The corresponding data for the last marker recovered was 22 days for the inorganic marker), while it was not possible to recover the last organic marker administrated.

These findings fall within the ranges estimated for juvenile green turtles in previous studies (Table 2.3). The differences in the results of these previous studies may result from experimental design and husbandry conditions. Furthermore, all of them indicated variations in the transit times within and between their experimental animals (Table 2.3). Authors attributed these variations to individual physiological and behavioural differences. This effect may also explain variations between individuals within our trials. Despite green turtles in this study being smaller in size (CCL) than those used in previous studies (Table 2.3), there is no consistent evidence in the literature suggesting that shorter digestive tracts in smaller animals, within the same age class of a species, are associated with shorter gastrointestinal transit times.

Table 2.3. Experiment details and summary of results from studies of gastrointestinal transit times on juvenile green turtles (*Chelonia mydas*). Gastrointestinal transit time values are expressed as percentage of markers expelled; where Ti is the time to expulsion of the first marker, and Tf is the time to the last marker recovered.

Reference	Sample size (N=turtles)	CCL[1] range (cm)	Water temperature, mean ± SD (°C)	Transit times, mean ± SD (days)	Diet composition
McDermott et al. (2006)	4	60.6 – 72.7	26.6 ± 1.9	Ti = 15.4 ± 0.5 Tf = >35	Natural diet on the red algae *Gracilariopsis lemaneiformis*
Brand et al. (1999)	3	50.3 – 55.2	24.1 ± 2.4	T50 = 6.5 - 13.5[3]	Natural diet as free-range turtles
Amorocho and Reina (2008)	6	52.0 – 62.2 (SCL)[2]	28.3 ± 0.3	Ti = 22.0 ± 6.3 Tf = 24.7 ± 6.0	Three different maintenance diets; (a) fish based (b) plant based (c) fish & plant based
Inorganic marker trial (present study)	6	33.7 – 47.0	23.4 ± 3.1	Ti = 14.6 ± 3.6 Tf = 22.5 ±4.2	Natural diet on green algae *Ulva sp.*
Organic marker trial (present study)	8	33.9 – 37.0	27.7 ± 1.2	Ti = 6.63 ± 1.6 Tf = 17.7 ± 3.7	Maintenance diet of mixed food

[1]CCL = curved carapace length

[2]SCL = straight carapace length

[3]Transit times reported in Brand et al., (1999) are given as a range of days for expelling of 50% of the markers.

In both trials, markers were expelled both individually and in batches. I assumed that markers travelled with food boluses as I found the markers embedded in faeces. During digestion in green turtles, distinct boluses are compacted by the peristaltic movements of turtle's gut (Bjorndal, 1997; Penry & Jumars, 1987).

Plastic markers are widely used for gastrointestinal transit time studies in reptiles (Amorocho & Reina, 2008; Brand et al., 1999; Hailey, 1997; McDermott et al., 2006; Spencer et al., 1998; Valente et al., 2008). They are low cost, or easy to manufacture, and do not suffer degradation or discoloration when passing through the digestive tract, making them reliable and easy to detect and recover. Apart from our organic marker trial, all the studies in Table 2.3 used plastic markers. Nevertheless, potential secondary effects arising from the use of plastic markers such as chemical leaching are yet to be assessed. Organic markers such as corn kernels provide an alternative for assessing digestive motility, avoiding issues of chemical leaching. However, corn is subject to degradation along the digestive process, which may hamper the detection of the last markers expelled, and consequently reducing their recovery rate. I observed this handicap in my study, with a recovery success of 72.5% using corn kernels as markers compared to 96% recovery success using plastic markers.

Water temperature is a factor that is likely to influence the gastrointestinal transit times and trials should ideally be conducted in the wild (see Amorocho & Reina 2008; Brand et al., 1999); or on captive turtles maintained at temperatures reflecting natural conditions as much possible (see McDermott et al., 2006, and the inorganic marker trial in this study). Additionally, I postulate that the higher and more constant temperatures of the organic marker trial may facilitate the digestion process, thereby shortening the gastrointestinal transit times.

Another factor that is likely to influence gastrointestinal transit time is the diet administered during the experimentation. Green turtles are predominantly herbivores and use a hindgut-fermentation strategy to digest the structural carbohydrates in plant cell walls (Bjorndal, 1980; Mackie, 2002).

However, Higgins (2003) observed in long-captive green turtles a feeding adaptability to carnivorous or mixed artificial diets, which are commonly used in turtle rehabilitation programs. The mixed diet of processed food in the organic marker trial might have facilitated faster digestion in comparison to the diet based on macroalgae species *Ulva* provided in the inorganic marker trial. This may partially also explain shortened gastrointestinal transit times in the organic marker trial.

In addition, excess animal handling might increase stress and consequently affect digestive processes and gastrointestinal transit times. Valente et al. (2007) observed that stress caused by excessive handling in loggerhead turtle, *Caretta caretta*, induced longer gastrointestinal transit times. I minimised handling the experimental animals to reduce this factor as much as possible, aside from the need to handle turtles for administering the inorganic markers.

On the other hand, it was not possible to recover any of the markers administered to turtle UY06 as this animal was diagnosed with a partial obstruction caused by plastic. Such incident is evidence that gastrointestinal transit time assessment is a useful non-intrusive and indirect approach for providing early warning of digestive system blockages. This approach could be particularly helpful for conservation and rehabilitation organisations that do not have convenient access to specialised equipment and techniques for accurate diagnoses due to budget constraints or proximity to facilities.

Gastrointestinal transit times also represent a parameter of relevance for future toxicology studies. There is increasing concern about the sub-lethal effects derived from plastic ingestion due to the leaching and absorption of toxic substances contained in, or adhered to, the ingested plastic. These toxic substances can lead to metabolic and endocrine malfunctions or fertility inhibition in males (Clukey et al., 2018; Sala et al., 2021; Savoca et al., 2018; White et al., 2018). These adverse effects are likely to be directly related to the time spent by the toxins inside the organism.

2.5 CONCLUSIONS

This study provides novel information on the gastrointestinal transit time on juvenile green turtles, particularly in a class size for which there is no previous data. Ingested items can last from one week up to three weeks to pass through the entire digestive tract of a healthy turtle. As an implication for my research, the faecal monitoring period should extend to at least 22 days to maximise the likelihood of collecting all plastic from the faeces of the live turtles examined in my study.

By estimating transit times using two different types of markers, inorganic (inert plastic discs) and organic (corn kernels), allowed me to compare their efficiency for such studies. Overall, inert plastic markers are more efficient since they not degraded or discoloured by the digestive process, enabling high recovery success. However, potential secondary effects such as chemical leaching from plasticisers should be considered. Other important factors affecting gastrointestinal transit times to be considered include water temperature and diet composition, which ideally should reflect natural conditions.

This baseline data on gastrointestinal transit times will contribute towards warning assessments of digestive motility disorders. Furthermore, this knowledge on transit times could be of interest for toxicology studies regarding time of exposure to toxic substances lixiviated from debris ingested.

PhD Chapter 3

Plastic ingestion by juvenile green turtles (*Chelonia mydas*) in Uruguayan waters; Insights from different studies approaches

Chapter objective

To assess plastic ingestion by juvenile green turtles in Uruguayan waters, using different sources of specimens and sampling methods to ultimately provide information about the strengths and limitations of these approaches.

Methodology

The incidence and annual trends of plastic ingestion were analysed in juvenile green turtles (N = 294) present in Uruguayan waters between 2014 - 2020. In addition, ingestion patterns were assessed based on the quantities and characteristics of ingested plastic. Turtles were from different sources of specimens (stranded and bycaught dead turtles, and live wild-captured and rescued turtles), and samples were collected using three techniques (necropsy, faecal matter monitoring, and gastric lavage), enabling the evaluation of the advantages and disadvantages of these different approaches.

Key Findings

- The overall incidence of plastic ingestion was 76% among the examined turtles, with quantities of ingested plastic recorded higher than those found in green turtles in the rest of the Southwestern Atlantic region.
- No identifiable annual trend was detected in the incidence of plastic ingestion, nor the quantities of plastic ingested across the sources of specimens.
- A negative relationship was observed between the quantities of ingested plastic and turtles' curved carapace length (CCL), indicating that larger turtles consumed proportionally less plastic.
- Laminar soft plastics in white and clear/transparent colours were the most consumed plastic types in number of pieces. This feeding selectivity could be driven by their resemblance to the most common dietary items for green turtles in Uruguayan waters: macroalgae and gelatinous macrozooplankton.

Conclusions

Uruguayan waters represent a significant hotspot for plastic ingestion among juvenile green turtles in the Southwestern Atlantic region. This situation is attributed to oceanographic features in these latitudes, aggregating high accumulations of plastic debris, and the feeding behaviour exhibited by early juveniles recently recruited to the neritic habitat. Urgent measures are required to mitigate the impacts of plastic pollution on this stock population.

Bycaught and wild-captured turtles are more reliable indicators of a population's overall exposure to plastic ingestion if sampling is systematic, while stranded dead and rescued turtles can provide valuable insights on the severity of the impact caused by plastic ingestion.
Necropsy examination remains the most reliable sampling technique for assessing plastic ingestion, enabling retrieval of all the digestive contents. While faecal monitoring enables live animals to be sampled, it provides adequate monitoring time for collecting all the ingested plastic. Gastric lavage proved inefficient for studying plastic ingestion in marine turtles.

Publication

González-Paredes, D., Vélez-Rubio, G.M., Marsh, H., & Hamann, M. (*in prep.*)
Plastic ingestion by green turtles (Chelonia mydas) in Uruguayan waters: Insights from different studies approaches.
Endangered Species Research (target journal)

3.1 INTRODUCTION

Uruguayan waters are a key feeding and development area for a mixed stock of juvenile green turtles within the Southwestern Atlantic region (López-Mendilaharsu et al., 2006, 2016; Vélez-Rubio et al., 2013, 2016, 2018b; see Chapter 1). The species exhibits a seasonal occurrence in these waters mainly driven by changes in the sea surface temperature (SST). The higher occurrences of green turtles occur during the austral summer, when the juveniles reach the foraging grounds within the neritic zone along the Uruguayan coast, looking to feed on macroalgae and gelatinous macrozooplankton (Vélez-Rubio et al., 2016, 2018b; see Chapter 1). Nevertheless, the high level of plastic pollution in these waters leads these turtles to a significant risk of plastic ingestion (González-Carman et al., 2014; Vélez-Rubio et al., 2018a; see Chapter 1). Assessing the impacts of plastic ingestion on this stock population is crucial for understanding its vulnerability to the threat of plastic pollution.

However, studying plastic ingestion in marine turtles is often challenging due to the complexity of factors involved in this threatening process. As outlined in Chapter 1, exposure to plastic ingestion can vary widely due to the intra- and interspecific variability in turtles' feeding behaviour (Duncan et al., 2019a; Lynch, 2018; Nelms et al., 2016; Schuyler et al., 2014a). The life stage of turtles represents another significant predictor of plastic ingestion. Post-hatchlings and juvenile turtles in oceanic stages are considered more susceptible to ingesting a wide range of plastics and higher volumes due to their opportunistic feeding strategy (Lynch, 2018; Schuyler et al., 2014b). Furthermore, the occurrence and abundance of plastic are not uniform in the environment, varying across space and time (Cózar et al., 2014; Kershaw & Rochman, 2015); consequently, the risk of plastic ingestion increases significantly when highly polluted areas overlap with marine turtles' habitats (González-Carman et al., 2014; Schuyler et al., 2016).

Approaches to assessing plastic ingestion are diverse and differ according to research objectives. These should be selected considering the accessibility of resources and capabilities (see Chapter 5).

Basic reports of plastic ingestion generally are based on turtles collected opportunistically through bycatch programs or stranding networks, in which plastic ingestion is detected after routine necropsy examinations (Da Silva et al., 2015; Gama et al., 2021; Vélez-Rubio et al., 2018a). Monitoring plastic ingestion over time requires more representative sample sizes and systematic data collection following standard procedures to infer incidence, trends and/or ingestion patterns (Choi et al., 2021; Domènech et al., 2019) (see Chapter 5 for more information about approaches and research aims concerning plastic ingestion in marine turtles). Among the factors influencing the efficiency and effectiveness of a research strategy to detect plastic ingestion are the source of specimens and the sampling method. For example, the necropsy examination of a stranded turtle enables the retrieval of all its digestive contents to detect and analyse ingested plastics. However, ingestion rates may be overestimated if it is not possible to determine whether the turtle was in poor health before being encountered, exhibiting abnormal feeding behaviour, as outlined in Chapter 2. Hence, understanding the advantages and disadvantages of the available approaches is central when designing research on plastic ingestion in marine turtles.

In this chapter, I aim to analyse plastic ingestion in juvenile green turtles (N = 294) present in Uruguayan waters between 2014 and 2020 to determine the incidence and assess ingestion patterns. For this purpose, I quantified and characterised the ingested plastic collected through three sampling techniques (necropsy, faecal matter monitoring, and gastric lavage) across different sources of specimens (stranded and bycaught dead turtles, and live wild-captured and rescued turtles). This allows me to evaluate the strengths and limitations of these approaches.

3.2 METHODS

3.2.1 Sample collection

The data and sample collection involved three different techniques: necropsy, monitoring faecal matter, and gastric lavages. The examined turtles were obtained from various sources, including

stranded and bycaught dead turtles, as well as live wild-captured and rescued turtles (Table 3.1). Turtles were all juveniles with a mean curved carapace length (CCL) of 38.9 ± SD 6.4 cm (range 28.6 to 70.7 cm), assuming a minimum size at maturity of CCL = 90 cm for green turtles in the Southwestern Atlantic region (Almeida et al. 2011).

Table 3.1. Sampling methods and sources of specimens used in this study.

Health status	*Source of specimens*	*Sampling technique*	*Number of turtles*
Dead	Stranded	Necropsy	124
Dead	Bycaught	Necropsy	18
Live	Captured	Faecal matter monitoring	59
Live	Rescued	Faecal matter monitoring	37
Live	Captured	Gastric lavage	56

3.2.1.1 Necropsy examination

I analysed plastic ingestion in stranded (n = 124) and bycaught (n = 18) dead turtles opportunistically collected by the marine turtle rescue and stranding network of Karumbé NGO (see procedures in Vélez-Rubio et al., 2013). Animals were all necropsied following the procedures described in Wyneken (2001). The digestive contents were retrieved from each animal's oesophagus, stomach and intestines and rinsed separately with filtered water onto consecutive multiple sieves of ten-, five- and one-mm mesh. The remnant materials were transferred to a sorting tray to inspect and collect any ingested plastics.

3.2.2.2 Faecal matter monitoring

The presence of ingested plastic within faecal matter was monitored in turtles intentionally captured in the wild (n = 59) and rescued turtles in rehabilitation treatment (n = 37). Turtles were provided by (i) Karumbé NGO's long-term marine turtle monitoring program, where wild turtles are systematically captured using approved scientific techniques and then held in captivity for the organisation's research purposes (see procedures in López-Mendilaharsu et al., 2016 and Vélez-

Rubio et al., 2016); and (ii) injured turtles rescued from bycatch or stranding events, opportunistically collected by the marine turtle rescue and stranding network of the organisation (see procedures in Vélez-Rubio et al., 2013).

Husbandry conditions followed established protocols approved by the National Committee for Animal Experimentation of Uruguay (CNEA). Each turtle was held individually in 500 L monitoring tanks and checked multiple times daily for the presence and collection of faeces. Their health status and feeding behaviour were under veterinarian observation during the monitoring period. The monitoring duration was determined based on the gastrointestinal transit times of juvenile green turtles estimated in Chapter 2. Thus, monitoring periods of at least 22 days were established, coinciding with the upper limit of ingesta passage time for these turtles (González-Paredes et al., 2021b). In the case of rescued turtles, the monitoring period was adapted according to rehabilitation treatment and extended until the animal's recovery, and no plastic was detected in its faeces. This protocol maximised the likelihood of collecting all potential plastic ingested in the natural environment before the capture or rescue. Faeces were transferred to a sorting tray and rinsed to inspect and collect any ingested plastics. I assumed no plastic was retained in the digestive tract, given that the examined turtles exhibited normal feeding behaviour and regular digestive motility by the end of the monitoring period.

Monitored turtles that died during the rehabilitation (n=6) were included in the necropsy group, adding the plastics found within their faeces to the total amount of ingested plastics retrieved from their digestive contents through necropsy.

3.2.2.3 Gastric lavage

One-time gastric lavages were conducted on turtles captured in the wild (n = 56) using approved scientific techniques (see procedures in Vélez-Rubio et al., 2016). Veterinarians assessed all turtles as healthy and suitable for this sampling technique. The gastric lavages were executed by experienced and qualified staff of Karumbé NGO, following the procedures described in Forbes and Limpus

(1999). The retrieved contents were rinsed separately onto a 1 mm mesh sieve to thoroughly inspect and collect any ingested plastics. However, no plastic was found in these animals, consequently this subgroup of turtles was excluded from subsequent analyses.

3.2.2 Sample examination

All the ingested plastic collected over 1 mm in maximum diameter (or alternatively retained by a 1 mm mesh sieve) were cleaned, air-dried, labelled, and stored for subsequent analysis. Plastic pieces were quantified and individually weighed (Analytical Balance Mettler Toledo Ms 105, +/- 0.01 mg), measured in three dimensions (TTI Digital Calliper, +/- 0.01 mm), and scored for colour based on a standard chart of the visible spectrum, which included eight colours, along with black, white, and clear/transparent (see methods in Duncan et al., 2019a). Plastics were classified based on their morphology, following procedures in Van Franeker et al. (2011) and the MSFD Technical Subgroup on Marine Litter (2013) (Appendix 3A in Supplementary Materials). In addition, I evaluated the flexibility and sharpness of each plastic piece using a flexibility and sharpness index (FSI). This index was based on a three-value scale for each characteristic and summing both scores. For instance, a soft plastic wrap corresponds to an FSI 2, representing the lowest value on each characteristic scale. In contrast, a lollipop stick was assigned an FSI 6 due to its rigid nature and pointed edges.

Due to time and resource constraints, I conducted different levels of analysis on random subsamples of necropsied turtles (proportional to the sample size per year): *'Stranded - Subsample A'* included 71 out of 124 stranded turtles for quantification and characterisation of ingested plastic. *'Stranded - Subsample B'* comprised 26 out of 71 turtles from subsample A for analysis of the quantities and categories of ingested plastic, coupled with analysis of colour, dimensions, and the flexibility and sharpness index of each plastic piece. *'Bycaught - Subsample C'* consisted of 10 out of 18 bycaught turtles for the same analysis described in subsample B. This last comprehensive analysis was also conducted on all the plastic pieces retrieved from turtles under faecal monitoring (Table 3.2).

Table 3.2. Analyses conducted on the ingested plastic retrieved from different samples/subsamples of examined turtles.

Sampling technique	*Source of specimens*	*Number of turtles*	*Analysis*
Necropsy	Stranded	*71 (Subs. A)*	*Basic*: quantification and categorization of ingested plastic
		26 (Subs. B)	*Comprehensive*: quantification and categorization of ingested plastic, coupled with analysis of the colour, dimensions, flexibility and sharpness of each plastic piece
	Bycaught	*10 (Subs. C)*	*Comprehensive*: quantification and categorization of ingested plastic, coupled with analysis of the colour, dimensions, flexibility and sharpness of each plastic piece
Faecal matter monitoring	Captured	59 *(all group)*	*Comprehensive*: quantification and categorization of ingested plastic, coupled with analysis of the colour, dimensions, flexibility and sharpness of each plastic piece
	Rescued	37 *(all group)*	*Comprehensive*: quantification and categorization of ingested plastic, coupled with analysis of the colour, dimensions, flexibility and sharpness of each plastic piece

3.2.3 Statistical analysis

I assumed the plastic retrieved is representative of the plastic consumed by the examined turtles. However, in this chapter, I have not taken into consideration potential variations in retention times associated with plastic types or dimensions, nor whether the health status of the turtle might influence the progression rates of plastic along the digestive tract.

The datasets were divided for statistical analysis into four subgroups based on the source of specimens (stranded, bycaught, captured, and rescued turtles). I used individual turtles as the sampling unit and the number of pieces as the reporting metric for comparison with related studies in the Southwestern Atlantic region. Results are presented as the average and standard deviation, range (minimum and maximum), and frequency of occurrence (%FO) of the number of pieces. Additionally, median values (number of pieces) and weights (grams) are provided for specific parameters.

I examined the incidence and quantity of plastic ingestion over the study period, assessing potential significant differences across the sources of specimens using chi-square and t-tests, respectively.

Following the methods described in Provencher et al. (2015) and Lavers et al. (2021), power analyses were conducted to determine the detectable percentage of change over the study period based on the sample sizes and coefficients of variation (CV) for each source of specimens (see the formula in Appendix 3B in the Supplementary Materials). Subsequently, trends in the incidence and quantities of plastic ingestion over the study period were analysed for each subgroup using Generalized Linear Models (GLMs).

Additionally, I employed a linear mixed-effects regression model (LMER) to examine the potential correlation between turtle size and quantities of ingested plastics. The analysis considered CCL as the fixed effect, while the source of specimens and year of turtle encounter were treated as random effects. The best-fitting model was determined based on generated Akaike Information Criterion (AIC) values.

Potential ingestion patterns were assessed based on the quantities and characteristics of plastic ingested.

All statistical analyses were conducted in R 4.2.2 (R Core Team, 2022).

3.3 RESULTS

3.3.1 Incidence and temporal trends in plastic ingestion

The overall incidence of plastic ingestion recorded was 76% of the 238 juvenile green turtles examined in Uruguayan waters between 2014 and 2020. This incidence varies slightly among the different turtle subgroups. Among the turtles examined by necropsy, plastic ingestion was detected in 71.8% of stranded turtles (89/124 animals) and 77.8% of bycaught turtles (14/18 animals). No significant difference was observed between the incidence of these two subgroups ($\chi^2(1) = 0.28, p =$

0.594). Regarding monitored turtles, plastic was found in the faecal matter of 86.4% of captured turtles (51/59 animals) and 73.0% of rescued turtles (27/37 animals). No significant difference was detected between the incidence of plastic ingestion of these two subgroups ($\chi^2(1) = 2.58$, $p = 0.108$). The annual incidence of plastic ingestion fluctuated among the different turtle subgroups over the study period (Fig. 3.1).

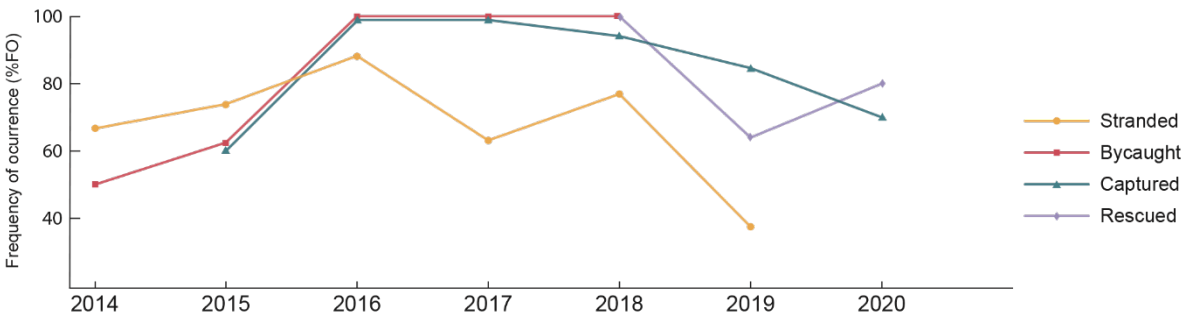

Figure 3.1. Incidence of plastic ingestion (reflected as Frequency of Occurrence, %FO) registered in each subgroup of turtles examined.

Although power analysis indicated that the sample sizes in each subgroup enabled reliable detection of inter-annual changes during the study period (Table 3.3), logistic regressions did not identify any trends in the annual incidence of plastic ingestion (Appendix 3C in Supplementary Materials).

Table 3.3. Results of the power analysis on the percentage of change detection in the annual incidence of plastic ingestion over the study period according to the sample size and coefficient of variation (CV) of each subgroup of turtles examined (see formula in Appendix 3B in Supplementary Materials).

Source of specimens	*Stranded*	*Bycaught*	*Captured*	*Rescued*
Sample size	124	18	59	37
CV (SD/mean*100)	26.3	23.4	24.0	29.5
Change detection (%)	12.5	33.8	13.2	28.6

Acronyms: CV (coefficient of variation), SD (standard deviation)

3.3.2 Analysis of the quantities of plastic ingested

The quantity of plastic ingested varies significantly among the different turtle subgroups. Stranded turtles exhibited an accumulation of plastic (median 208 pieces of plastic) an order of magnitude greater than the rest of the subgroups: bycaught (median 14 pieces of plastic), captured (median 11 pieces of plastic), and rescued turtles (median 5 pieces of plastic) (Fig. 3.2 and Appendix 3D in Supplementary Materials).

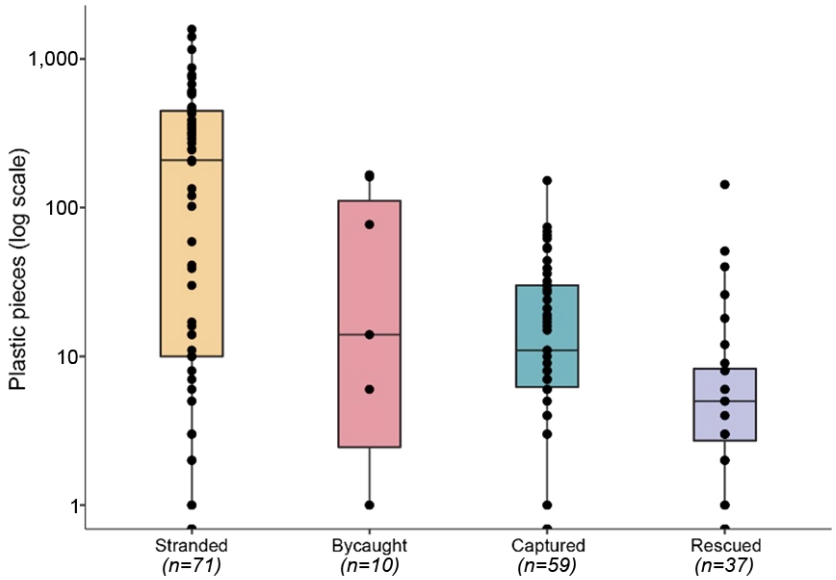

Figure 3.2. Quantities of ingested plastics registered in each subgroup of turtles examined over the study period. The y-axis represents the accumulation of total number of plastic pieces in logarithmic scale. The x-axis shows the sources of specimens. Lower and upper box boundaries 25th and 75th percentiles, respectively, horizontal line median, and circles the total number of plastic pieces ingested by individual turtles.

During necropsy examinations, a total of 18,464 pieces of plastic (548.3 g) were retrieved from the digestive contents of 63 out of 71 stranded turtles within *Subsample A*; and 270 pieces of plastic (7.45 g) were collected from 7 out of 10 bycaught turtles within *Subsample C* (Appendix 3D in Supplementary Materials). The average accumulation of plastic in stranded turtles within *Subsample A* was 260.4 ± SD 343.7 pieces, while the bycaught turtles within *Subsample C* consumed an average of 42.6 ± SD 67.8 pieces of plastic. There was a significant difference between the average quantities

of plastic ingested by these two subgroups ($t(71) = -4.72, p < 0.001$). Most of the plastics ingested by stranded turtles were found in the intestines (mean 139.0 ± SD 229.7 pieces of plastic), followed by the stomach (mean 114.4 ± SD 183.2 pieces of plastic), while the oesophagus contained much smaller quantities (mean 6.6 ± SD 20.7 pieces of plastic) (Appendix 3D in Supplementary Materials). Similarly, the quantities of plastics found in the intestines of bycaught turtles are larger (mean 21.1 ± SD 48.1 pieces of plastic) than in the stomach (mean 5.6 ± SD 11.8 pieces of plastic). Only a few pieces were found in their oesophagus (mean 0.3 ± SD 0.7 pieces of plastic) (Appendix 3D in Supplementary Materials).

Throughout the faecal matter examinations, a total of 1,141 pieces of plastic (27.46 g) were collected from the faeces of 51 out of 59 captured turtles; and 376 pieces of plastic (5.40 g) were retrieved from the faecal matter of 27 out of 37 rescued turtles (Appendix 3E in Supplementary Materials). The captured turtles exhibit an average accumulation of 19.3 ± SD 26.2 pieces of plastic, while rescued turtles consumed an average of 10.2 ± SD 25.0 pieces of plastic. No significant difference was observed between the mean quantities of plastic ingested by these two subgroups ($t(79) = 1.72, p = 0.089$).

The quantities of plastic ingested varied over time and across the turtle subgroups (Fig. 3.3).

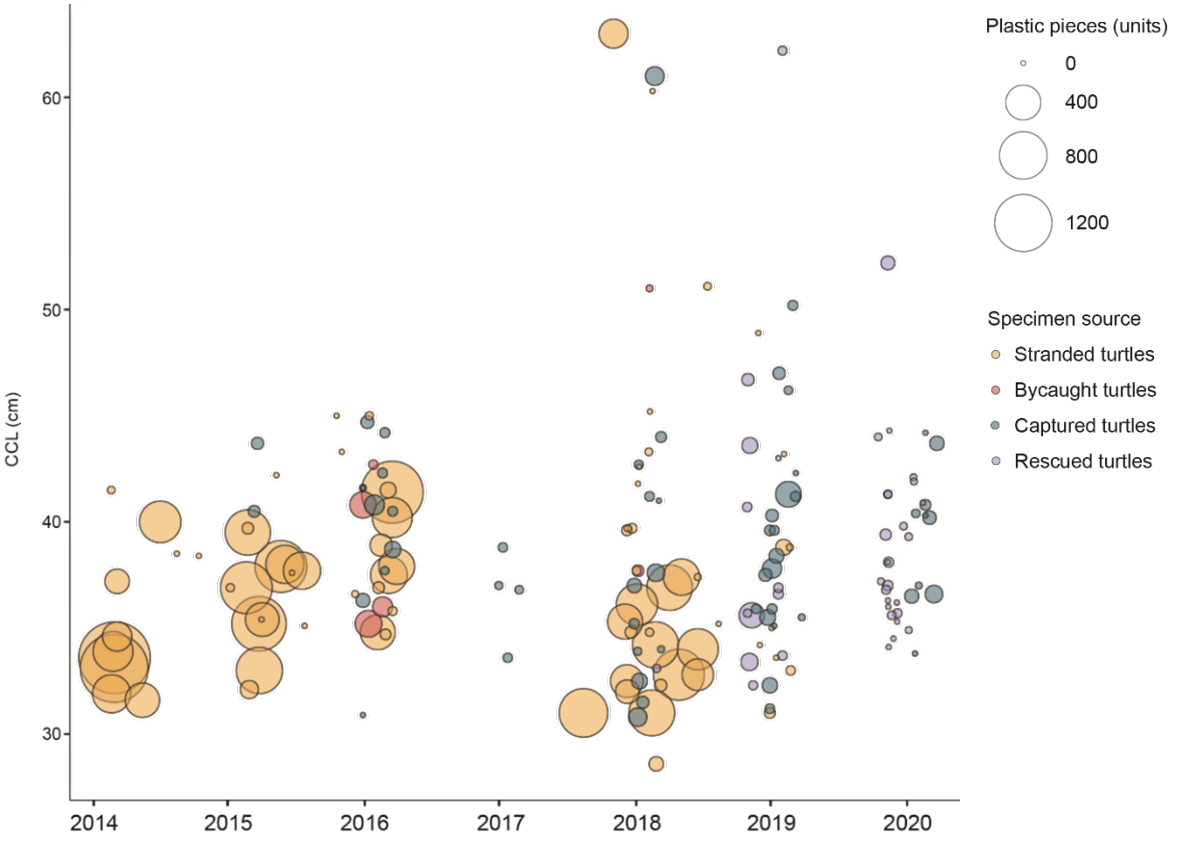

Figure 3.3. Quantities of ingested plastics recorded over time in relation to the source of specimen and turtle size. The y-axis represents the turtle size, reflected as curved carapace length (CCL). The x-axis shows the study period. Colours represent the different sources of specimens and circles the total number of plastic pieces ingested by individual turtles.

The power analysis showed that reliable inferences on temporal trends of the mean quantities of plastic ingested across the study period were not feasible, except for captured turtles (Table 3.4). Hence, I performed a Poisson logistic regression for this subgroup to identify annual trends in the mean quantities of plastic ingested, however, no identifiable trend was detected in captured turtles (Appendix 3F in Supplementary Materials).

Table 3.4. Results of the power analysis on the percentage of change detection in the mean quantity of plastic ingested by examined turtles over the study period according to the sample size and coefficient of variation (CV) of each subgroup of turtles examined (see formula in Appendix 3B in Supplementary Materials).

Source of specimens	*Stranded*	*Bycaught*	*Captured*	*Rescued*
Sample size	71[a]	10[c]	59	37
CV (SD/mean*100)	127.7	91.6	31.3	138.7
Change detection (%)	-	-	23.0	-

Acronyms: CV (coefficient of variation), SD (standard deviation)

[a] Stranded turtles - Subsample A

[c] Bycaught turtles - Subsample C

In addition, the linear mixed-effects regression model, LMER, indicates an inverse relationship between the size of the turtles and plastic ingestion; larger turtles consumed proportionally less plastic (Fig. 3.4). The best-fitted model (Formula: *lmer(number of pieces ~ ccl + (1 | source of specimen) + (1 | year)*) revealed a significant negative association between turtle size (fixed effect), reflected as CCL, and quantity of ingested plastic in terms of number of pieces (dependent variable) (*Estimate = -6.194, SE = 2.818, df = 134, t = -2.198, p = 0.029*), with no significant effect of the source of specimen or year of turtle encounter (random effects) (Appendix 3G in Supplementary Materials). These results are preliminary and should be interpreted with caution due to the small number of individuals with CCL ≥ 50 cm (see Chapter 4 for further analysis of the effect of CCL on plastic ingestion).

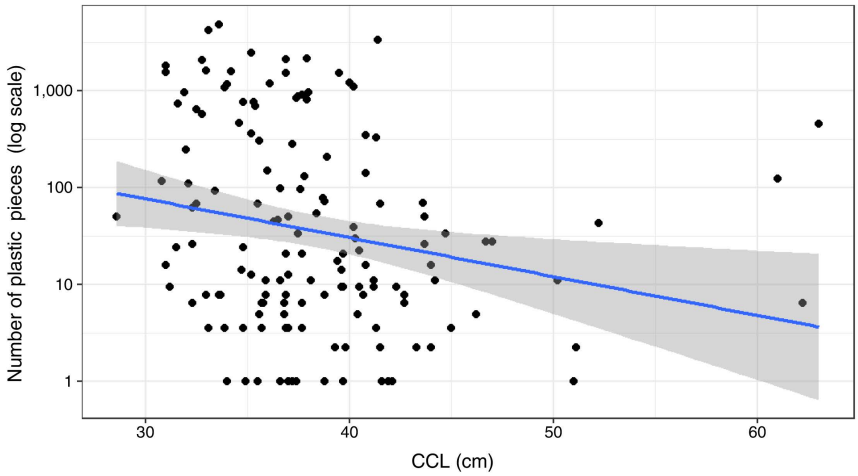

Figure 3.4. Relationship between curve carapace length (CCL) of green turtles and quantities of ingested plastic (number of pieces).

3.3.3 Analysis of the characteristics of ingested plastic

Among the turtles examined by necropsy, laminar soft plastics (SHE) were the most consumed plastic-type, accounting for 40.2% and 54.1% of the total plastic retrieved from stranded and bycaught turtles, respectively (Fig. 3.5). Pieces within the 'white' and 'clear/transparent' categories comprised over 55% of the total pieces collected in both subgroups (Fig. 3.5) (Appendix 3D in Supplementary Materials).

Similarly, laminar soft plastics (SHE) were the most consumed plastic-type by captured turtles, amounting to 50% of the pieces retrieved from these turtles (Fig. 3.5). In contrast, rescued turtles exhibited a primary consumption of threads and fibres (THR), followed by laminar soft plastics (SHE), constituting respectively 62.0% and 29.8% of the total plastic collected in this subgroup (Fig. 3.5). The categories 'white' and 'clear/transparent' accounted for over 50% of the total pieces retrieved in both subgroups (Fig. 3.5). I found that the 'black' category was also significantly consumed in captured turtles, comprising 15.0% of the total plastic ingested; as well as 'blue' plastics in rescued turtles, accounting for 20.5% of the total plastic ingested (Fig. 3.5) (Appendix 3E in Supplementary Materials).

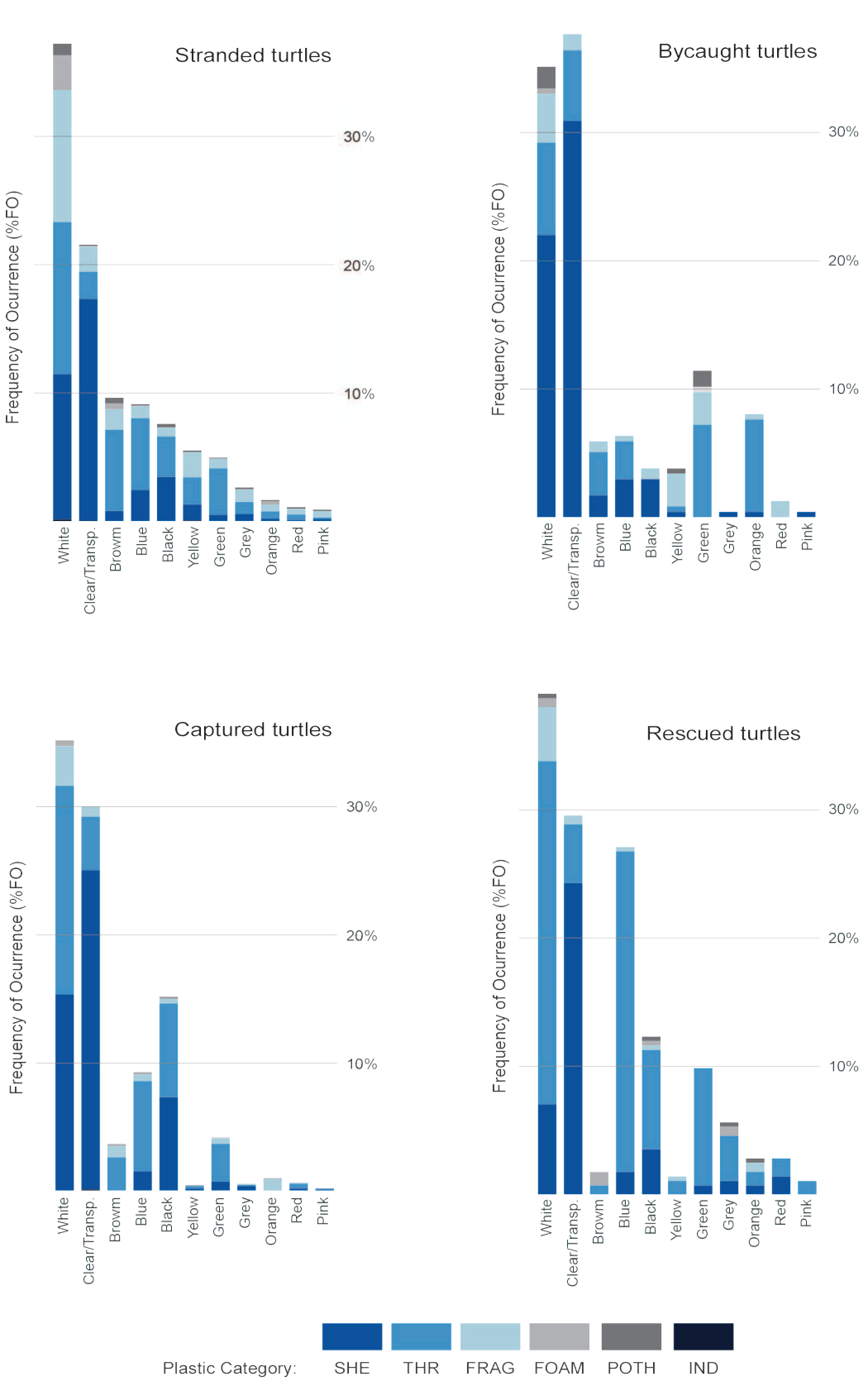

Figure 3.5. Colours and plastic-type categories of the ingested plastics retrieved from the examined turtles. Acronyms: IND (industrial plastics), SHE (plastics sheet-like), THR (plastics thread-like), FRAG (rigid plastic fragments), FOAM (foam), and POTH (other plastics).

Stranded turtles exhibited a primary consumption of soft plastics with the lowest sharpness and flexibility index, FSI 2, followed by the hardest plastics within FSI 6 category, constituting respectively 69.1% and 14.3% of the total plastic retrieved from this subgroup (Fig. 3.6). Bycaught turtles showed higher consumption of soft plastics within the categories FSI 2 and FSI 3, accounting respectively for 58.1% and 31.4% of the plastic collected (Fig. 3.6). Plastics with FSI 3 were the most consumed by captured turtles, with 57.1 % of the total plastic retrieved; followed by the softest plastics with FSI 2, with 32.8 % of the total plastic retrieved (Fig. 3.6). Inversely, softest plastics with FSI 2 were the most consumed by rescued turtles followed by intermediate pieces with FSI 3, amounting respectively to 54.0 % and 31.4 % of the total plastic collected in this subgroup (Fig. 3.6) (Appendixes 3D and 3E in Supplementary Materials).

Macro plastics (> 25mm in diameter) and meso-plastics (5 – 25 mm in diameter) were the most frequently ingested plastic size classes, representing approximately 90% of the total pieces collected across all subgroups (Fig. 3.6) (Appendixes 3D and 3E in Supplementary Materials).

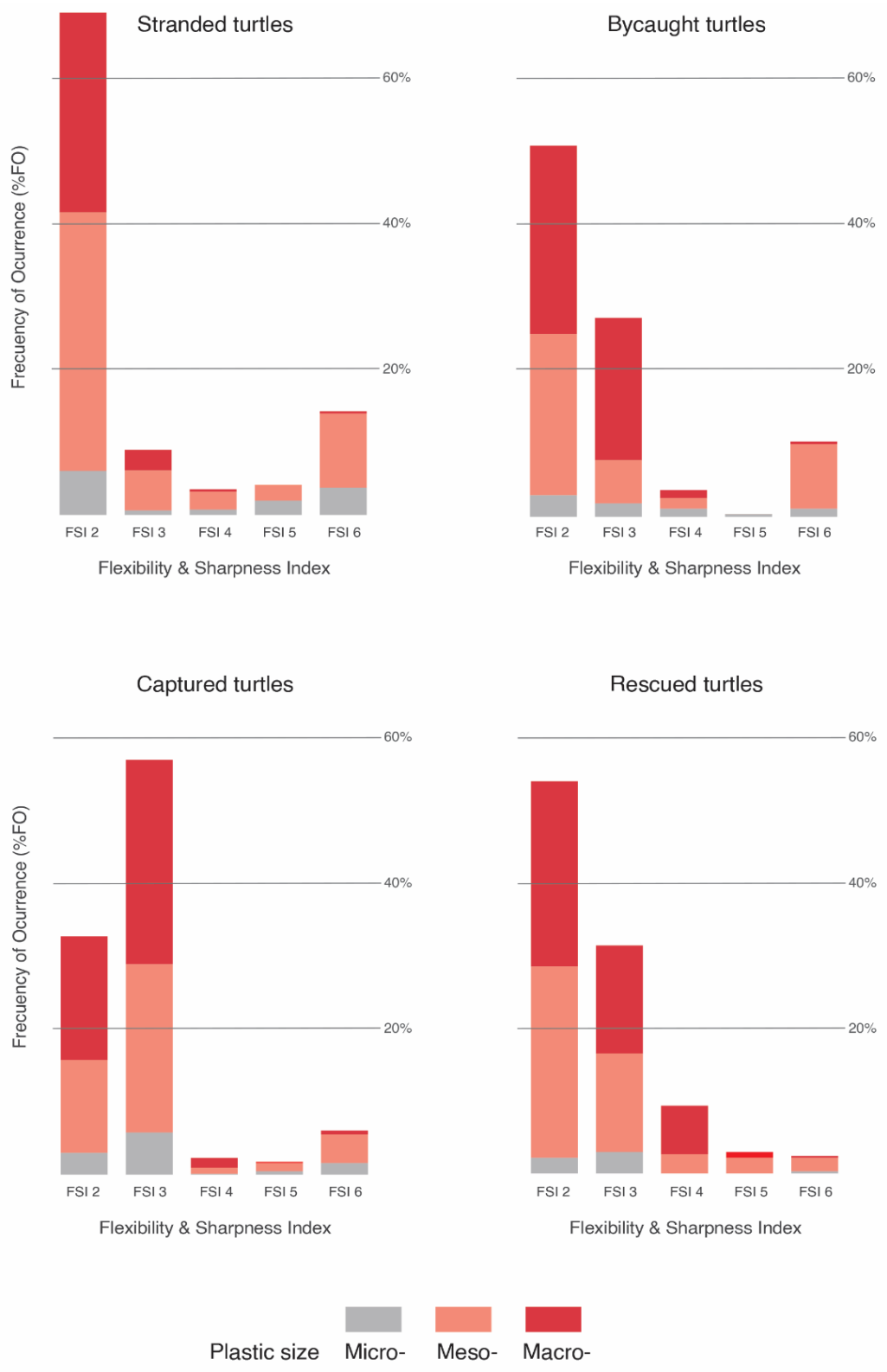

Figure 3.6. The size classes and FSI (flexibility and sharpness index) of the ingested plastics retrieved from the examined turtles. Microplastics refer to pieces of <5 mm, meso-plastics to 5 - 25 mm, and macro plastics to pieces >25 mm.

3.4 DISCUSSION

3.4.1 Incidence and temporal trends in plastic ingestion

The overall incidence of plastic ingestion was 76% among juvenile green turtles examined in Uruguayan waters between 2014 and 2020 with slightly variations across the subgroups, ranging from 71.8% in stranded turtles to 86.2% in captured turtles (Table 3.5). Furthermore, the ingestion rate observed in stranded turtles indicated it remained relatively consistent in Uruguay over the last 15 years, when comparing with related previous studies (Table 3.5). Such values remain within the range of incidence reported for the last decade in juvenile green turtles (70 – 93%) along the Southwestern Atlantic region (Table 3.5).

However, the quantities of ingested plastic recorded in this study were higher than those found in juvenile green turtles across the region (Table 3.5). These higher ingestion rates could be primarily attributed to the high levels of plastic pollution in Uruguayan waters. This situation originates from the sub-tropical convergence of the Brazil and Malvinas currents, transporting debris from other latitudes and creating an aggregation zone offshore Uruguay (Franco et al., 2020; Manta et al., 2022; Mello et al., 2022), in addition to a benthic salinity front within the Rio de la Plata estuary acting as a barrier and trawling wastes toward the coast (González-Carman et al., 2014; Lozoya et al., 2015, 2016; Rodriguez et al., 2020, see Chapter 1).

Table 3.5. Reports of plastic ingestion by green sea turtles (*Chelonia mydas*) in the Southwestern Atlantic Ocean during the last decades.

Study	Area	Period	Sample source	Sample size	CCL range (cm)	*Plastic ingestion*		
						Incidence (%FO)	Mean ± SD (pieces/turtle)	Range (pieces/turtle)
González-Carman et al., 2014	Argentina	2008/11	Bycaught	62	31.3 - 52.2	90.0	13	0 - 591
Santos et al., 2015	South Brazil	2009/13	Stranded	265	26.1 - 78.4	70.0	47.5 ± 120	0 - 965
Vélez-Rubio et al., 2018a	Uruguay	2005/13	Stranded	93	29.8 - 62.0	70.0	220.7 ± 320.8	0 - 1364
Rizzi et al., 2019	South Brazil	2013/17	Stranded	48	28.0 - 60.0	81.3	N/A	0 - 544
Nunes et al., 2020	South Brazil	2014/15	Stranded	40	29.8 - 57.0	92.7	116.3 ± 198.9	0 - 873
Present study	Uruguay	2014/20	Stranded	124	28.6 – 70.7	71.8	260.1 ± 343.7	0 - 1580
Present study	Uruguay	2014/20	Bycaught	18	33.3 – 55.3	77.8	27.0 ± 53.7	0 - 165
Present study	Uruguay	2014/20	Captured	59	30.8 – 61.0	84.7	19.3 ± 26.2	0 - 152
Present study	Uruguay	2014/20	Rescued	37	32.3 – 62.2	73.0	10.2 ± 25.0	0 - 143

Acronyms: %FO = frequency of occurrence; SD = standard deviation

No trend was identified in the annual incidence of plastic ingestion over the study period. This could be related to the fluctuation in the number of green turtles monitored per year. The annual research efforts carried out by the Karumbé NGO in Uruguay are subject to changes due to resource accessibility, staff and volunteer availability, weather conditions, and occasional climate events, among other constraints. Another potential factor influencing this result could be the fluctuation of the seasonal occurrence of green turtles in Uruguayan waters (see Chapter 1), which is subject to alterations during warm and cold periods related to El Niño - Southern Oscillation (ENSO) (Ortega & Martínez 2007).

The power analysis used in this study also enables the estimation of the sample sizes required to detect annual changes in plastic ingestion incidence at a predefined level, based on the coefficient of variation (CV) in each subgroup of turtles examined (Table 3.6). The results indicate that larger sample sizes are required for stranded and bycaught turtles to infer variations in the incidence of plastic ingestion due to the high coefficient of variation (CV) in these subgroups of turtles.

Table 3.6. Sample sizes required to detect annual changes in plastic ingestion incidence (reflected as frequency of occurrence, %FO) in the basis of the coefficient of variation (CV) in each subgroup of turtles examined.

Source of specimens (CV)	***Stranded*** (0.569)	***Bycaught*** (0.414)	***Captured*** (0.133)	***Rescued*** (0.221)
Changes in %FO		Sample size required (no. individuals)		
5	2574	1592	164	454
10	706	437	45	124
25	146	90	9	26
50	53	32	3	9
100	23	14	1.5	4

3.4.2 Quantities of plastic ingested

The accumulation of plastic in stranded turtles was significantly higher compared to the quantities recorded in the other subgroups of turtles examined (bycaught, captured, and rescued turtles) (Fig. 3.2). A previous study by Vélez-Rubio et al. (2013) identified plastic ingestion as one of the primary causes of stranding for green turtles in Uruguayan waters. The ingestion of substantial amounts of plastic generally hinders turtles' ability to dive, compelling them to feed exclusively at the sea surface, where the concentration of plastic is usually higher. Consequently, these turtles remain exposed to the risk of ingestion, accumulating an increasing quantity of plastic and contributing to the progressive degeneration of the turtle's health, which ultimately leads to stranding. Therefore, the recorded quantities of ingested plastic in stranded turtles could be overestimated in relation to the overall rate of the stock of juvenile green turtles under study.

No annual trend was detected in the quantities of plastic ingested by the examined turtles, as expected due to the high variability of number of pieces ingested by a relatively small sample size of turtles per year in each subgroup.

Necropsy examinations showed that the largest quantities of plastic accumulated in the intestines, followed by the stomach. The higher retention of plastic in the intestines might be explained by the longer length of this section and the hindgut fermentation strategy of green turtles (Brand et al., 1999; Bjorndal, 1980; Mackie, 2002; Wyneken, 2001). The morphology of the stomach of green turtles in a 'J' shape, ending with a sphincter, also facilitates the retention of plastic in this section (Colferai et al., 2017; Magalhães et al., 2012; Wyneken, 2001).

Larger turtles registered lower accumulations of ingested plastic. This could be associated with the feeding strategies exhibited by juvenile green turtles in Uruguayan waters. According to Vélez-Rubio et al. (2016), two size classes of juvenile green turtles coexist in these waters: 'resident' turtles, which are large juveniles within the neritic habitat showing a primarily herbivorous diet on the benthos; and 'new settlers', which are early juveniles, smaller turtles, recently recruited to the

neritic zone reflecting a relict omnivorous diet from their pelagic stage. This opportunistic feeding behaviour makes the 'new settlers' potentially more exposed to the risk of plastic ingestion due to their low discrimination in their selection of dietary items.

3.4.3 Characteristics of ingested plastic

The ubiquitous prevalence of laminar soft plastics (SHE), coinciding with plastics scoring FSI 2 and FSI 3, in white and clear/transparent colours retrieved from all turtle subgroups, suggests a preference for these specific plastics. If it occurs, this feeding selectivity could be influenced by the similarities in shape and appearance of plastics with the commonest dietary items for green turtles in Uruguayan waters, such as macroalgae and gelatinous macrozooplankton (Vélez-Rubio et al., 2016). Other relevant studies have provided evidence of selectivity patterns in marine turtles driven by similarities of plastics to dietary items (Casale et al., 2016; Choi et al., 2021; Clukey et al., 2018; Duncan et al., 2019a; Godoy & Stockin, 2018; Nelms et al., 2016; Schuyler et al., 2012, 2014b). However, validating these selective behaviours requires an assessment of the availability of plastic debris in the environment to distinguish between a genuine selective pattern and a higher prevalence of these specific plastics in the study area.

Meso- and macro-plastics were the size classes most frequently consumed among the examined turtles. Studies on the diet of green turtles indicate that the optimal particle size for ingestion falls within the range of 24-26 mm in diameter (Gulick et al., 2021), which coincides with the size boundary between meso- and macro-plastics. On the other hand, the lower threshold for plastic size in this study was set at 1 mm in maximum diameter (or alternatively retained by a 1 mm mesh sieve) due to equipment and protocol limitations (see Chapter 5 about plastic size thresholds and required equipment and protocols). Thus, pieces smaller than this threshold could not be collected, potentially leading to an underestimation of microplastic quantities. Nevertheless, the size of the plastic pieces retrieved may differ from the originally ingested sizes due to turtle mastication and

digestion processes. As mentioned above, it is important to consider the availability of different plastic size classes in the environment before validating any assumptions regarding preferences.

3.4.4 Evaluation of the methodological approaches

The source of specimens is a determining factor when assessing plastic ingestion in marine turtles (see Chapter 5). Using a particular source of specimens depends on the research objectives. If the aim is to analyse the incidence of plastic ingestion, bycaught and wild-captured turtles are more representative sources of specimens for understanding the overall exposure to plastic ingestion in a population if the sampling is systematically and/or part of routine programs (e.g., monitoring programs capturing turtles in the wild for scientific purposes, or bycatch programs retrieving regularly turtles from fishing gear). Usually, the lower variability in ingestion rates in these turtles allows for the inference of reliable temporal trends in plastic ingestion with relatively small sample sizes. Hence, it is recommended to conduct power analyses, considering sample sizes and coefficients of variation, to assess and validate the reliability of the derived inferences, or alternately, for pre-determining minimum sample sizes.

In contrast, stranded dead and rescued turtles constitute a subset of animals mostly in poor health conditions, which are usually sampled opportunistically. Therefore, biases in the quantities of ingested plastic can occur due to pre-existing abnormal feeding behaviour or habitat use. Nevertheless, these turtles can provide valuable insights when the aim is to evaluate the severity of the impact resulting from plastic ingestion (see Chapter 4).

Sampling techniques are another crucial factor in researching plastic ingestion in marine turtles. Each technique has its own advantages and disadvantages that should be considered when planning the research and defining the objectives (see Chapter 5). Necropsy examinations are the most reliable method, enabling retrieval of all the plastic ingested by a turtle, but are restricted to dead animals. While faecal matter monitoring allows plastic ingestion to be assessed in live animals,

provided adequate monitoring periods upper the limit of ingesta passage time to allow all ingested plastics to be collected. Ideally, a comprehensive assessment of plastic ingestion should include necropsy samples from diverse sources of specimens, coupled with the examination of faecal matter in a representative subset of live turtles.

Gastric lavages proved to be a non-efficient method to detect plastic ingestion. This study found no plastic in the examined turtles using this technique. Even if some plastic pieces were collected by this method, they might represent only a tiny portion of the total ingested amount since plastic tends to accumulate in the stomach and intestines.

3.5 CONCLUSIONS

The overall incidence of plastic ingestion was 76% of the sampled juvenile green turtles in Uruguayan waters between 2014 and 2020. Furthermore, the quantities of ingested plastic recorded in the examined turtles were higher than those found in juvenile green turtles across the region. Hence, Uruguayan waters represent a hotspot for plastic ingestion by juvenile green turtles in the region. This situation is enhanced by (i) oceanographic features in these latitudes that aggregate significant accumulations of plastic debris, and (ii) the feeding behaviour exhibited by early juvenile green turtles recently recruited to the neritic habitat, which reflects a relict opportunistic behaviour. The low discrimination in selecting dietary items makes these smaller turtles potentially more exposed to ingesting a wide variety and higher quantities of plastics.

Using a particular source of specimens depends on the research objectives. Bycaught and wild-captured turtles are more representative sources of specimens for assessing the overall incidence of plastic ingestion in a population if the sampling is conducted systematically. Conversely, stranded and rescued turtles are sources of specimens that can provide valuable insights into the severity of the impact caused by plastic ingestion on the turtles' health.

In terms of sampling techniques, necropsy remains the most reliable procedure for assessing plastic ingestion. It allows for the separate collection of digestive contents from each section of the tract for the examination of ingested plastics. Additionally, faecal matter examination enables the sampling of live animals if monitoring periods over the ingesta passage time are feasible. Gastric lavage proved to be a non-efficient method for these types of studies.

PhD Chapter 4

Impact severity of plastic ingestion on juvenile green turtles (*Chelonia mydas*) in relation to the volumes and characteristics of ingested plastic

Chapter objective

To analyse factors influencing the severity of the impact caused by plastic ingestion on juvenile green turtles in Uruguay, considering the volumes and characteristics of ingested plastic.

Methodology

The severity of the impact associated with plastic ingestion was assessed in a subsample of turtles (n = 150) examined in Chapter 3, based on their necropsy reports and health assessments. The ingested plastic was quantified and characterised to explore potential factors contributing to the severity of the impact. Variables for analysis included the cumulative volume of ingested plastic, the size of plastic particles, and the type of plastic retrieved from the examined turtles. Additional analyses evaluated the relationship between the severity of impact and turtle size, expressed as individual curved carapace length (CCL).

Key Findings

- There was a positive relationship between the ingested volume of plastic and the severity of the impact, with a highly significant difference in the volumes of ingested plastic between non-affected and impacted turtles.
- Meso-particles were the most consumed plastic particle size.
- Ingested plastic accumulates increasingly along the digestive tract: oesophagus < stomach < intestines.
- Laminar soft plastics were the most consumed plastic-type. These plastics pose a particular risk for turtles due to their pliability. Large and malleable pieces can act as a mesh, entangling other plastic items and part of the solid fraction of the digestive contents, which results in fecaloma compaction and gut obstruction.
- Juvenile green turtles with a curved carapace length (CCL) below 40 cm were more susceptible to the impacts of plastic ingestion than larger turtles. Their opportunistic feeding behaviour related to their previous oceanic stage makes these smaller turtles potentially more exposed to ingesting a wider range of plastic and higher volumes.

Conclusions

This study contributes valuable insights into the severity of the impact caused by plastic ingestion on green turtles in Uruguay. Additional research efforts should be directed toward a better understanding of the impaction process and the full extent of the impacts caused by plastic ingestion on turtles' health, enabling more comprehensive risk assessment of plastic ingestion in marine turtles.

Related publication

González-Paredes, D., Jones R., De la Fuente, A., Ferrando, V., Vélez-Rubio, G.M., Hamann, M., & Marsh, H. (*in prep.*)

Impact severity associated with plastic ingestion in juvenile green turtles (Chelonia mydas) in relation to the volumes and characteristics of ingested plastics

Marine Pollution Bulletin (target journal)

4.1 INTRODUCTION

The impacts of plastic ingestion on marine turtles' health are diverse, ranging from negligible to lethal (Choi et al., 2021; Clukey et al., 2017; Santos et al., 2015; Vélez-Rubio et al., 2018a). In this respect, one of the main factors influencing the severity of these impacts is the quantity of plastic ingested. Relatively low volumes of plastic can be retained within a turtle's digestive system with low or no impact (Hoarau et al., 2014; Lutz, 1990). For example, routine monitoring of turtles in captivity commonly reports plastic within faecal matter with no apparent adverse effect on the animal's health (Hoarau et al., 2014; González-Paredes et al., 2021b). Nonetheless, the displacement of dietary items by ingested plastic can reduce stomach capacity and feeding stimulus, leading to malnutrition and diminished nutrient gains (McCauley & Bjorndal, 1999; Santos et al., 2020; Tourinho et al., 2010). In other cases, veterinarians have reported blockages of the digestive tract caused by large pieces of plastic or fecalomas resulting from the compaction of substantial volumes of plastic with part of the solid fraction of the digestive contents (Rizzi et al., 2019; Vélez-Rubio et al., 2018a). These fecalomas can eventually result in ischemic necrosis and septicaemia with lethal consequences (Mashkour et al., 2020; Tagliolatto et al., 2020).

Another factor influencing impact severity is the characteristics of the ingested plastic. Rigid and sharp plastics have the potential to cause abrasions or even lacerate the digestive tract (Camedda et al., 2014; Derraik, 2002; Lazar & Gračan, 2011). While soft plastics may adhere to the intestinal walls more easily than hard plastics, hindering their expulsion and prolonging the retention time within the digestive tract (Colferai et al., 2017; Rizzi et al., 2019). The risk of blockage is also influenced by the size of the ingested plastics. Small plastics are expected to pass through the digestive tract and be easily expelled; in contrast, larger plastic pieces are more likely to get stuck in the digestive tract, leading to obstructions (González-Paredes et al., 2021b; Santos et al., 2015; Vélez-Rubio et al., 2018a).

On the other hand, exposure to plastic ingestion varies according to inherent factors related to the biology and ecology of marine turtles, such as species, feeding behaviour and life stage (see Chapter 1). Omnivorous turtles (e.g., loggerheads) or juvenile individuals in oceanic stages exhibiting an opportunistic feeding behaviour (e.g., juvenile green turtles) are potentially exposed to consuming larger quantities and wider ranges of plastics (Duncan et al., 2021; Lynch, 2018; Nelms at al., 2016). Furthermore, hatchlings and juvenile turtles have smaller-diameter digestive tracts than adults, which may hinder the passage of large pieces and potentially make these turtles more susceptible to retaining plastics (Colferai et al., 2017; Magalhães et al., 2012). The health condition of turtles might also influence their feeding behaviour and/or habitat use and, consequently, the risk of plastic ingestion (Casale et al., 2016; Rice et al., 2021). For instance, individuals exhibiting positive buoyancy due to any pathology such as pneumonia or even plastic ingestion will be restricted to feeding at the sea surface, where the concentration of plastic and the risk of ingestion is usually higher.

Improving our knowledge of the factors influencing the impacts of plastic ingestion and how the impaction process occurs would allow more accurate assessments of the vulnerability of marine turtles to this threat. In this chapter, I assess the severity of the impact caused by plastic ingestion on juvenile green turtles in relation to the cumulative volumes, particle size and characteristics of ingested plastic.

4.2 METHODOLOGY

4.2.1. Sample collection

I assessed the impact severity caused by plastic ingestion in a subsample of the juvenile green turtles examined in Chapter 3 (n = 150), for which complete necropsy reports or health assessments were available. Plastics were retrieved through necropsy technique (n = 54 turtles) or faecal matter monitoring (n = 96 turtles). Turtles were all juveniles (curved carapace length, CCL, mean 39.7 ± SD

6.1 cm; range 30.8 to 70.7 cm), assuming a minimum size at maturity of CCL = 90 cm for green turtles in the Southwestern Atlantic region (Almeida et al. 2011).

4.2.1.1 Necropsied examination

I examined plastic ingestion in stranded dead (n=45) and bycaught turtles (n=9) collected opportunistically by the Karumbé NGO - Marine Turtle Rescue and Stranding Network, which were either dead recently on collection or died after unsuccessful recovery (see procedures in Vélez-Rubio et al., 2013). Karumbé veterinarians conducted necropsies following Wyneken (2001), documenting turtle condition and determining the cause of death when possible. Additionally, digestive contents were retrieved separately from each animal's oesophagus, stomach, and intestines for assessing plastic ingestion.

4.2.1.2 Faecal matter monitoring

I monitored the presence of plastic within the faecal matter of turtles captured in the wild (n = 59) and (ii) rescued turtles (n = 37) provided by Karumbé NGO. These turtles are the same subset examined in Chapter 3. The animals were kept individually in monitoring tanks, and their faeces were collected multiple times a day to check for ingested plastics (see details of procedures and methods in Chapter 3, section 3.2.2.2).

4.2.2 Assessment of plastic ingestion

The impact and severity of plastic ingestion were assessed for each of the examined animals based on their necropsy report (dead turtles) or health assessments (alive turtles). Subsequently, turtles were classified according to an Impact Severity Index (IPS). The index, created for this study, includes the following groups: **IPS 3**: plastic ingestion was deemed to be the cause of death by the obstruction of the digestive tract; **IPS 2**: plastic ingestion was considered as a driver of adverse effects, inducing satiety leading to emaciation and DTS (chronic debilitated turtle syndrome)

(Mashkour et al., 2020; Stacy et al., 2018), which eventually cause of death to the necropsied turtles; **IPS 1**: plastic caused no apparent adverse effects; the cause of death was determined to be different from plastic ingestion or indeterminate in necropsied turtles; and **IPS 0**: no evidence of plastic ingestion; and the cause of death was different from plastic ingestion or indeterminate in necropsied turtles (Table 4.1).

Table 4.1. The impact severity scale related to plastic ingestion on the examined turtles.

Impact Severity Index	Diagnosis
IPS 3	Plastic ingestion determined as the cause of death by the obstruction of the digestive tract.
IPS 2	Plastic ingestion induces satiety, which led to emaciation and DTS (chronic debilitated syndrome). Plastic ingestion considered as a driver of/ factor related to the cause of death.
IPS 1	Plastic ingestion caused no adverse effects. Cause of death determined as different from plastic ingestion or indeterminate.
IPS 0	Detected no plastic ingestion. Cause of death determined as different from plastic ingestion or indeterminate.

4.2.3 Sample examination

All the ingested plastic retrieved over 1 mm in maximum diameter (or alternatively retained by a 1 mm mesh sieve) were cleaned, air-dried, labelled, and stored for subsequent analysis. The analysis was constrained to specific attributes deemed likely to contribute to the severity of the impact caused by plastic ingestion and its influence on the turtles. These attributes include (i) physical characteristics and composition of the ingested plastics, (ii) individual volume of each plastic piece, and (iii) cumulative ingested volume.

Plastic pieces were categorized based on their physical characteristic, following the classification proposed by Van Franeker et al. (2011) and adapted by the MSFD Technical Subgroup on Marine

Litter (2013). The volume of each plastic piece (mm^3) was calculated by multiplying its length, width, and depth. The water displacement method for calculating induvial volume was considered unsuitable due to the minuscule dimensions of each plastic piece, which is hardly measurable using this technique. These pieces were then classified into three different volume categories (own classification): micro-particles (< 100 mm^3), meso-particles (100 - 1,000 mm^3), and macro-particles (> 1,000 mm^3). Cumulative total volumes of ingested plastic (mm^3) were recorded for each examined turtle, which I consider better reflects the plastic burden within the digestive tract than the number of pieces (see further discussion on reporting metrics in Chapter 5). Additionally, cumulative volumes were calculated for each plastic-type.

4.2.4 Statistical analysis

Turtles were grouped based on their Impact Severity Index (IPS 0–3) for the analyses. Individual turtles were the sampling unit for statistical analysis, using ingested volume (mm^3) as the metric. In order to assess plastic distribution along the digestive tract and impacts associated, the variables under analysis were: (i) total cumulative volume of plastic ingested, (ii) cumulative volume of three different volume categories (micro-, meso-, and macro-particles), (iii) cumulative volume of plastic-type consumed, and (iv) turtle size, expressed as CCL. Exploration of the factors influencing the impact severity on turtles was conducted using logistic regression and analysis of deviance.

All the statistical analyses were conducted in R 4.2.2 and R Studio 2022.12.0 (R Core Team, 2022).

4.3 RESULTS

4.3.1 Assessment of plastic ingestion

Plastic ingestion was detected in 70.7% of turtles examined (106/150 animals).

Turtles within the IPS 3 group (n = 10), for which plastic ingestion was determined as the cause of

death, necropsy reports indicated obstructions in the digestive tract. Such obstructions, primarily in the intestines, were caused by fecalomas compacting large volumes of plastic along with a portion of the solid fraction of the digestive contents (Fig 4.1). The obstruction irreversibly led the turtles to severe emaciation, associated with starvation and morbidity, ultimately resulting in death. In one of the turtles, fecalomas also caused internal compression of the bowel wall and vessels, leading to ischemic necrosis and massive septicaemia, with fatal consequences (see summary of necropsies in Appendix 4A in Supplementary Materials).

Turtles within the IPS 2 group (n = 14) includes animals diagnosed with emaciation associated with plastic ingestion. In the case of necropsied turtles (n = 10), veterinarians reported high accumulations of plastic in the digestive tract, mostly in the intestines, presumably inducing satiety and diminishing nutrient gains, which resulted in chronic debilitated syndrome (DTS) and morbidity (see summary of necropsies in Appendix 4A in Supplementary Materials). Additionally, health assessments of monitored animals indicated a low feeding stimulus in affected turtles (n = 4), presumably due to the displacement of dietary items by ingested plastic, reducing their stomach capacity. One of these turtles was admitted to rehabilitation with severe emaciation and chronic debilitated syndrome (DTS) associated with plastic ingestion, from which it could not recover, ultimately leading to its death. The other three turtles recovered after receiving rehabilitation treatments.

Turtles within the IPS 1 group (n = 82) exhibited plastic ingestion without apparent adverse effects. The necropsied turtles (n = 8) had different clinical diagnoses (e.g., pneumonia, septicaemia, etc.), which eventually led to death. Consequently, the cause of death in these turtles was considered unrelated to plastic ingestion (see summary of necropsies in Appendix 4A in Supplementary Materials). Most of the turtles under faecal matter monitoring within group IPS 1 (n = 74) showed normal digestive motility and feeding behaviour. Several plastic pieces were found in their faeces without causing any apparent impact on the turtles' health.

The IPS 0 group includes necropsied turtles (n = 26) and monitored turtles (n = 18) where plastic

ingestion was not detected. This group was excluded from the subsequent analysis because it does not contribute data on ingested plastic.

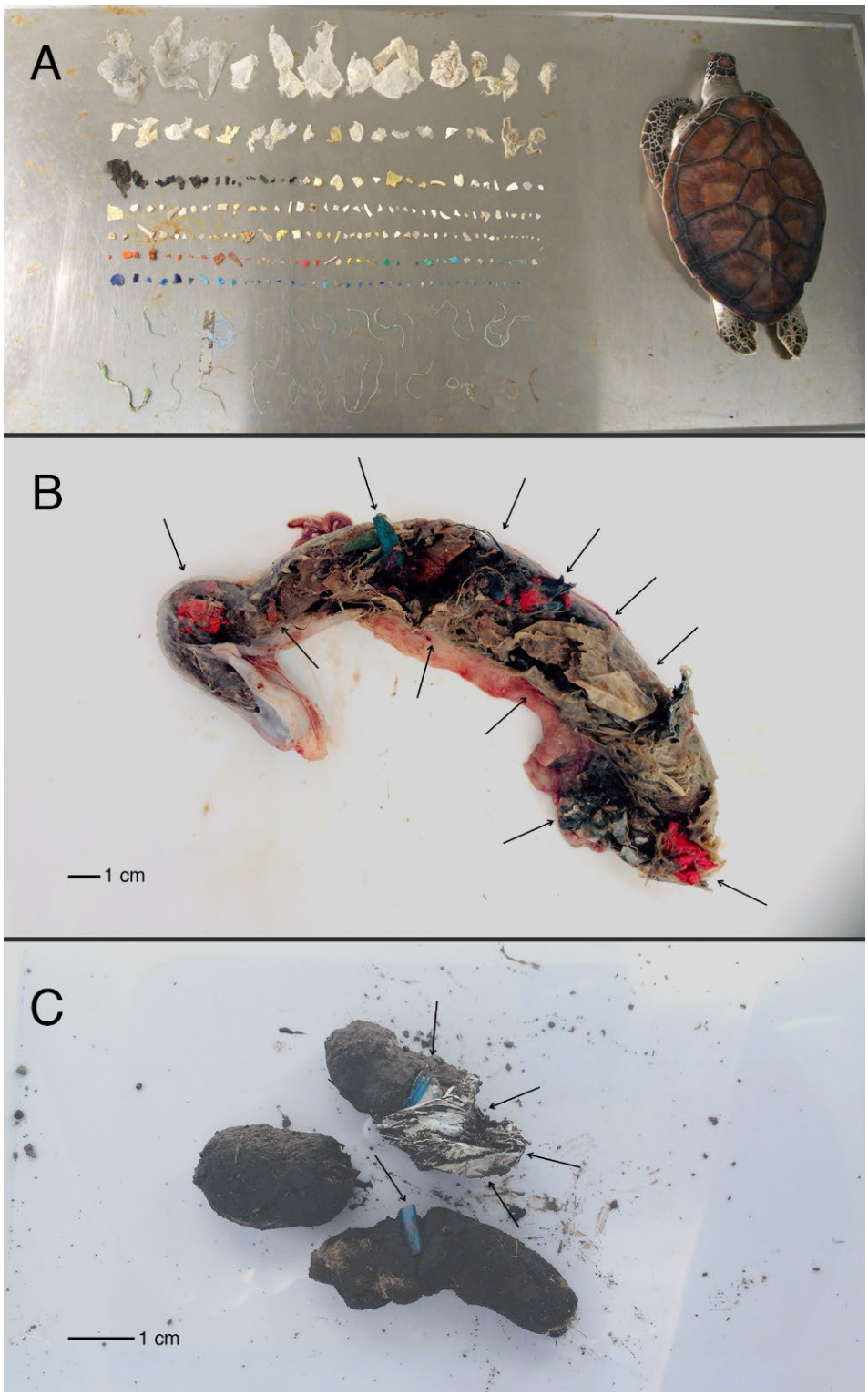

Figure 4.1. Necropsy examination of a turtle impacted by plastic ingestion: (A) plastic collected during a necropsy; (B) obstruction caused by plastic in the digestive tract of a necropsied turtle; (C) plastic within the faeces of a monitored turtle. Arrows indicate plastic. Pictures copyright © Karumbé NGO.

4.3.2 Analysis of total cumulative volume of plastic ingested

A total volume of 838,960 mm^3 of plastic (10,317 pieces) was retrieved from the examined animals. The quantities of ingested plastic differed according to the IPS. The IPS 3 group showed the highest volumes of ingested plastic (median 31,422 mm^3), followed by turtles within the IPS 2 group (median 18,286 mm^3) and far below was the IPS 1 group (median 373 mm^3) (Fig. 4.2).

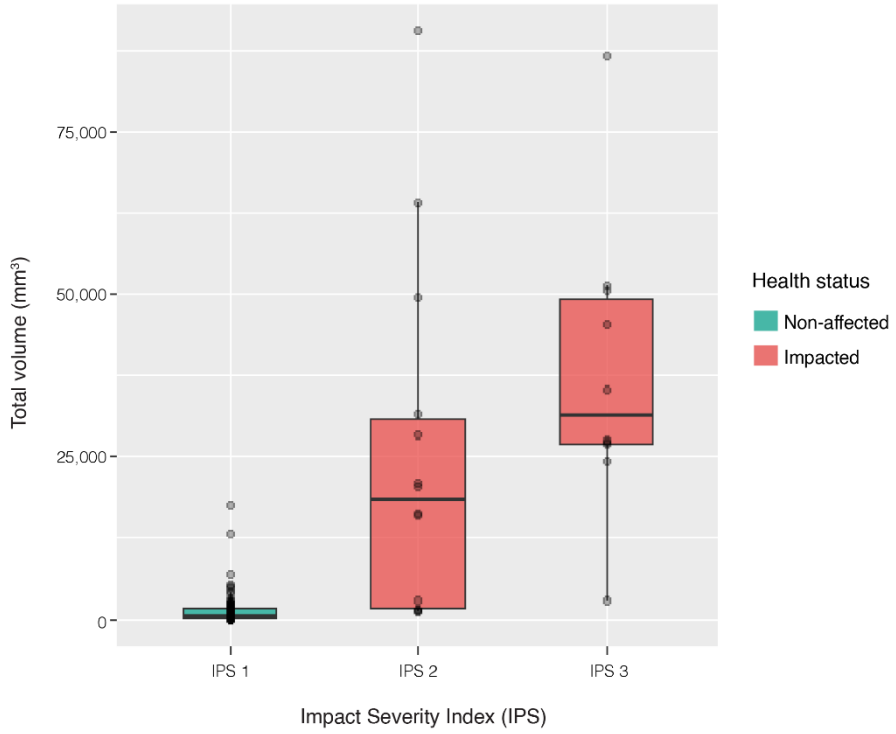

Figure 4.2. Cumulative volumes of plastic recorded in the examined turtles. The y-axis represents the total cumulative volume of plastic ingested (mm^3). The x-axis shows the turtles grouped according to their impact severity index (IPS). Lower and upper box boundaries 25th and 75th percentiles, respectively, horizontal line median, and circles the cumulative volume ingested by individual turtles.

The multinomial logistic regression analysis indicated there is a positive relationship between the volume of ingested plastic and the severity of the impact caused by plastic ingestion (IPS), ($\chi^2(2) = 79.92$, $p < 0.001$) (Appendix 4B in Supplementary Materials). Post hoc analysis using Tukey's pairwise comparisons revealed significant differences between IPS groups. Specifically, the odds of belonging to the IPS 2 group were significantly higher than the IPS 1 group ($p = 0.020$), as were the odds of the

IPS 3 group compared to the IPS 1 group (*p = 0.004*). However, the difference between IPS 2 and IPS 3 groups was not significantly different (*p = 0.059*) (Fig. 4.2) (Appendix 4B in Supplementary Materials). Therefore, I segregated the studied animals into non-affected, IPS 1 group, (n = 82) and impacted turtles, IPS 2 and IPS 3 groups, (n = 24) to increase the power of subsequent analyses.

4.3.3 Analysis of plastic ingestion across three distinct volume categories

Analysis of plastic ingestion across the three volume categories — micro-particles (< 100 mm^3), meso-particles (100–1,000 mm^3), and macro-particles (> 1,000 mm^3) — revealed meso-particles comprised the highest ingested volume of 414,907 mm^3 (1,496 pieces of plastic) (Table 4.2 and Fig. 4.3), with the lowest accumulation within the IPS 1 group (median 182 mm^3), followed by the IPS 2 group (median 9,492 mm^3), and nearly double the volume in the IPS 3 group (median 18,390 mm^3).

Macro-particles represent the second most consumed particle size class comprising 292,512 mm^3 (125 pieces of plastic). These particles accumulated less in the IPS 1 group (median 0.001 mm^3), followed far above by the IPS 3 group (median 5,078 mm^3), and the IPS 2 group (median 6,138 mm^3) (Table 4.2 and Fig. 4.3).

Micro-particles amounted to 131,542 mm^3 (8,696 pieces of plastic), accumulating in ascending order within the IPS 1 group (median 81 mm^3), followed by the IPS 2 group (median 2,466 mm^3), and the IPS 3 group (median 5,644 mm^3) (Table 4.2 and Fig. 4.3).

Table 4.2. Cumulative volumes of plastic retrieved from examined turtles regarding particle size classes. Turtles are grouped according to their Impact Severity Index (IPS); results are expressed as volume ingested (mm^3).

	***IPS 1* (n = 82)**			
	[Mean ± SD]	**[Range]**	**[Total / %FO]**	**[No. turtles]**
Cumulative vol.	1,401 ± 2,652	1 – 17,518	114,886 / 100%	82
Particle size				
Micro	175 ± 217	1 – 973	13,625 / 11.9%	78
Meso	614 ± 1,105	0 – 5,959	50,376 / 43.8%	52
Macro	621 ± 1,951	0 – 14,569	50,885 / 44.3%	16
	***IPS 2* (n = 14)**			
	[Mean ± SD]	**[Range]**	**[Total / %FO]**	**[No. turtles]**
Cumulative vol.	24,712 ± 26,936	1,187 – 90,750	345,970 / 100%	14
Particle size				
Micro	3,562 ± 3,638	472 – 12,089	49,862 / 14.4%	14
Meso	12,590 ± 13,655	234 – 49,695	176,262 / 50.9%	14
Macro	8,560 ± 11,607	0 – 38,532	119,846 / 34.6%	9
	***IPS 3* (n = 10)**			
	[Mean ± SD]	**[Range]**	**[Total / %FO]**	**[No. turtles]**
Cumulative vol.	37,810 ± 22,428	2,821 – 86,593	378,104 / 100%	10
Particle size				
Micro	6,806 ± 3,190	1,390 – 11,775	68,055 / 18.0%	10
Meso	18,827 ± 8,975	1,432 – 34,540	188,269 / 49.8%	10
Macro	12,178 ± 16,541	0 – 52,075	121,781 / 32.2%	8

Acronyms: %FO (frequency of occurrence), SD (standard deviation)
Micro-particles (< 100 mm^3); Meso-particles (100 - 1,000 mm^3); Macro-particles (> 1,000 mm^3)

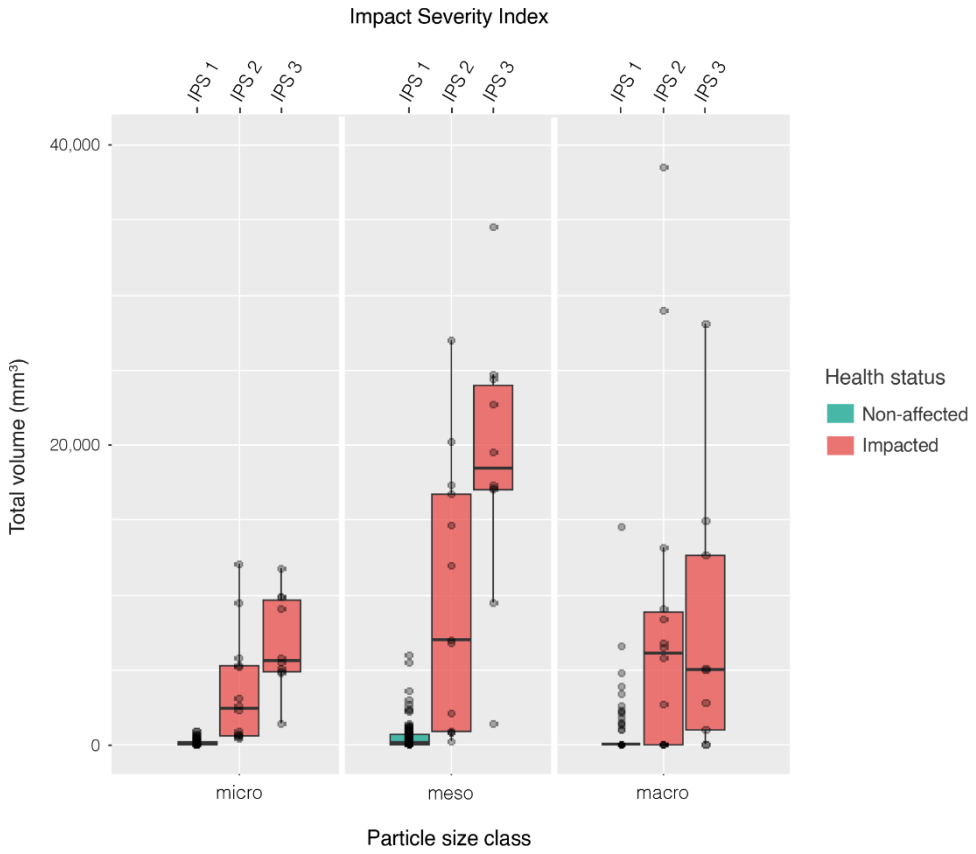

Figure 4.3. Volumes of the three size classes of plastic particles recorded in the examined turtles. The y-axis represents the total cumulative volume of plastic ingested (mm³). The upper X-axis shows the turtles grouped according to their impact severity index (IPS); the lower x-axis shows plastic particle size classes: micro-particles (<100 mm³), meso-particles (100 – 1,000 mm³), and macro-particles (>1,000 mm³). Lower and upper box boundaries 25th and 75th percentiles, respectively, horizontal line median, and circles the cumulative volume ingested by individual turtles.

The logistic regression detected a significant positive relationship between the accumulation of micro-particles and the impact caused by plastic ingestion *(Estimate = 8.334e-03, SE = 2.973e-03, df = 3, z = 2.804, p = 0.005)* (Appendix 4C in Supplementary Materials). Impacted turtles (IPS 2 and IPS 3 groups) accumulated significantly higher volumes of micro-particles compared with the combined accumulation of meso- and macro-particles (Fig. 4.4). In contrast, non-affected turtles (IPS 1 group) exhibited a more consistent ratio of micro-particle accumulation with lower combined volumes of meso- and macro-particles (Fig. 4.4).

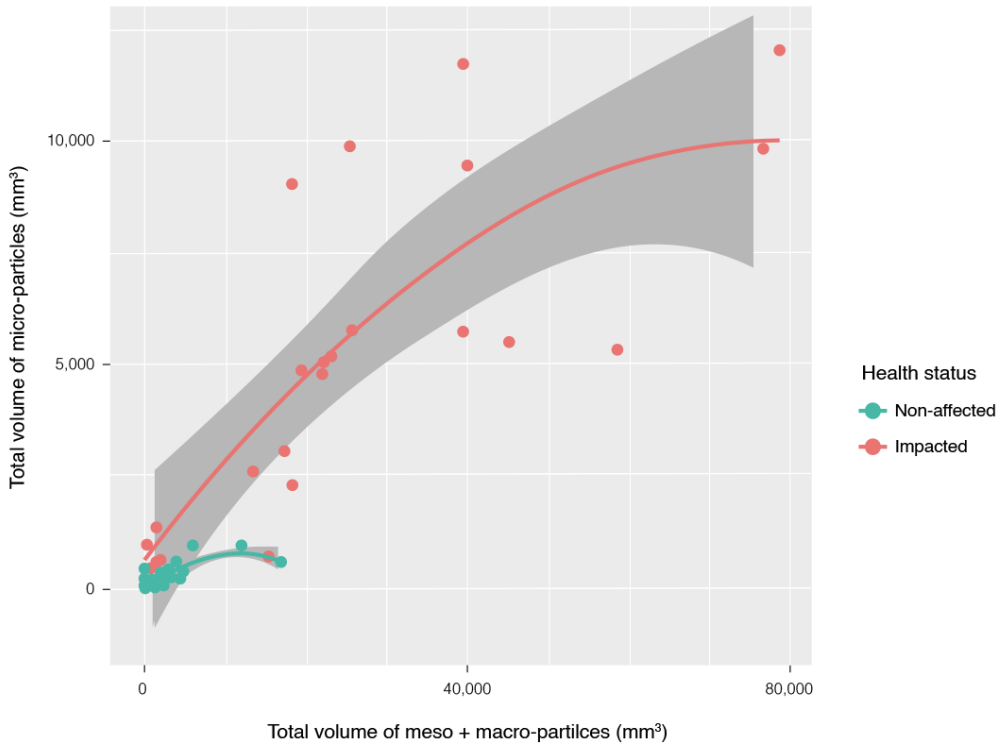

Figure 4.4. Accumulation of micro-particles by volume relative to the combined accumulation of meso- and macro-particles. The red line shows the cumulative volumes in impacted turtles, and the aqua line corresponds to non-affected turtles. Shaded areas represent the 95% confidence interval.

4.3.4 Analysis of the distribution of plastic particles along the digestive tract

The distribution of plastic particles along the digestive tract was assessed in all the necropsied turtles (n = 28) across the IPS groups by analysing the digestive contents collected separately from each animal's oesophagus, stomach, and intestines. The three distinct volume categories showed a similar pattern of increasing accumulation along the digestive tract: oesophagus < stomach < intestines (Fig. 4.5). Furthermore, the analysis of deviance indicated a significant effect of location ($\chi^2(2) = 71.07$, $p < 0.001$) (Appendix 4D in Supplementary Materials).

This differentiated distribution is especially evident in impacted turtles necropsied within IPS 3 group (n= 10) and IPS 2 group (n = 10), as they ingested proportionally higher volumes of plastic than non-affected turtles within IPS 1 group (n = 8), in which only a single turtle ingested macro-particles (Fig. 4.5). However, the small sample size precluded a robust conclusion.

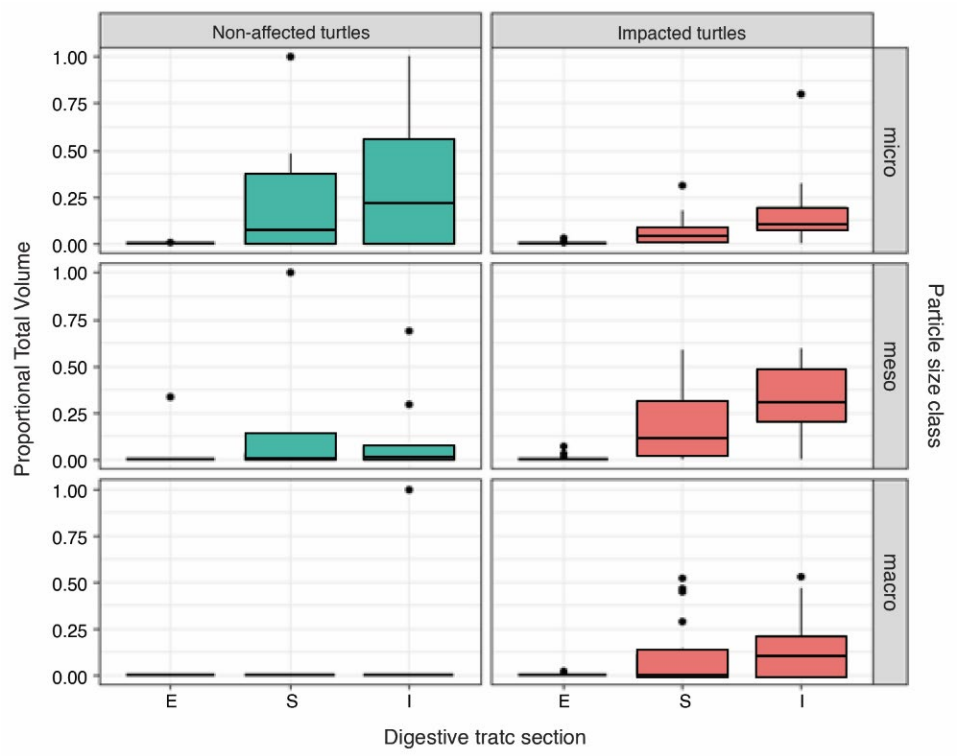

Figure 4.5. Cumulative volume of the plastic particle size classes along the digestive tract. The left y-axis represents the proportional total volume of plastic ingested; the right y-axis displays the three particle size classes: micro-particles (<100 mm³), meso-particles (100 – 1,000 mm³), and macro-particles (>1,000 mm³). The upper X-axis represents the turtles grouped as non-affected (aqua boxplots) and impacted turtles (red boxplots); the lower x-axis shows the sections of the digestive tract oesophagus (E), stomach (S) and intestines (I). Lower and upper box boundaries 25th and 75th percentiles, respectively, horizontal line median, and circles the cumulative volume ingested by individual turtles.

4.3.5 Analysis of plastic ingestion characteristics

Laminar soft plastics (SHE), plastic fragments (FRAG), and other plastics (POTH) followed a pattern of increasing accumulation across the IPS groups, while threads and fibres (THR) and foam (FOAM) accumulated randomly. The accumulation of industrial plastics or 'pellets' (IND) was not significant in any of the IPS groups (Fig. 4.6 and Table 4.3). Laminar soft plastics (SHE) were the most consumed plastic types across the IPS groups, with a mean volume of 12,355 ± 8,263 mm^3 in the IPS 3 group; 8,687 ± 9,114 mm^3 in the IPS 2 group; and 922 ± 1,610 mm^3 in the IPS 1 group (Fig. 4.6 and Table 4.3).

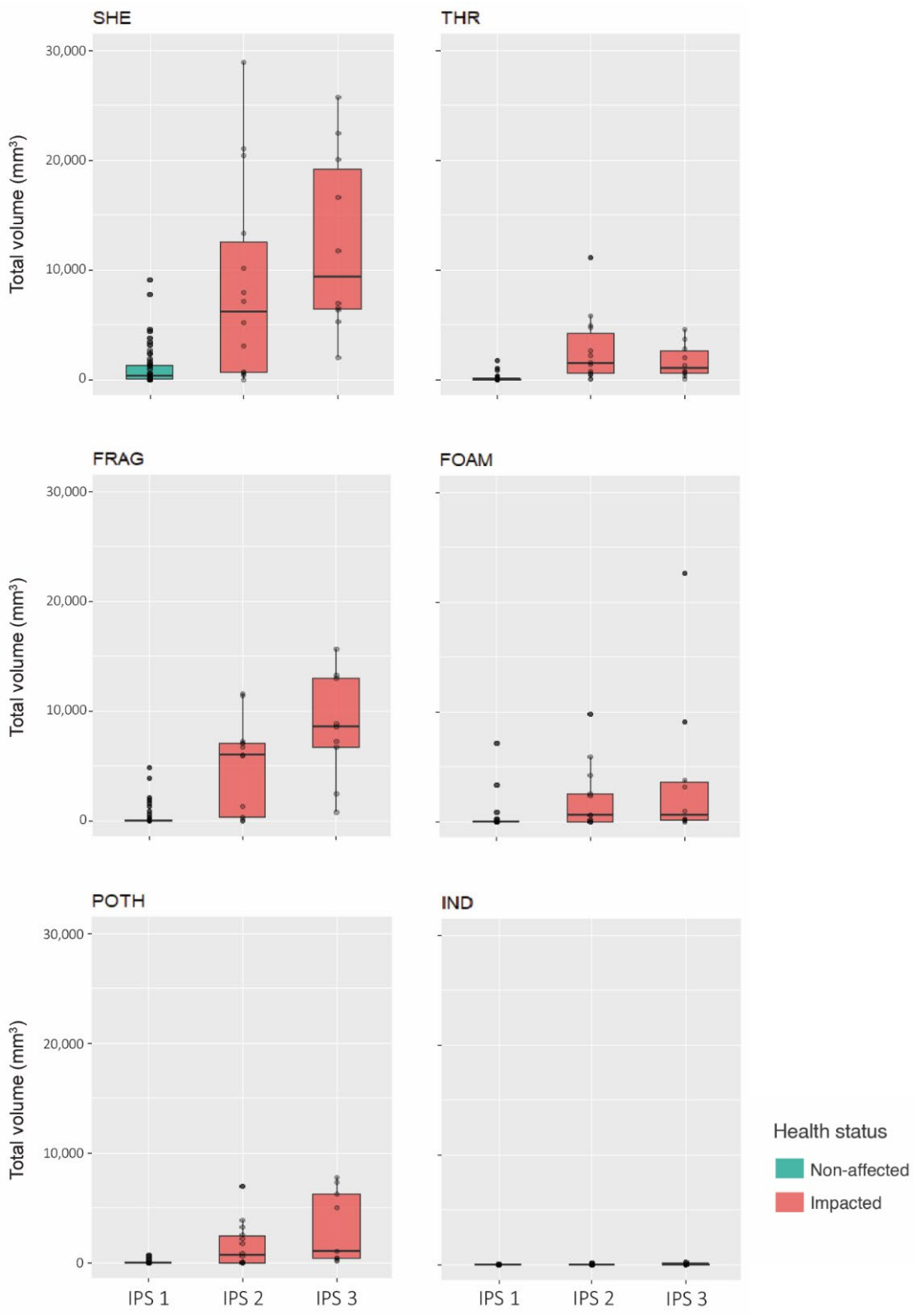

Figure 4.6. Total cumulative volumes of plastic types ingested by the examined turtles. Each chart represents a specific plastic-type: SHE (laminar soft plastics), THR (threads and fibres), FRAG (plastic fragments), FOAM (foam), POTH (other plastics), and IND (industrial plastics or 'pellets'). The y-axis represents the total cumulative volume of the plastic ingested (mm³). The upper x-axis shows the impact severity groups of turtles (IPS). Lower and upper box boundaries 25th and 75th percentiles, respectively, horizontal line median, and circles the cumulative volume ingested by individual turtles.

Table 4.3. Total cumulative volumes of plastic ingested by examined turtles and their respective volumes regarding plastic-type. Turtles are grouped according to their Impact Severity Index (IPS); results are expressed as volume ingested (mm^3).

	***IPS 1* (n = 82)**			
	[Mean ± SD]	**[Range]**	**[Total / %FO]**	**[No. turtles]**
Cumulative vol.	1,401 ± 2,652	1 – 17,518	114,886 / 100%	101
Plastic type				
SHE	922 ± 1,610	0 – 9,052	75,619 / 65.8%	74
THR	76 ± 242	0 – 1,719	6,211 / 5.4%	63
FRAG	238 ± 786	0 – 4,832	19,538 / 17.0%	26
FOAM	143 ± 868	0 – 7,120	11,691 / 10.2%	5
POTH	22 ± 103	0 – 645	1,817 / 1.6%	5
IND	0.0 ± 1	0 – 10	10 / 0.0%	1
	***IPS 2* (n = 14)**			
	[Mean ± SD]	**[Range]**	**[Total / %FO]**	**[No. turtles]**
Cumulative vol.	24,712 ± 26,936	1,187 – 90,750	345,970 / 100%	14
Plastic type				
SHE	8,687 ± 9,114	0 – 28,939	121,618 / 35.2%	13
THR	2,470 ± 3,181	6 – 11,127	34,580 / 10.0%	14
FRAG	7,812 ± 11,384	0 – 44,809	109,370 / 31.6%	12
FOAM	4,161 ± 8,538	0 – 32,103	58,256 / 16.8%	10
POTH	1,558 ± 2,041	0 – 6,942	21,814 / 6.3%	10
IND	24 ± 56	0 – 170	332 / 0.1%	3
	***IPS 3* (n = 10)**			
	[Mean ± SD]	**[Range]**	**[Total / %FO]**	**[No. turtles]**
Cumulative vol.	37,810 ± 22,428	2,821 – 86,593	378,104 / 100%	10
Plastic type				
SHE	12,355 ± 8,263	1964 – 25,694	123,553 / 32.7%	10
THR	1,669 ± 1,550	34 – 4,582	16,692 / 4.4%	10
FRAG	11,983 ± 12,027	724 – 43,553	119,830 / 31.7%	10
FOAM	4,000 ± 7,126	0 – 22,585	40,003 / 10.6%	9
POTH	7,765 ± 14,868	93 – 49,119	77,653 / 20.5%	10
IND	37 ± 76	0 – 249	374 / 0.1%	5

Acronyms: SD (standard deviation), %FO (frequency of occurrence)
Plastic type: SHE (laminar soft plastics), THR (threads and fibres), FRAG (plastic fragments), FOAM (foam), POTH (other plastics), IND (industrial plastics or 'pellets').

I observed significant variability in both the plastic types consumed and their respective cumulative volumes in turtles within the same IPS group, as well as across IPS groups (Fig. 4.6 and Table 4.3). The mean volume of each plastic-type (except IND) consumed by impacted turtles (IPS 2 and IP3 groups) was at least an order of magnitude higher than the mean volumes consumed by non-affected turtles (IPS 1 group) (Fig. 4.6 and Table 4.3). As expected, the Wilcoxon test results indicated significant differences in the means of ingested volumes for all plastic types between non-affected and impacted turtles. Given these differences, a model of impactability to analyse the effect of plastic-type (independent variable) on the severity of the impact (response variable) would be unreliable when comparing non-affected and impacted turtles. Therefore, to assess the influence of specific plastic types on the severity of impact caused by plastic ingestion, I considered only turtles within the IPS 2 and IPS 3 groups, which exhibit different degrees of impact severity. The result of the logistic regression model suggests that the ingested volume of laminar soft plastics (SHE) (*Estimate = 2.737e-04, SE = 1.385e-04, df = 7, z = 1.976, p = 0.048*) and threads and fibres (THR) (*Estimate = -1.349e-03, SE = 6.762e-04, df = 7, z = -1.995, p = 0.046*) contribute to explaining changes in the probability of severe impact (Appendix 4E in Supplementary Materials). Nevertheless, these results should be interpreted cautiously due to the low sample size (n = 24).

4.3.6 Analysis of impact severity in relation to turtle size/age

The results of the logistic regression model indicated a statistically significant negative relationship between turtle size, represented by curved carapace length (CCL), and the likelihood of severe impacts caused by plastic ingestion (*Estimate = -0.187, SE = 0.068, df = 1, z = -2.751, p = 0.006*) (Appendix 4F in Supplementary Materials), suggesting that an increase in CCL is associated with a decrease in the probability of impact. Impacts of plastic ingestion are imperceptible in turtles over 50 cm of CCL, even when there were substantial volumes of plastic in their digestive tracts (Fig. 4.7).

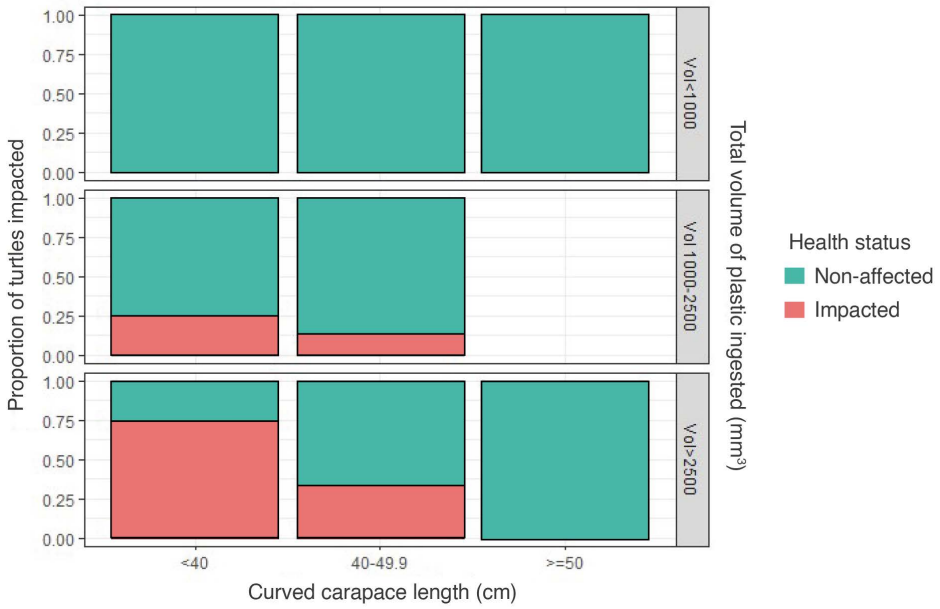

Figure 4.7. The proportion of turtles impacted by plastic ingestion in relation to their curved carapace length (CCL). The left y-axis represents the proportional of turtles impacted: non-affected (aqua box) and impacted turtles (red box); the right y-axis displays three levels of plastic ingestion: low volume (<1,000 mm³), medium volume (1,000 – 2,500 mm³), and high volume (>2,500 mm³). The x-axis represents three turtle size classes (CCL).

4.4 DISCUSSION

4.4.1 Assessment of plastic ingestion

After evaluating the necropsy reports and health assessments, only 24 turtles were assessed as impacted (14 turtles as IPS2, and 10 turtles as IPS3), all of them by obstruction of the digestive tract caused by the ingestion of plastic ingestion. Ideally, a model incorporating all key explanatory variables should have been used for assessing the impact severity of plastic ingestion in the examined turtles. However, this approach was not feasible due to the limitations imposed by the number of "events" in the dataset. Therefore, I had to minimise the number of explanatory variables and the degrees of freedom associated with each one as much as possible by (i) leaving explanatory variables as continuous where possible, (ii) minimizing the number of levels for categorical variables, (iii) leaving out explanatory variables that did not have a substantial effect on impact, and (iv) omitting the consideration of interaction effects.

Consequently, the statistical power of the analyses in this chapter was compromised due to the low sample size of impacted turtles. Nevertheless, along with the observations made during the necropsies and health assessments, the results enabled me to draw the meaningful conclusions below regarding the factors influencing the impact of plastic ingestion on the examined turtles.

4.4.2 Cumulative volumes of plastic ingested

The analysis showed a positive relationship between the cumulative volume of ingested plastic and the severity of the impact; the more plastic ingested, the more severe the impact. I observed a significant disparity in the volumes consumed between non-affected and impacted turtles. Non-affected turtles (n = 82) consumed only 12% of the retrieved plastic (114,886 mm^3 / 1,231 pieces), while impacted turtles (n = 24) ingested the remaining 88% of the plastic collected (724,074 mm^3 / 9,086 pieces). This disparity, coupled with an absence of turtles with intermediate values of plastic ingestion in the dataset, prevented a more detailed dose-response analysis. Controlled laboratory experiments could contribute to obtaining more diverse and stratified datasets for this type of analysis. However, experimentation with marine turtles is challenging and usually restricted due to ethical considerations. Hence, additional research efforts should be addressed to explore alternative approaches for accurately determining the minimum ingested volume causing severe impacts.

The significant difference observed might be attributed to impacted turtles continuing to feed despite experiencing total or partial obstruction (Rice et al., 2021; Santos et al., 2020). As a result, impacted turtles remain exposed to the risk of plastic ingestion, retaining an increasingly higher volume of plastic due to the obstructions and the difficulty in expelling the ingested material. This potential 'cumulative side-effect' might hinder accurately determining the minimum volume of plastic ingestion required to cause severe impacts.

4.4.3 Plastic ingestion across three distinct volume categories

Despite the significantly higher accumulation of micro-particles in impacted turtles compared to non-affected turtles, I consider it unlikely that micro-particle accumulation is a reliable predictor of impact severity by itself. Given the small individual volume of these particles, they should pass through the digestive tract of juvenile turtles without difficulty and be expelled easily. Veterinarians reported blockages in the intestines of impacted turtles by fecalomas resulting from the compaction of meso- and/or macro-particles, along with a large cumulative volume of micro-particles and other digestive contents. Micro-particles may contribute to the formation of fecalomas, but it is unlikely that they are the particles initially causing them. Furthermore, the mean cumulative volume of micro-particles found in the digestive tract of impacted turtles was three times less than the mean cumulative volume of meso-particles and two times less than the mean cumulative volume of macro-particles (Table 4.2).

The examined turtles primarily consumed meso-particles, followed by macro-particles. This preference might be attributed to juvenile green turtles' optimal ingesta particle volume for dietary items, aiming to maximise digestive efficiency and fermentation rates (Gulick et al., 2021). It should be also considered that characteristics such as type and shape of plastic may influence the particle volume for ingesta. For instance, the dimensions of solid plastic fragments are constrained by the turtle's mouth gape or bite width due to their stiffness, while soft plastics can be swallowed without such restrictions because of their flexibility.

4.4.4 Distribution of plastic along the digestive tract

I also observed an increasing accumulation of plastic in all particle sizes along the digestive tract: oesophagus < stomach < intestines. As outlined in Chapter 3, among the main factors contributing to plastic retention in the final sections of the tract include: (i) the hindgut fermentation strategy of green turtles, resulting in extended retention times in the intestines for the fermentation and

absorption of nutrients from a plant-based diet (Brand et al., 1999; Bjorndal, 1980; Mackie, 2002); and (ii) specific anatomical features of the digestive tract in juvenile green turtles, characterized by long-narrow intestines, and a stomach in 'J' shape (Colferai et al., 2017; Magalhães et al., 2012; Wyneken, 2001).

4.4.5 Characteristics of plastic ingested

Laminar soft plastics (SHE) were the most consumed plastic-type among the examined turtles in terms of ingested volume, which aligns with the findings in the previous chapter regarding the total number of pieces (see Chapter 3, section 3.4.2). As outlined in Chapter 3, the prevalence of soft plastics might be attributed to selectivity in feeding behaviour driven by the resemblance of these plastics to dietary items such as macroalgae and gelatinous macrozooplankton, which are the main food sources for green turtles in Uruguay (Vélez-Rubio et al., 2016). I recall that a thorough validation of this selective behaviour would require an assessment of the availability of different plastic types in the environment.

In addition, the analysis revealed that the total ingested volume of laminar soft plastics (SHE) could serve as potential predictor of severe impact caused by plastic ingestion. Since these plastics were the most consumed plastic-type, also exhibiting an increasing accumulation across the IPS groups (see Fig. 4.6 and Table 4.3), it suggests that laminar soft plastics (SHE) might influence the impact severity to a larger extent than other plastic types. Furthermore, such plastics represent a potentially high risk for turtles due to their pliability characteristics. Turtles can ingest large pieces of soft plastics without restriction of their mouth gape. Once in the intestines, these large and malleable pieces of plastic can act as a mesh, tangling up other plastic items and digestive contents and facilitating the compaction of fecalomas, extending retention times within the digestive tract which eventually can result in obstruction, as observed during the necropsied of severely impacted turtles within IPS 3.

The model also detected an adverse effect related to the volume of threads and fibres (THR) ingested by the examined turtles probably because of the lower volume of this plastic-type consumed by turtles in the IPS 3 group compared to the IPS 2 group (see Fig. 4.6 and Table 4.3). Considering that the total volume of threads and fibres (THR) consumed by these turtles is marginal compared with the ingested volume of laminar soft plastics (SHE), I didn't consider the ingested volume of threads and fibres (THR) as a predictor of impact severity in this study.

4.4.6 Severity of impact in relation to turtle size/age

Turtles with CCL <40 cm exhibited a proportionally higher impact ratio than larger turtles, aligning with the analysis in the previous chapter (see Chapter 3, section 3.3.2). This result might be associated with the feeding behaviour exhibited by early juveniles recently recruited to neritic habitats in Uruguay. As outlined in Chapter 3, these smaller turtles still reflect a relict opportunistic behaviour related to their previous oceanic stage (Vélez-Rubio et al., 2016). This feeding behaviour makes them potentially more susceptible to ingesting a wide range of plastics and higher volumes due to their lower discrimination of dietary items. In addition, such behaviour leading early juveniles to ingest higher volumes of plastics could represent an evolutionary trap for the species, as theorised by Duncan et al. (2021) and Santos et al. (2021).

4.5 CONCLUSIONS

The severity of impact caused by plastic ingestion depends largely on the quantities and characteristics of ingested plastics. The analysis conducted on juvenile green turtles present in Uruguayan waters revealed a positive relationship between the cumulative volume of ingested plastic and the severity of the impact. Ingested plastic accumulates increasingly along the digestive tract: oesophagus < stomach < intestines. In addition, necropsies revealed intestinal obstructions by

fecalomas in the severely impacted turtles, where large malleable plastics played a critical role, acting as a mesh and entangling other plastics and digestive contents. Furthermore, green turtles with CCL <40 cm exhibited a higher impact ratio than larger turtles, presumably due to their opportunistic feeding behaviour.

This study contributes valuable insights into the severity of the impact caused by plastic ingestion on juvenile green turtles in relation to the volumes and characteristics of ingested plastic. Nevertheless, additional research is required to better understand the full extent of the impacts caused by plastic ingestion on turtles' health, as well as how the impaction process occurs, elucidating how turtles transition from a non-affected state to undergoing progressive degeneration due to plastic ingestion.

PhD Chapter 5

A best practice framework for assessing plastic ingestion in marine turtles

Chapter objective

To establish an effective framework for designing and conducting research on plastic ingestion in marine turtles, aiming to assist researchers in articulating optimal strategies and standard methods aligned with research objectives, while considering the accessibility of resources and capabilities

Methodology

This globally applicable framework outlines standard methods and established approaches in the context of the resources available and capabilities required to guide practitioners, and stakeholders from the early stages of research design through data collection and analysis to reporting and publication of the results. The framework was informed by the literature, the collective experiences of collaborators, and discussions among experts, also incorporating the insights and learnings gained from conducting my PhD research.

Key findings

- Key aspects that conform to the best practice framework include setting clear research objectives and defining the research scope, selecting appropriate approaches and methods for data collection and analysis, as well as understanding the potential biases associated with these, and assessing available resources and capabilities.

Conclusions

There is still limited consensus on adopting consistent research protocols and methodologies for investigating plastic ingestion in marine turtles. This lack of agreement hampers the comparison of results, hindering efforts to obtain broader impact assessments. In this context, this best practice framework provides guidance for designing and implementing research to assess plastic ingestion in marine turtles based on common research protocols and standardised methods.

Related publication

González-Paredes, D., Duncan, E., Godley B. J., Marsh, H., & Hamann, M. (*in prep.*)
A best practice framework for assessing plastic ingestion in marine turtles.
Conservation Biology (target journal)

5.1 INTRODUCTION

Plastic ingestion is recognised as an emerging threat and a priority conservation concern for marine turtles (Fuentes et al., 2023; Hamann et al., 2010; Nelms et al., 2016; Vegter et al., 2014). Understanding the impacts caused by plastic ingestion is crucial to assessing the vulnerability of marine turtles to this threat. In recent years, reports on plastic ingestion in marine turtles have increased, transitioning from largely opportunistic observations to more systematic and structured studies. Several research efforts have focused on gathering and analysing meta-data to outline and provide a broader view of the issue of plastic pollution affecting marine turtles on a global scale (Lynch, 2018; Moon et al., 2023; Nelms et al., 2016; Schuyler et al., 2014a, 2016). Consistent, long-term data collection has played a crucial role in understanding the scope and trends of plastic ingestion in marine turtles, such as those conducted through stranding networks (Choi et al., 2020; Domènech et al., 2019) or bycatch programs (Clukey et al., 2018; Fukuoka et al., 2016). On the other hand, in Europe, regional efforts such as the Marine Strategy Framework Directive (MSFD) and the OSPAR Convention (Convention for the Protection of the Marine Environment of the North-East Atlantic) have established guidelines for monitoring the impact of plastic pollution on marine megafauna. These protocols were adapted for marine turtles by Galgani et al. (2013). Additionally, the INDICIT Consortium employs loggerhead turtles (Caretta caretta) as bioindicators to assess plastic pollution levels in the Mediterranean basin, in alignment with the objectives of the Barcelona Convention (Darmon et al., 2022; Fossi et al., 2018; Matiddi et al., 2019). Similarly, in the United States, the project BEMAST (Biological and Environmental Monitoring and Archival of Sea Turtle Tissues), a long-term biobanking initiative, archives marine turtle blood and tissue samples for real-time and retrospective contaminant analysis related to plastic pollution (Savoca et al., 2023; Shaw et al., 2021). On a global scale, international initiatives like the Global Plastic Ingestion Bioindicators (GPIB) promote the use of marine turtles as bioindicators to generate critical insights into the trends, risks, and impacts of plastic pollution on species and ecosystems (Savoca et al., 2024).

All these initiatives establish protocols and methodologies for assessing plastic ingestion in marine turtles. Nevertheless, in regions or countries where limited consensus persists on adopting consistent procedures and standard protocols, disparities in reporting plastic ingestion in marine turtles remain. This lack of harmonization hampers the comparability of results across studies and reduces the applicability of data for long-term monitoring and assessments of plastic pollution as a population- or species-level threat, particularly when these are transboundary in nature (Casale et al., 2016; Fuentes et al., 2023; Hamann et al., 2010; Nelms et al., 2016; Provencher et al., 2017; Senko et al., 2020).

Understanding the impacts caused by plastic ingestion is crucial to assessing the vulnerability of marine turtles to this threat. A coherent project design aligned with available resources and capabilities, coupled with the use of standard methods and protocols, along with the sharing of data and results in open-access repositories, is crucial for a broader impact assessment of plastic pollution on marine turtles at both the population and species levels (Fuentes et al., 2023; Hamann et al., 2010; Nelms et al., 2016; Senko et al., 2020). In this chapter, I develop a globally applicable best practice framework for designing and implementing research to assess plastic ingestion in marine turtles. The literature has informed this document, alongside with the collective experience of collaborators, and discussions amongst experts (I have led the Workshops on Sea Turtles and Plastic Pollution held in the last four International Sea Turtle Symposiums), also incorporating the insights and learnings gained from conducting my PhD research. I discussed fundamental components for establishing and fulfilling research objectives and outlined strategies for best practices to strengthen monitoring and research initiatives, aiming to guide practitioners and stakeholders in assessing plastic ingestion in marine turtles.

5.2 A BEST PRACTICE FRAMEWORK

Achievement of research goals largely depends on establishing a well-designed research plan before project commencement. In this context, a framework represents a conceptual structure for the theoretical and technical background essential to designing efficient research plans by articulating strategies based on common research methods and replicable techniques. I discuss below key aspects of a best practice framework, including setting clear research objectives and defining the scope, selecting appropriate approaches and methods for data collection and analysis as well as understanding the biases associated with these approaches, and evaluating resources and capabilities (Fig. 5.1).

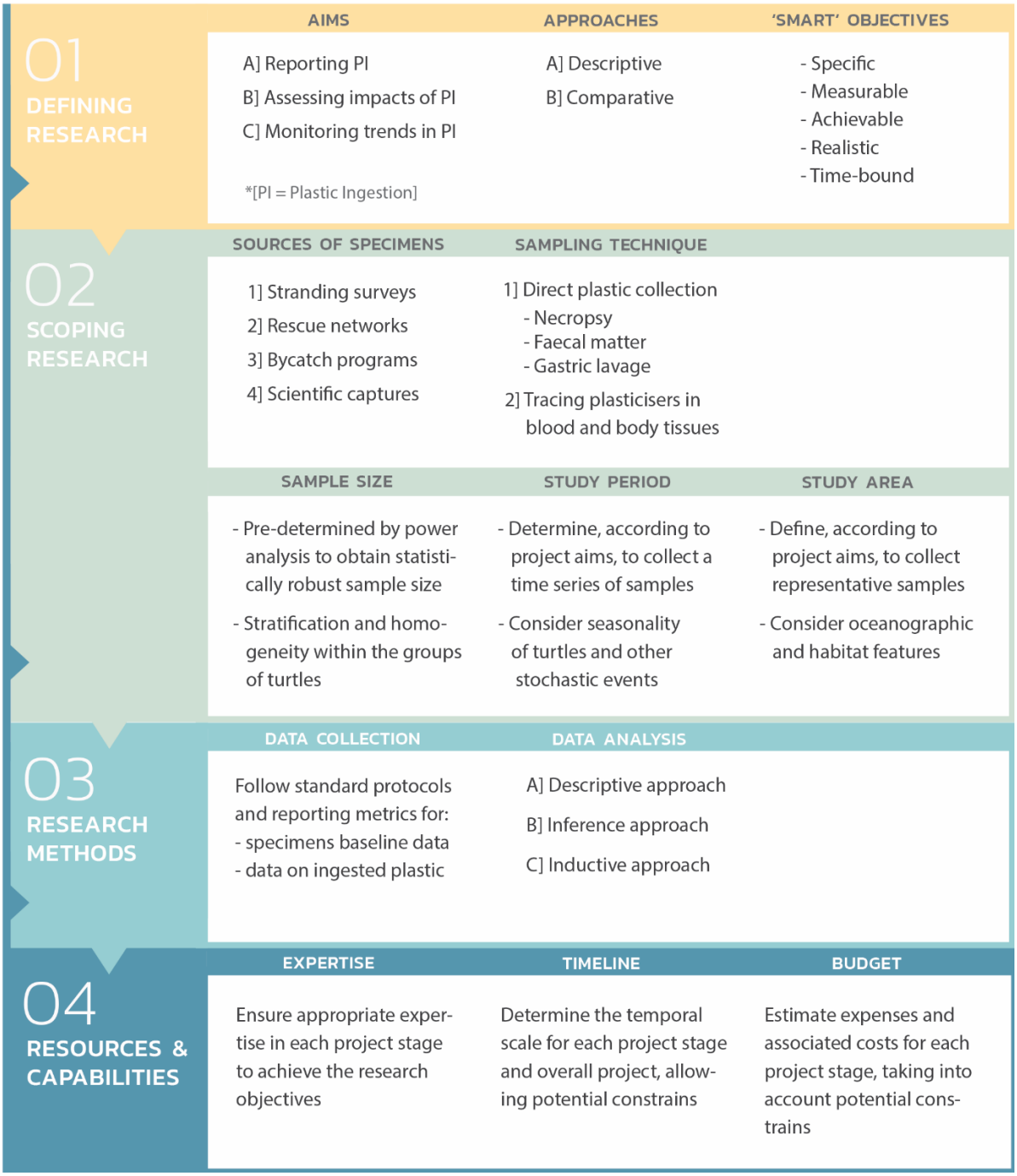

Figure 5.1. Conceptual map of the proposed best practice framework for assessing the impact of plastic ingestion in marine turtles.

5.2.1 Define research aims, objectives and approaches

An initial and central component of designing a research project is determining the study's aim(s). Given the wide diversity of research aims concerning plastic ingestion in marine turtles, I identified three possible primary purposes, which will serve as reference points throughout this chapter:

A] Reporting plastic ingestion. This aim can be addressed by projects ranging from opportunistic to extended and systematic studies. Along with physical descriptions and measurements of quantities of the plastic ingested, parameters such as frequency of occurrence, incidence, and associated mortality enable inferences regarding rates and patterns of ingestion, provided a representative sample size (Duncan et al., 2019a; Gama et al., 2021; Vélez-Rubio et al., 2018a; see Chapter 3).

B] Assessing impacts of plastic ingestion on the health of turtles. These studies aim to reveal and quantify adverse effects caused by plastic ingestion at individual or group levels and evaluate the factors involved in the impact process. According to the research scope, these studies may involve multiple disciplines, such as toxicology, veterinary diagnostic assessments, and evaluation of environmental pollution levels, among others (Sala et al., 2021; Savoca et al., 2018; see Chapter 4).

C] Monitoring plastic ingestion over time. These studies build upon the approaches in [A] or [B] above but require long-term, systematic, standardised data collection and appropriate sample sizes to detect trends across time in the data gathered (Choi et al., 2021; Domènech et al., 2019).

These three aims can be approached from a descriptive or comparative perspective. Descriptive reports on the incidence of plastic ingestion [type A aims] or reporting injuries at the individual level [type B aims] are frequently opportunistic and associated with studies where assessing plastic ingestion is not the primary goal (e.g., bycatch monitoring programs or stranding networks collecting and examining dead turtles, in which plastic ingestion is detected after routine necropsy examinations; see Da Silva et al., 2015). Opportunistic information can help establish evidence based on the hazard of plastic pollution on marine turtles. However, the meaningful inferences about plastic ingestion are usually limited to individuals or constrained groups of turtles in these studies

(Casale et al., 2016; Lynch, 2018). On the other hand, descriptive approaches to evaluate plastic ingestion patterns [type A aims] or assess lethal and sub-lethal effects caused by plastic ingestion [type B aims] at large scales or the population level require a higher degree of planning and research complexity, as well as to include representative subset of animals to allow the elaboration of conclusive results and/or infer cause-and-effect relationships (e.g., toxicology studies revealing endocrine disruptions associated with high levels of phthalates related to plastic ingestion; see SanJuan et al., 2023; Savoca et al., 2018).

Comparative analyses use systematic methods to understand the general principles of plastic ingestion by identifying differences and similarities among distinct groups of turtles. These could also apply to analysis based on understanding variation or interpretation of diversity to establish statistical relationships between two or more datasets (e.g., analysis of plastic ingestion patterns in different marine turtle species caught as bycatch in pelagic longline fisheries; see Clukey et al., 2018).

In addition, descriptive or comparative approaches can serve as a basis for long-term monitoring purposes [type C aims], involving systematically assessing a subset of animals over time. The transition from research to monitoring becomes more likely and feasible by establishing longer-term objectives and adhering to well-established and common methods. Using standard procedures and systematically extending them over time and/or space enables researchers to evaluate the impact of plastic ingestion on the study group across different temporal and spatial scales (e.g., evaluation of trends in plastic ingestion by green turtles in the Gulf of Mexico for three decades; see Choi et al., 2021).

Establishing clear, unambiguous research objectives must underpin project aim(s). SMART objectives (**S**pecific, **M**easurable, **A**chievable, **R**ealistic and **T**ime-bound) enable a focus on the specific and achievable research question(s), which can be addressed using a specific approach(s) to attain quantitatively and/or qualitatively measurable data in a defined timeframe (Doran, 1981). In

addition, these objective(s) must be realistic and feasible, hence the need to assess potential limitations and biases and evaluate the availability of resources and capabilities (concepts developed in the sections below).

It is equally important to ensure that results are statistically robust. Comprehensive data collection, combined with a large and stratified sample size, allows reliability and representativeness in the inferences drawn from analyses. In the early stages of the project design, I recommend using power analyses to estimate the minimum sample size required to accomplish the specific SMART objectives, given a desired significance level, effect size, and statistical power (Lavers et al., 2021; Provencher et al., 2015; see Chapter 3).

5.2.2 Scoping the research or monitoring project

The scope of the research or monitoring project describes the extent to which the field of study will be explored, defining the parameters within which the study will be developed, such as source of samples, sampling frequency, sample size, extent of the study area, project duration, types of data and subsequent analysis.

5.2.2.1 Source of specimens

There are multiple sources of specimens for research on plastic ingestion in marine turtles, including (1) stranded turtles collected through directed beach surveys, (2) injured turtles that have been rescued, (3) turtles obtained via bycatch, or (4) turtles intentionally caught in the wild by researchers. Stranded and rescued turtles haphazardly collected might have been in poor health conditions before encountering; consequently, exhibiting abnormal feeding behaviours and/or habitat use. Thus, the recorded quantities of plastic ingestion in these turtles are subject to biases, either underestimating or overestimating the overall ingestion rates of a stock or population (Casale et al., 2016; Vélez-Rubio & Tomás, 2016) (Table 5.1) (see Chapter 3). Nevertheless, these turtles can provide valuable insights when the aim is to evaluate the extent of the impact resulting from plastic

ingestion (Table 5.1) (see Chapter 4). In contrast, bycaught turtles or turtles captured in the wild are likely to be more reliable sources of samples if sampling is systematic (e.g., bycatch programs retrieving turtles from fishing gears, or monitoring programs which capture turtles in the wild), as they better represent the overall exposure of a population to plastic ingestion (Table 5.1) (see Chapter 3).

It should also be considered that the target species could be a potential predictor of plastic ingestion, since the likelihood of plastic ingestion is closely linked to interspecific feeding strategies (Lynch, 2018; Schuyler et al., 2014a). Opportunistic foraging species are potentially exposed to higher incidences of plastic ingestion because of low discrimination in feeding behaviour (e.g., loggerhead turtles, *Caretta caretta*, with an omnivorous feeding on a wide range of prey). At the same time, some specialist foragers are also considered to be more likely to ingest plastic debris resembling their diet items (e.g., leatherback turtle, *Dermochelys coriacea*, feeding primarily on gelatinous organisms) (Bjorndal, 1997; Mrosovsky et al., 2009; Schuyler et al., 2014b; see Chapter 3). Age and size classes represent another significant predictor of plastic ingestion. Post-hatchlings and juvenile turtles exhibiting an opportunistic foraging strategy in pelagic waters (Lynch, 2018; Schuyler et al., 2014b) are considered more susceptible to ingesting a wide range of plastics and higher volumes due to their lower discrimination in the selectivity of dietary items (Lynch, 2018; Schuyler et al., 2014b; see Chapter 4). Furthermore, early life stages are potentially more vulnerable to internal injuries from plastic ingestion because of their narrow digestive tract relative to the size of plastic particles (Boyle, 2006; Schuyler et al., 2012). Other authors also suggest that the longer digestive tracts of adults and sub-adults could retain greater amounts of plastic debris for longer than small animals (Casale et al., 2016). While species and age/size class can serve as predictors of plastic ingestion, specific individual-level differences may occur in relation to habitat use, feeding behaviour, and diet (Duncan et al., 2019b, 2021; Casale et al., 2016; Lynch, 2018; Nelms et al., 2016; Schuyler et al., 2014b).

5.2.2.2 Sampling technique

The sampling technique is a key factor when scoping the research and developing SMART objectives. The sampling technique will define the analytical methods and determine the conclusions that can be drawn (Lavers et al., 2021). Sample sources for assessing plastic ingestion in marine turtles can be grouped into two main categories according to the methods used for their collection: (1) direct collection of ingested plastic and (2) tracing plasticisers in blood and body tissues.

1] Direct collection of ingested plastic. The ingested plastic can be collected from gastrointestinal contents removed during necropsy or by examining faecal matter and/or gastric lavages in live animals (Casale et al., 2016; Nelms et al., 2016; see Chapters 3 and 4). Plastic retrieved through any of these techniques can be considered representative of the plastic consumed by the examined turtle. However, potential variations in retention times associated with types of plastic or their dimensions, and/or influences of the turtle's health status on the progression rates of plastic along the digestive tract, can result in higher accumulations of specific plastics. These factors should be taken into consideration in the analyses, otherwise, assumptions must be explicitly stated.

As outlined in Chapter 3, necropsy remains the most reliable procedure for extracting and examining the entire digestive contents (see methods in Wyneken 2001). This technique allows the examination of all digestive tract sections to ascertain the presence and distribution of ingested plastics (see methods in Matiddi et al., 2017; Duncan et al., 2021). However, this technique is not applicable to live animals; consequently, the analyses could be subject to biases depending on the source of specimens (Table 5.1).

Faecal matter examination allows sampling of live animals for assessments of plastic ingestion. This methodology can be applied to those turtles in rehabilitation facilities or caught in the wild and held in captivity for research purposes (see methods in Casale et al., 2016; Fukuoka et al., 2016; Hoarau et al., 2014; see Chapters 3 and 4). This sampling technique requires monitoring periods longer than the upper limit of the ingesta passage time to maximise the likelihood of collecting from the faeces

all potential plastic previously ingested in the environment (González-Paredes et al., 2021b; Valente et al., 2008; see Chapter 2). This methodology is more efficient when applied to turtles that exhibit certain digestive motility and defecate regularly; otherwise, it could serve as an early warning of digestive disorders or obstructions (Table 5.1) (see Chapter 2).

Gastric lavage can also be used as a method for examining digestive contents in live turtles (see methods in Forbes & Limpus, 1993; Stokes et al., 2008; see Chapter 3). However, this technique is not recommended, as it only allows for the collection of an unknown proportion of the oesophagus and stomach contents, preventing to ascertain the quantity of plastics remaining in the intestines, where accumulation is primarily observed (Camedda et al., 2014; Duncan et al., 2019a). Furthermore, it involves a risk of internal lacerations or perforations in the digestive tract if not conducted carefully and is thus limited to people with appropriate training and expertise (Manire et al., 2017). Nevertheless, this sampling technique only allows an unknown proportion of the oesophagus and stomach contents to be collected, being not possible to ascertain the proportion of plastics remaining in the intestines, where the majority of ingested plastic accumulates (Table 5.1) (Camedda et al., 2014; Duncan et al., 2019a). As outline in Chapter 3, this technique proved to be a non-efficient method for assessing plastic ingestion in marine turtles.

Ideally, a comprehensive assessment of plastic ingestion would include necropsy samples covering a representative subset of animals from different sources, along with the inclusion of faecal analysis in the routine examination of live turtles, enabling healthy turtles to be included for a more robust evaluation of ingestion patterns.

The collection of ingested plastics must be meticulous to obtain reliable and accurate results. Generally, the collected plastics are classified based on their size; commonly used boundaries are micro-plastics (<5 mm in diameter), meso-plastics (5–25 mm in diameter), and macro-plastics (>25 mm in diameter) (OSPAR Commission, 2020). Alternatively, they can be classified according to their volume as micro-particles (<100 mm^3), meso-particles (100 - 1,000 mm^3), and macro-particles (>

1,000 mm^3), as proposed in Chapter 4 to reflect the burden within the digestive tract better. It is essential to determine the minimum and maximum thresholds and the relative breakdown by size (volume) class to assess and differentiate impacts associated with them. Once study objectives are settled, establishing these thresholds early is important because they help determine the scale and accuracy of the equipment required for sample collection (e.g., sieve mesh size, Vernier callipers' precision, balance accuracy, microscopy lens). In addition, the analytical methods required due to the procedures for examining and minimising contamination of micro-plastics differ from those used for meso- and macro-plastics (Duncan et al., 2019b). Macro and meso-plastics are cleaned to remove biological material before drying and storage in appropriate, cleaned, and labelled containers (Provencher et al., 2019). While micro-plastics often need enzymatic digestion or potassium hydroxide (KOH) to remove organic material and biofilm (Duncan et al., 2019b; Joon Shim et al., 2017; Kühn et al., 2017). Care must be taken throughout the cleaning and storing to eliminate potential sample contamination. Among the most common sources of contamination are atmospheric contamination from airborne plastic particles, water contamination, equipment contamination and cross-sample contamination when multiple samples are being processed simultaneously (Bogdanowicz et al., 2021). Other specific analyses may require particular protocols and equipment (e.g., polymer identification of ingested plastic; see methods in Jung et al., 2018).

2] Tracing plasticisers in blood and body tissues. Polymer bonds of plastic are subject to breakage due to photochemical and mechanical forces when exposed for a long time to UV light and the physical pressure of waves and wind in the marine environment (Andrady, 2015). This polymer fragmentation might facilitate the lixiviation of toxic substances of ingested plastic and eventually their absorption into the tissues or blood. Furthermore, the digestive process could also influence the chemical leaching and absorption of these toxins into the turtle's body (Sala et al., 2021; Savoca et al., 2018). It has been speculated that chronic exposure to plasticisers could lead to sub-lethal effects such as metabolic and endocrine malfunctions, disorders in somatic rates, or fertility inhibition (Andersson et al., 2016; Clukey et al., 2018; Marn et al., 2020; Nelms et al., 2016; Ryan et

al., 2016). Such cryptic effects, however, are still poorly understood in marine turtles, as outlined in Chapter 4.

The plasticisers most studied in marine turtle toxicology include organophosphate esters (OPEs) and phthalates. Generally, analysis to determine the accumulation levels of OPEs is restricted to dead turtles, as muscular tissue is required (see methods in Sala et al., 2021). In contrast, phthalates can be detected in both dead turtles by sampling gonads and liver, and in live turtles through biopsy of fat tissues (samples must be large enough, >0.5g wet weight, for allowing detection of low concentrations; see methods in Savoca et al., 2018). Both techniques will potentially enable the assessment of cryptic sub-lethal effects caused by the accumulation of toxic substances in plastic. However, these techniques are all in the development phases, and at present, defining harmful levels of absorbed plasticisers is a key challenge, as well as discriminating the accumulation of toxic substances from ingested plastics or their assimilation from background ocean pollution (Koelmans et al., 2021).

Table 5.1. Strengths and limitations of the multiple approaches for assessing plastic ingestion in marine turtles.

Approach	Strengths	Limitations
Specimen source		
• Bycaught and wild-capture turtles	Indicators of the overall population's exposure to plastic ingestion	Sampling must be systematic
• Stranded and rescued turtles	Provide valuable insights into the severity of the impact of plastic ingestion	Potential biases in plastic ingestion rates due to pre-existing health issues, leading to abnormal feeding behaviour and/or habitat use
Sampling method		
• Necropsy	Allow to retrieve all the digestive contents for assessing plastic ingestion	Only applicable to dead turtles, potential biases according to the specimen source
• Faecal matter monitoring	Allow to assess plastic ingestion in live turtles	The minimum monitoring period needs to be adjusted to the upper limit of gastrointestinal transit time No data is provided regarding the location of plastics along the digestive tract
• Gastric lavages	Allow to assess plastic ingestion in live turtles	Only a small portion of the digestive content can be retrieved from the oesophagus and stomach Non-efficient method for assessing plastic ingestion
• Tracing plasticisers in blood and body tissues	Allow to assess sub-lethal effects associated with the chemical leaching of plastic into blood or tissues	Harmful levels of plasticiser accumulation remain unclear Need to differentiate from the assimilation of chemicals from background ocean pollution

5.2.2.3 Sample size

Ideally, the sample size (or the minimum required) must be pre-determined according to the source of specimens, the sampling technique and potential analyses (Lavers et al., 2021; Provencher et al., 2015). Power analyses can pre-determine the size and study period required to collect samples for statistically robust analysis and reliable results. Additionally, power analysis can predict the

percentage of change over the study period based on the sample sizes and coefficients of variation (Kraemer & Blasey, 2015; Provencher et al., 2015; see Chapter 3). Post-hoc power analysis can validate the data collection and approach and set the monitoring framework for ongoing research (Gillett, 1994; Lavers et al., 2021). A key consideration regarding sample sizes that include a large number of turtles from different sources is segregating the dataset into homogeneous groups (e.g., age classes, habitat uses, dead and live turtles, stranded/rescued and bycaught/captured turtles, etc.) to enable comparisons across them.

Descriptive or opportunistic studies on plastic ingestion are generally less focused on obtaining pre-determined sample sizes, as the key component of these studies is the use of consistent and well-documented approaches to enable data collection to be expanded more systematically if resources or time permits. Quantitative studies generally require larger and more representative sample sizes, allowing assessments of incidence and ingestion rates. In this respect, monitoring projects require systematic sample collection using standard methods across time to generate datasets with sufficient statistical power to infer reliable trends and differences among groups. Establishing cause-effect relationships between ingestion rates and impacts on turtles' health remains challenging in the absence of controlled dose-response trials due to ethical restrictions related to experimenting on marine turtles. Small and highly variable sample sizes may compromise the power of analysis by limiting the number of variables to consider in these studies (see Chapter 4). In this context, large and well-stratified datasets are crucial for ensuring reliable results and drawing meaningful conclusions about the factors involved in the impaction process.

5.2.2.4 Study period

Defining the study period should be a central component of the research design to obtain the samples and dataset required to achieve the project objectives. This is particularly relevant when developing monitoring programs, which require sufficient time to collect a time series of representative sample sizes to assess incidence and trends in plastic ingestion. The periods

necessary for descriptive reports of plastic ingestion or impact assessments will depend on the research aims; these may vary according to the source of specimens and/or sampling technique (see sections 5.2.2.1 and 5.2.2.2).

It should be considered when defining the study period that the presence and aggregation of turtles may vary across time, even at small temporal scales, due to breeding and nesting seasons and migratory patterns among other stochastic events (Godley et al., 2010; Meylan et al., 2011; Schuyler et al., 2014a). Furthermore, the occurrence and abundance of plastic are also subject to variations at temporal and spatial scales due to abiotic factors (wind patterns, ocean currents, coastal fronts, river discharges) or episodic and casual events (heavy rainfalls, cyclones, natural or anthropogenic disasters) (Cózar et al., 2014; Kershaw & Rochman, 2015).

5.2.2.5 Study area

The study area can range from a few kilometres of coastline, where stranding surveys are usually conducted, reporting plastic ingestion in dead or rescued turtles, to specific foraging grounds, where trends in plastic ingestion over a stock can be evaluated, or even larger ecologically relevant scales to determine the impact of plastic ingestion on a population (Duncan et al., 2021; Mascarenhas et al., 2004; Petry et al., 2021; Stahelin et al., 2012).

Since the distribution and concentration of plastics are not uniform in the environment, the study area and its defining biophysical features should be assessed and considered as potential predictors of plastic ingestion. Plastics are often concentrated in oceanic gyres and coastal fronts by the combined actions of winds and currents (see Chapter 1). Land-based sources of wastes, discharging rivers and frontal systems also generate aggregation zones of debris. As a result, the risk of plastic ingestion increases significantly when these areas with high loads of plastic overlap with habitats occupied by marine turtles (González-Carman et al., 2014; Schuyler et al., 2016; see Chapter 3).

5.2.3 Research methods

Research methods are devised to provide appropriate techniques and tools for sample and data collection, and procedures for subsequent analysis and interpretation of results. Standard protocols and reporting metrics enable robust statistical analysis, repeatability, and the comparison of results. Before deciding on the most suitable method for achieving the research goals, exploring and considering the scope, purpose, and applicability of the available techniques is essential.

5.2.3.1 Sample and data collection methods

Diverse sample and data collection methods can be applied in researching plastic ingestion in marine turtles. Initially, standard techniques are used to generate baseline data of the studied animals (e.g., biometry, geolocation, health status or necropsy report) and samples of the specimens (e.g., digestive contents, blood, and body tissues). These methods may vary according to the source of specimens and sampling technique (see sections 5.2.2.1 and 5.2.2.2) but are generally based on common fieldwork protocols, standard veterinary assessments, or necropsy procedures (see methods in Eckert et al., 1999; Matiddi et al., 2019; Rodríguez-Baron et al., 2016; Wyneken et al., 2013). Additional methods may apply depending on the research objectives; for example, studies using satellite telemetry data to analyse overlaps of turtle habitats with aggregation zones of plastic debris (González-Carman et al., 2014) or experimental methods to obtain data about responses of turtles to airborne odorants emanating from biofouled plastic (Pfaller et al., 2020).

A standardised dataset of the ingested plastic is required for subsequent analysis, allowing comparison among studies. Quantification of ingested plastic is commonly reported on the number of plastic pieces. This can be done by counting fragments independently, which is recommended as an objective method; or by grouping fragments from the same ingested item, which may lead to subjectivity. Nevertheless, plastic pieces may vary in dimensions and characteristics, the ingestion of which entails different risks for turtles. Another potential bias of using the number of pieces is that plastics are subject to fragmentation during feeding or the digestive process. Hence some authors

recommend the use the mass of ingested plastic (Camedda et al., 2014; Mattidi et al., 2017; Domènech et al., 2019). However, plastics have different densities, and as a result, this metric may inaccurately reflect the plastic burden if the aim is to assess the impact such those caused by partial or total blockage of the digestive tract. In order to evaluate the impact caused by ingested plastic, in addition to the quantity, the dimensions and density (associated with the type of plastic) should also be considered. An alternative metric, proposed in Chapter 4, to better reflect the plastic burden within an animal's digestive tract would be the volume of ingested plastic. Categorisation of ingested plastic is usually based on its morphology and typology, such as the protocol established by Van Franeker et al. (2011) and adopted by the MSFD Technical Subgroup on Marine Litter (2013). The ingested plastic can also be classified according to its colour (see protocol in Duncan et al., 2019) or flexibility and sharpness characteristics (see protocol proposed in Chapter 3). These protocols are among the multiple options regarding the most common standard methods and reporting metrics for analysing ingested plastic (Table 5.2).

Also essential is determining the range of dimensions of ingested plastic to differentiate associated impacts. Commonly used size boundaries are micro-size (<5 mm in diameter), meso-size (5–25 mm in diameter), and macro-size (>25 mm in diameter) (OSPAR Commission, 2020). If the metric to be use is the volume of ingested plastic, as recommended for assessing impacts, plastics can be classified according to their individual volume as micro-volume (<100 mm^3), meso-volume (100 - 1,000 mm^3), and macro-volume (> 1,000 mm^3) (González-Paredes, 2024).

It is equally important to consider and report cases of individuals exhibiting no plastic ingestion to avoid overestimating ingestion rates (Lynch, 2018; Provencher et al., 2017). Turtles in which ingested plastic was not found after analysis should be recorded as zero-plastic, provided the presence/absence of plastic was adequately investigated.

The standardisation and consistent use of protocols when collecting samples and datasets are required for quality assurance, objectivity, and minimising systematic or random errors. Quality

Assurance (QA) and Quality Control (QC) are techniques usually used to maximise the likelihood of data integrity (Batini et al., 2009; Reynolds et al., 2011). While quality assurance applies in the research design process by deploying specific procedures to avoid jeopardising the data collection methods, quality control refers to protocols focused on ensuring the integrity of collected data (see procedures in Konieczka & Namieśnik, 2018).

Table 5.2. Standard methods and reporting metrics for the classification, characterization, and quantification of ingested plastic.

Analysis	Reporting metric	Objective	References
Categorisation	Plastic category	Classification of ingested plastic based on their typology.	Matiddi et al. (2019); MSFD Technical Subgroup on Marine Litter (2013); Van Franeker et al. (2011)
Dry weight	Grams of ingested plastic	Mass of ingested plastic (total, per plastic category or per piece) by a single turtle or sampled group.	Camedda et al. (2014); Colferai et al. (2017); Pham et al. (2017); Schuyler et al. (2012)
Body burden	Grams of ingested plastic/Kilograms of turtle weight	Relation between mass of ingested plastic and turtle weight.	Clukey et al. (2017); Duncan et al. (2021); Doménech et al. (2019); White et al. (2018)
Quantification	Units of plastic pieces	Number of plastic pieces retrieved (total or per digestive tract section) ingested by a single turtle or a sampled group.	Hoarau et al. (2014); Rice et al. (2021); Wilcox et al. (2018); Yaghmour et al. (2018); Gonzalez-Paredes et al. (*in preparation*).
Occurrence	Frequency of Occurrence (%FO)	Proportion of sampled turtles presenting plastic ingestion or percentage of a plastic category over the entire sample.	Choi et al. (2021) Doménech et al. (2019); Matiddi et al. (2017); Rizzi et al. (2019)
Colour	Colour category	Colour of plastic pieces retrieved based on standard charts, including the visible spectrum, black, white and clear/transparent.	Duncan et al. (2019); Eastman et al. (2020); Fukouka et al. (2016); Santos et al. (2016); Schuyler et al. (2012)
Dimensions	Millimetres (1D); or square millimetres (2D)	Length, width and depth measurement of plastic particles retrieved, or surface area (total, per plastic category or per piece) ingested by a single turtle or a sampled group.	Colferai et al. (2017); Duncan et al. (2021); Jâms et al. (2020); Santos et al. (2016)
Volume	Cubic millimetres (3D)	Volume of plastic ingested (total, per plastic category or per piece) by a single turtle or a sampled group.	Clukey et al. (2017); Doménech et al. (2019); Godoy et al. (2018); Vélez-Rubio et al. (2018a); Gonzalez-Paredes et al. (*in preparation*).
Sharpness and Flexibility	Sharpness Index and flexibility Index, based on a three value-scale for each characteristic	Shape and stiffness of each plastic particle retrieved as index of impact severity	Rizzi et al. (2019); Schuyler et al. (2014b); Yaghmour et al. (2021); Gonzalez-Paredes et al. (*in preparation*).
Buoyancy	Positive (floats at surface), negative (sinks), or neutral (remains floating in the water column).	Buoyancy of plastic particles retrieved as an indicator of where in the water column the plastic was ingested (surface, bottom or in the water column)	Fazey & Ryan (2016); Reisser et al. (2015); Rumbold et al. (2020) Vélez-Rubio et al. (2018a)
Polymer composition	Polymer category	Characterization of the polymer composition of the plastic particles retrieved through FT-IR or Raman spectrophotometry analysis.	Camedda et al. (2022); Digka et al. (2020); Jung et al. (2018); Rice et al. (2021); Rizzi et al. (2019);

5.2.3.2 Data analysis methods

The available analytical methods are diverse, and the approach will depend on the research aim(s). Reporting rates of plastic ingestion from opportunistic or unstructured sampling generally applies a *descriptive approach* to examine the presence/absence, frequency of occurrence and incidence of ingested plastics using a cross-sectional strategy to gather the dataset (data collection in a particular point of time) (Barreiros & Barcelos, 2001; Digka et al., 2020; Stahelin et al., 2012). Researching and monitoring plastic ingestion trends require quantitative analytical methods within an *inferential approach* to deduce patterns over time on a dataset collected systematically in a longitudinal manner (Choi et al., 2021; Domenech et al., 2019; Schuyler et al., 2014a). To assess the impact of plastic ingestion on turtles' health, methods can be either quantitative or qualitative but applied using an *inductive approach* to evaluate and reveal patterns between and among variables and possibly deduce cause-effect relationships (Franzen-Klein et al., 2020; Rice et al., 2021; Wilcox et al., 2018).

Analytical methods must be carefully assessed, and potential biases and constraints must be explicitly identified and considered to determine the most suitable analysis to derive meaningful insights from the generated dataset. Methods should be selected based on data type (quantitative or qualitative), research question(s) and project objective(s). Ultimately, the reliability, validity, and accuracy of the results discerned through the analytical method shall be evaluated for the quality assurance of the research conducted (see methods in Batini et al., 2009).

5.2.4 Resources and capabilities

Assessing the accessibility of resources and capabilities required for the research project, along with considering related constraints and limitations, is crucial during the planning and execution stages of the project.

5.2.4.1 Expertise

Research projects usually comprise different stages, which probably require different expertise. Hence, it is essential to ensure that appropriate expertise is available to complete all project stages, including design, approvals and permits, securing funding, using special equipment, data collection, data analysis and reporting.

Reporting plastic ingestion on opportunistic samples generally requires basic technical expertise. Monitoring ingestion trends over time demands a higher level of knowledge and expertise, as this type of research involves systematic sampling, continuous (re)assessment, and analytical skills to ensure reliable results. Assessing the impacts of plastic ingestion on turtles' health requires an even higher level of expertise, in addition to knowledge of veterinary medicine and pathology (Table 5.3).

5.2.4.2 Timeline

The project timeline should be pre-determined and scheduled according to the planned sequence of actions. It is crucial to consider both the overall time needed for completing the project and the time required for each stage, allowing for delays and contingencies (e.g., bureaucracy and permit approvals, availability of equipment and materials, weather constraints, etc.).

Setting a timeline is particularly crucial for monitoring projects, which require data to be collected for an appropriate period to enable thorough analysis and derive robust results from observed patterns and trends (Table 5.3).

5.2.4.3 Project Budget

Financial planning is central to ensure that all research expenses and associated costs can be covered across all project stages, ideally for the entire project duration. Consideration should be given to the costs of human resources, field trips, materials and special equipment, advanced techniques, and infrastructure. In general, research projects involving longer temporal scales and established expertise require larger budgets (Table 5.3).

Table 5.3. Resources and capabilities required to achieve research objectives when researching plastic ingestion in marine turtles.

Research objective	**Expertise and research capability**				**Temporal scale**	**Budget**
	[Logistics]	**[Study design]**	**[Data Collection]**	**[Data Analysis]**		
Descriptive reporting of plastic ingestion	Low	Low	Medium	Medium	Variable	Low
Monitoring incidence of plastic ingestion	High	Medium	Medium	Medium	Variable	High
Assessing health impacts of plastic ingestion	Medium	High	High	High	Variable	Medium/ High

TABLE KEY

Expertise

Low	Achievable with minimal training
Medium	Require specific training in the targeted field and/or in sample collection.
High	Requires higher level training and could require input from external experts

Temporal scale

Small	Month/s
Medium	Months to Years
Large	Years
Variable	Applicable to small, medium and large spatial scales

Budget (USD $)

Low	<$1,000
Medium	$1,000 - $10,000
High	>$10,000

5.3 CONCLUSIONS

Plastic ingestion is an ever-growing threat affecting all seven species of marine turtles. Understanding the full extent of this threat requires increasing research and monitoring efforts, as well as collaborative efforts among scientists, agencies, and stakeholders. Bringing together diverse disciplines such as biology, ecology, veterinary pathology, toxicology, and oceanography will allow a more comprehensive understanding of the threat process of plastic ingestion and its impacts. Establishing a global common framework, outlining standard methods and research strategies that underpin best practices, will contribute towards a comprehensive assessment of plastic ingestion hazards for marine turtle populations. Furthermore, open access data repositories will contribute significantly towards a broad and global evaluation of the plastic ingestion threat for marine species.

General discussion

Chapter objective

To summarize the key findings of previous chapters in the context of the thesis objectives and discuss the vulnerability of juvenile green turtles to plastic ingestion in Uruguay, along with its implications for the conservation status of green turtles in the South Atlantic Ocean. In addition, it provides recommendations for future research and conservation plans.

Methodology

The results of the previous chapters have been synthesized and integrated, highlighting my original contribution to our understanding of the impact of plastic ingestion on the stock of juvenile green turtles in Uruguayan waters. The conservation implications of these findings for the green turtle subpopulation in the South Atlantic - Regional Management Unit (RMU) are discussed. In this regard, several approaches are proposed for a more comprehensive examination of this concern, aiming to inform future listing assessments of this subpopulation alongside recommendations to address the marine plastic pollution issue. Additionally, recommendations are provided for ongoing research in Uruguay and further investigations in this field.

Key findings:

- Uruguayan waters are a hotspot of plastic ingestion for juvenile green turtles, affecting 76% of the stock population present in the area.
- The future viability of the green turtle subpopulation in the South Atlantic - Regional Management Unit (RMU) may be compromised because plastic ingestion considerably affects the early juvenile stages of the species in the region. This concern needs a comprehensive examination to inform future listing assessments of this subpopulation.

Conclusions

Assessments of the conservation status within the different regional management units for marine turtles (RMUs) should incorporate impact evaluations for specific threats affecting these subpopulations to provide a more realistic understanding of their conservation status.
Urgent measures should be implemented to reduce the input of plastic waste into the marine environment. This includes plans to remove existing plastic debris, mitigation strategies aimed at decreasing plastic emissions, and raising awareness among the population to empower them to turn the tide of plastic pollution.

Related publication

González-Paredes, D., Fallabrino, A., Godley B. J., Marsh, H., & Hamann, M. (*in prep.*)
Impacts of plastic ingestion on green turtles (Chelonia mydas) in Uruguayan waters
Report to be submitted to the IUCN-SSC Marine Turtle Specialist Group.

6.1 PhD thesis summary and results

Researchers are becoming genuinely concerned about the impacts of plastic pollution on marine turtles (Hamann et al., 2010; Kühn & Van Franeker, 2020; Schuyler et al., 2014a; Vegter et al., 2014). While efforts to understand these impacts are primarily focused on individual and small scales, there is a growing number of research and conservation organisations indicating that this threat is likely to affect entire cohorts or populations of marine turtles (Fuentes et al., 2023; Nelms et al., 2016; Senko et al., 2020; Vegter et al., 2014). However, evaluating these impacts at the population scale is challenging due to marine turtles' complex life history and extensive distribution (Duncan et al., 2021; Santos et al., 2015; Schuyler et al., 2014a; Senko et al., 2020). Over their long life spans, marine turtles exhibit highly migratory behaviour, occupying distinct habitats during different life stages (Bolten, 2003; Godley et al., 2010; Mansfield et al., 2017; Meylan et al., 2011). Consequently, the exposure to plastic pollution and its potential impacts varies throughout their lifecycle and across different habitats. (Lynch, 2018; Schuyler et al., 2014a). Furthermore, one of the challenges in this research field involves assessing plastic ingestion in early life stages, which are considered the most affected but also the hardest to survey technically and logistically in open waters (Duncan et al., 2021; Lynch, 2018; Santos et al., 2021; Schuyler et al., 2014a).

In this context, my PhD research aimed to assess plastic ingestion in the stock of juvenile green turtles present in Uruguayan waters, evaluating the severity of the impacts in relation to the volume and characteristics of the plastic ingested. As outlined in Chapter 1, Uruguayan waters are considered a key feeding and developing area for green turtles in the Southwestern Atlantic Ocean (López-Mendilaharsu et al., 2006, 2016; Vélez-Rubio et al., 2013, 2016, 2018b). These waters host a mixed stock of juveniles of the species originating from the main rookeries in the region (Caraccio, 2008; Proietti et al., 2012; Prosdocimi et al., 2012). The green turtle exhibits a markedly seasonal occurrence in Uruguay driven by changes in sea surface water temperatures throughout the year (González-Carman et al., 2012; Vélez-Rubio et al., 2018b). The highest aggregations occurs during

the austral summer when the juvenile green turtles reach the coastal waters of Uruguay to feed on macroalgae and gelatinous macrozooplankton (Vélez-Rubio et al., 2016, 2018b).

My research provides evidence that Uruguayan waters are a hotspot of plastic ingestion for the green sea turtle in the Southwestern Atlantic Ocean, registering the higher quantities of plastic ingested by the species in the region (see Chapter 3). This situation is primarily attributed to (i) oceanographic features in the coastal waters of Uruguay and (ii) the transient feeding behaviour of the green turtles in these waters:

i] Oceanographic features: Uruguayan waters exhibit high levels of plastic pollution. This considerable accumulation of plastic mainly results from the subtropical convergence of the Brazil current and the Malvinas current, transporting debris from other latitudes and aggregating them in Uruguayan waters (Franco-Fraguas et al., 2014; Ortega & Martínez 2007; Manta et al., 2020; Piola et al., 2008; Simionato et al., 2006) (see Chapter 1). This situation is enhanced by a turbidity and salinity front within the Rio de la Plata estuary, acting as a barrier and dragging wastes from tributaries and densely populated areas towards the oceanic zone (Acha et al., 2003; González-Carman et al., 2014; Lebreton et al., 2017; Lozoya et al., 2015, 2016; Rodríguez et al., 2020) (see Chapter 1).

Feeding behaviour: the early juveniles of green turtles (CCL <40 cm), recently recruited to neritic habitats in Uruguay, exhibit a relict opportunistic feeding behaviour related to their previous oceanic stage (Vélez-Rubio et al., 2016). The low discrimination in the selectivity of dietary items makes these smaller turtles potentially more exposed to ingesting a wider range of plastics and higher quantities (see Chapters 3 and 4). These turtles showed a higher consumption of laminar soft plastics, the ingestion of which represents a potentially high risk. Due to the pliability and malleable characteristics of these plastics, they are likely to act as a mesh within the intestines, entangling other ingested plastics and digestive contents, thereby extending retention times in the gut. This contributes to the compaction of fecalomas, which can obstruct the digestive tract and, eventually, lead to death, as observed in severely impacted turtles. (see Chapter 4).

The specific results of my PhD research in relation to the settled objectives were:

Objective 1: Generate baseline data on gastrointestinal transit times in juvenile green turtles in Uruguayan waters to determine the monitoring period required for detecting ingested plastic in the faecal matter of live animals.

To obtain more representative samples, I decided to include live animals in the assessment of plastic ingestion on the stock of juvenile green turtles in Uruguayan waters. Faecal matter examination could represent a reliable approach for assessing plastic ingestion in live turtles, provided adequate monitoring time is allocated to collect all ingested plastic. In Chapter 2, I conducted an experiment using inert plastic markers to estimate the gastrointestinal transit times of juvenile green turtles in Uruguay. Coinciding with the upper limit of the gastrointestinal transit time of these turtles, I established a minimum period of 22 days for monitoring the faecal matter of live turtles in my study, maximising the likelihood of collecting all plastic pre-ingested in the environment. I consider this baseline data on gastrointestinal transit times to have the potential to contribute to assessing digestive motility disorders and toxicology studies related to exposure to toxic substances leached from ingested debris.

Objective 2: Analyse the incidence and temporal trends of plastic ingestion on green turtles in Uruguayan waters and quantify and characterise the ingested plastic to assess ingestion patterns.

In Chapter 3, I conducted a comprehensive analysis of plastic ingestion in the stock of green turtles present in Uruguayan waters using different approaches, which include multiple sources of specimens: stranded and bycaught turtles (dead animals), and rescued and wild-captured turtles (live animals); as well as three distinct sampling techniques: necropsy, faeces monitoring and gastric lavage.

The results of Chapter 3 indicate a high incidence of plastic ingestion among green turtles in Uruguayan waters during the period 2014 – 2020, affecting over 70% of turtles examined from each of the four specimen sources examined. Furthermore, the quantities of ingested plastic recorded were higher than those reported for green turtles across the Southwestern Atlantic region. No

annual trends were detected in the incidence of plastic ingestion, or the quantities of plastic ingested in any of the specimen sources assessed.

The examined turtles predominantly consumed laminar soft plastics (SHE) in white and clear/transparent colours, in terms of number of pieces. This may suggest a selective behaviour towards soft plastics, possibly due to their resemblance to macroalgae and gelatinous macrozooplankton, which are the main food sources for green turtles in Uruguayan waters (Vélez-Rubio et al., 2016). An assessment of the availability of different plastics in the environment would be required to completely validate selective behaviour.

Using multiple specimen sources and sampling techniques allowed me to assess the strengths and limitations of these approaches. I conclude that bycaught and wild-captured turtles are more reliable indicators of the overall population baseline exposure to plastic ingestion, if sampling is conducted systematically. In contrast, stranded dead and rescued turtles may lead to biases in the analysis of plastic ingestion due to potential pre-existing health issues influencing their feeding behaviour and/or habitat use. These animals, however, can provide valuable insights into the severity of the impact caused by plastic ingestion, as outlined in Chapter 4.

Among the sampling techniques, necropsy is the most reliable method for assessing plastic ingestion, enabling all the digestive contents to be retrieved for inspection of plastic and differences in their concentration along the digested tract to be investigated. Faecal monitoring allows live animals to be sampled, provided the monitoring periods are sufficiently long to collect all the ingested plastic. Gastric lavage proved to be a non-efficient method for assessing plastic ingestion in marine turtles.

Objective 3: Assess the severity of the impacts caused by plastic ingestion on green turtles in Uruguayan waters in relation to the volumes and characteristics of ingested plastics.

In Chapter 4, I assessed the impact severity of plastic ingestion by analysing the cumulative volumes and characteristics of plastics ingested in a subset of animals (N = 150) from the stock of juvenile

green turtles examined in Chapter 3. I detected a positive relationship between ingested volume and severity of the impact, the more plastic ingested, the more severe the impact. Severely impacted turtles (n = 10) exhibited intestinal obstruction by fecalomas, which led to eventual death. In addition, plastic ingestion caused emaciation and chronic debilitation syndrome in another 14 turtles. The accumulation of plastic followed an increasing pattern along the digestive tract (oesophagus < stomach < intestines). Meso-particles (plastic pieces with a volume between 100 and 1,000 mm^3) were the most frequently ingested plastic, while laminar soft plastics (SHE) were the predominant plastic-type consumed, reinforcing the findings presented in Chapter 3. There was a positive relationship between the ingested volume of laminar soft plastics and the severity of the impact, which suggests these plastics might influence the impact severity to a larger extent than other plastic types. Laminar soft plastics (SHE) represent a potentially high risk for turtles due to their pliability. Large and malleable ingested plastics can act as a mesh, entangling other plastic items along with part of the solid fraction of the digestive contents. This process facilitates the compaction of fecalomas, eventually leading to intestinal obstructions, as observed in severely impacted turtles.

I also observed that early juveniles with a curved carapace length (CCL) below 40 cm are more susceptible to the impacts of plastic ingestion. These smaller turtles exhibit a relict opportunistic feeding behaviour related to their previous oceanic stage (Vélez-Rubio et al., 2016), potentially exposing them to ingesting a more comprehensive range of plastics and higher volumes. This could represent an evolutionary trap for the species, as Duncan et al. (2021) and Santos et al. (2021) theorised about specific ecological behaviours leading early life stages to increased exposure to the impacts caused by plastic ingestion.

Objective 4: Develop a best practice framework of strategies and standard methods suitable for fulfilling research objectives, considering the accessibility of resources and capabilities required.

In Chapter 5, I developed a best practice framework for designing and implementing research to assess plastic ingestion in marine turtles to assist researchers, practitioners and stakeholders. This framework includes key aspects such as (i) defining aims and realistic objectives, (ii) selecting specimen sources, sampling techniques, sample sizes, duration of the study period and extent of the study area appropriate to the capacity of the research, (iii) outlining the research methods most appropriate for each situation and project, and (iv) evaluating the availability of resources such as the budget and timeline in the context of the expertise necessary for each stage of the project.

Objective 5: Inform the vulnerability of green turtles to plastic pollution and the implications for its conservation status at the South Atlantic Regional Management Unit (RMU).

In the present chapter, I highlight Uruguayan waters as a significant hotspot of plastic ingestion for juvenile green turtles in Uruguay (see section 6.1) and discuss the implications of my thesis results for the conservation of the species in the South Atlantic Ocean - Regional Management Unit (RMU) (Wallace et al., 2023) (see section 6.2). Additionally, I suggest different approaches for a more comprehensive examination of this concern, aiming to inform future listing assessments of this subpopulation, alongside recommendations to address the marine plastic pollution issue (see sections 6.2 and 6.3).

6.2 Conservation implications

In 2019, the conservation status of the green sea turtle subpopulation in the South Atlantic Ocean was down-listed from 'Endangered' to 'Least Concern' and conservation-dependent on the IUCN Red List (Broderick & Patricio 2019). This assessment was based on long-term nesting data sets (≥10 years) using annual clutch counts at nesting sites as the index of population abundance to analyse trends in population size (criterion A2 of the IUCN Red List). The assessment revealed a significant overall increase of 188% relative to the estimated population size three generations ago (see procedures for estimating the historical population size in Broderick and Patricio, 2019).

Nevertheless, one of the recognised challenges of the IUCN Red List assessment of marine turtles is that the metrics used to assess changes in population size are based on fluctuations in the abundance of adult females and their nesting activity. Relying solely on these two parameters as indicators of population trends can mask or underestimate other potential threats affecting the male subset or early life stages, in the latter case, the overall impacts of which cannot be ascertained until turtles reach sexual maturity.

Considering that female green turtles reach sexual maturity at >25 years of age (Almeida et al., 2011; Colman et al., 2015), the assessment conducted by Broderick and Patricio (2019) was based on nesting females from cohorts hatched in the South Atlantic Ocean before 1994. These cohorts experienced lower levels of plastic pollution during their juvenile stage compared to the current levels. Consequently, this assessment may not accurately reflect the impact of plastic pollution on the earliest life stages of this green turtle subpopulation at present (Fig. 6.1).

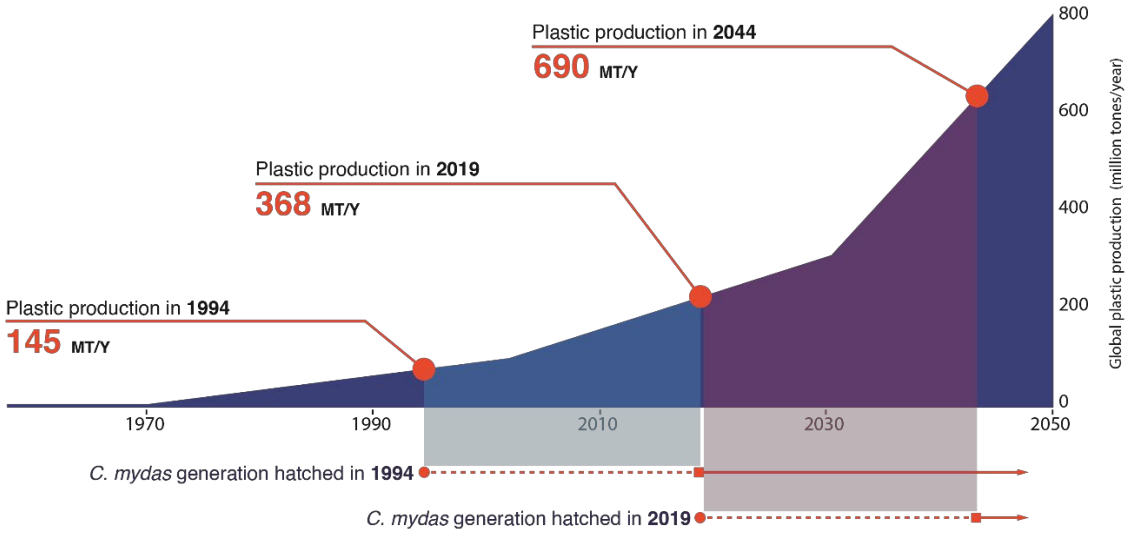

Figure 6.1. Global plastic production and potential exposure to plastic pollution to which different generations of green turtles are exposed. The light-blue area refers to the exposure of juvenile green turtles (0 – 25 years old) of the generation hatched in the year ≤1994, whose conservation status was assessed by Broderick & Patricio (2019). The red-garnet area refers to the exposure of juvenile green turtles (0 – 25 years old) of the current generation hatched in year ≥2019. Data collected from PlasticEurope (2022).

Since plastic ingestion is mainly affecting the juveniles in the Southwestern Atlantic region (incidence ranging from 70% to 92.7%, see Table 3.2) and considering the current levels and future projections of plastic pollution (Fig. 6.1), the future viability of the green turtle subpopulation in the South Atlantic - Regional Management Unit (RMU) may be compromised (Wallace et al., 2023).

Research efforts must be extended across the entire regional unit to gain a more comprehensive understanding of the processes and full extent of the impacts caused by plastic ingestion in this subpopulation. This baseline data could then serve as a basis for re-assessing the conservation status of the green turtle subpopulation in the South Atlantic Ocean. Such re-assessment could be based on criterion A3 of the IUCN Red List (Population reduction projected, inferred or suspected to be met in the future, up to a maximum of 100 years in future), using an index of abundance appropriate to the taxon that considers threats across all life-cycle stages, among other demographic parameters (criterion A3b) (Fig. 6.2); or demonstrating that plastic pollution is causing a decline in the habitat quality (criterion A3c) (Fig. 6.2). Alternatively, it could be based on the predictions of plastic pollution impacts on this subpopulation in relation to current/future pollution levels, following criterion A4 of the IUCN Red List (An observed, estimated, inferred, projected or suspected population reduction where the time period must include both the past and the future, up to a maximum of 100 years in future, and where the causes of reduction may not have ceased or may not be understood or may not be reversible) (Fig. 6.2).

Another less explored option for assessing the conservation status of marine turtle populations is to employ Population Viability Analysis (PVA) (Boyce, 1992). Under Criterion E of the IUCN Red List (Quantitative analysis indicating the probability of future extinction in the wild) (Fig. 6.2), a PVA could help us understand and assess the long-term effects of the plastic ingestion threat on the subpopulation by estimating the survival probability of green turtles of different life stages under different scenarios of exposure to plastic pollution. As a stochastic population model, PVA would consider factors such as plastic ingestion rates and associated mortality alongside fundamental reproductive and demographic parameters (e.g., age at maturity, generation length, survivorship

across life stages, adult and hatchling sex ratios, population size, migratory patterns, population threats and stressors, among others) to determine the potential impacts of plastic ingestion at a population level.

A. Population size reduction. Population reduction (measured over the longer of 10 years or 3 generations) based on any of A1 to A4

	Critically Endangered	Endangered	Vulnerable
A1	≥ 90%	≥ 70%	≥ 50%
A2, A3 & A4	≥ 80%	≥ 50%	≥ 30%

A1 Population reduction observed, estimated, inferred, or suspected in the past where the causes of the reduction are clearly reversible AND understood AND have ceased.

A2 Population reduction observed, estimated, inferred, or suspected in the past where the causes of reduction may not have ceased OR may not be understood OR may not be reversible.

A3 Population reduction projected, inferred or suspected to be met in the future (up to a maximum of 100 years) *[(a) cannot be used for A3]*.

A4 An observed, estimated, inferred, projected or suspected population reduction where the time period must include both the past and the future (up to a max. of 100 years in future), and where the causes of reduction may not have ceased OR may not be understood OR may not be reversible.

based on any of the following:

(a) direct observation *[except A3]*
(b) an index of abundance appropriate to the taxon
(c) a decline in area of occupancy (AOO), extent of occurrence (EOO) and/or habitat quality
(d) actual or potential levels of exploitation
(e) effects of introduced taxa, hybridization, pathogens, pollutants, competitors or parasites.

B. Geographic range in the form of either B1 (extent of occurrence) AND/OR B2 (area of occupancy)

	Critically Endangered	Endangered	Vulnerable
B1. Extent of occurrence (EOO)	< 100 km²	< 5,000 km²	< 20,000 km²
B2. Area of occupancy (AOO)	< 10 km²	< 500 km²	< 2,000 km²
AND at least 2 of the following 3 conditions:			
(a) Severely fragmented **OR** Number of locations	= 1	≤ 5	≤ 10
(b) Continuing decline observed, estimated, inferred or projected in any of: **(i)** extent of occurrence; **(ii)** area of occupancy; **(iii)** area, extent and/or quality of habitat; **(iv)** number of locations or subpopulations; **(v)** number of mature individuals			
(c) Extreme fluctuations in any of: **(i)** extent of occurrence; **(ii)** area of occupancy; **(iii)** number of locations or subpopulations; **(iv)** number of mature individuals			

C. Small population size and decline

	Critically Endangered	Endangered	Vulnerable
Number of mature individuals	< 250	< 2,500	< 10,000
AND at least one of C1 or C2			
C1. An observed, estimated or projected continuing decline of at least (up to a max. of 100 years in future):	25% in 3 years or 1 generation (whichever is longer)	20% in 5 years or 2 generations (whichever is longer)	10% in 10 years or 3 generations (whichever is longer)
C2. An observed, estimated, projected or inferred continuing decline AND at least 1 of the following 3 conditions:			
(a) (i) Number of mature individuals in each subpopulation	≤ 50	≤ 250	≤ 1,000
(ii) % of mature individuals in one subpopulation =	90–100%	95–100%	100%
(b) Extreme fluctuations in the number of mature individuals			

D. Very small or restricted population

	Critically Endangered	Endangered	Vulnerable
D. Number of mature individuals	< 50	< 250	**D1.** < 1,000
D2. *Only applies to the VU category* Restricted area of occupancy or number of locations with a plausible future threat that could drive the taxon to CR or EX in a very short time.	-	-	**D2.** typically: AOO < 20 km² or number of locations ≤ 5

E. Quantitative Analysis

	Critically Endangered	Endangered	Vulnerable
Indicating the probability of extinction in the wild to be:	≥ 50% in 10 years or 3 generations, whichever is longer (100 years max.)	≥ 20% in 20 years or 5 generations, whichever is longer (100 years max.)	≥ 10% in 100 years

Figure 6.2. Summary of the five criteria (A-E) used to evaluate if a taxon belongs in an IUCN Red List Threatened category (Critically Endangered, Endangered or Vulnerable) (Adapted from IUCN Red List, 2023).

6.3 Recommendations and further research

6.3.1 Recommendations for future research on plastic ingestion by green turtles in Uruguay

As my PhD research highlights, plastic pollution poses a significant threat to green turtles in Uruguayan waters. Consequently, ongoing research and conservation efforts are imperative to preserve the species in this region. Below, I propose further investigations and provide a set of recommendations in this regard:

A Population Viability Analysis (PVA) would be crucial for assessing the impact of plastic ingestion on this stock in Uruguay and its conservation implications for the green turtle subpopulation in the South Atlantic - Regional Management Unit (RMU), as outlined above.

The spatio-temporal occurrence of plastic debris aggregation in Uruguayan waters would be also an informative line of inquiry. These waters are highly dynamic due to the influence of the subtropical convergence, where the seasonal shifts in the prevalence of the Brazil Current and the Malvinas Current (see Chapter 1) likely drive seasonal changes in the aggregation of plastic debris offshore Uruguay. These shifts in oceanic currents and the associated changes in sea surface temperature also drive the seasonal occurrence of green turtles in Uruguay, with the highest concentrations during the austral summer (see Chapter 1). By examining the spatio-temporal dynamics of plastic debris aggregations throughout the year and comparing them to the seasonal migratory patterns of green turtles in these latitudes, it would be possible to determine the specific periods and locations where the exposure to plastic ingestion is higher. This information would enable a risk assessment of plastic ingestion by green turtles according to specific levels of plastic pollution in Uruguayan waters.

In addition, the Rio de la Plata and adjacent waters are known as one of the most polluted estuarine systems in the world (Lebreton et al., 2017). Additional research should be addressed to assess the origin of plastic waste and primary sources of pollution in the area. Various methodologies can be applied, such as modelling drift trajectories using ocean current simulationsto trace the movement of debris (Galaiduk et al., 2020; Pilechi et al., 2022), deploying tagged materials in rivers or drainage

systems to follow their path into marine environments (CARTHE, 2016; Duncan et al., 2020), and analysing the polymers within the plastic through techniques like FTIR or Raman spectroscopy to link them to specific industrial or regional uses (Fahrenfeld et al., 2019; Helm, 2017). Understanding these sources is crucial for developing effective mitigation strategies aimed to reducing plastic waste emissions into these waters.

Equally important is raising awareness among the population in Uruguay about the environmental impacts of plastic pollution, as well as promoting responsible plastic use and the proper disposal of plastic waste to empower citizens to turn the tide of plastic pollution and preserve marine ecosystems (González-Paredes & Estrades, 2021a).

6.3.2 Further investigation on plastic ingestion in marine turtles

In recent years, there has been a notable increase in research efforts focused on plastic ingestion by marine turtles. Despite this, there are still multiple knowledge gaps that need to be addressed:

Assessing species: Studies have been disproportionately focused on loggerhead and green turtles (Nelms et al. (2016) and Lynch (2018) and references therein). Since the impacts of plastic ingestion are likely related to specific feeding behaviours of species (Duncan et al., 2019a; Schuyler et al., 2014a), research on other species should be increased to assess their interspecific variability in vulnerability. To gain a more comprehensive and accurate understanding of vulnerability at the population scale, assessments of plastic ingestion should encompass all seven marine turtle species throughout their life stages and extend across different marine turtle regional management units (RMUs) (Wallace et al., 2023).

Assessing life stages: Age and size classes represent another significant predictor of plastic ingestion (Nelms et al., 2016; Lynch, 2018; Schuyler et al., 2014a). Therefore, the vulnerability of turtles across all life stages and habitats occupied throughout their lifespan needs to be determined, with a particular focus on early life stage turtles as they are considered particularly vulnerable to the

impacts of plastic ingestion (Eastman et al., 2020; Pham et al., 2017; Rice et al., 2021; Ryan et al., 2016; Wildermann et al., 2018; see Chapters 3 and 4).

Evaluating geographic areas: Marine turtles are globally distributed in tropical and temperate waters. However, most of the studies on plastic ingestion have occurred in the North Atlantic and the Mediterranean (Nelms et al. (2016) and Lynch (2018) and references therein). Expanding studies to under-researched geographical areas, including those within the Southeastern Atlantic, Pacific, and Indian Ocean, will provide a broader perspective on the plastic pollution threat across different regional management units for marine turtles (RMUs) (Wallace et al., 2023). This is particularly important in highly polluted and/or under-researched geographical areas, aiming to identify plastic ingestion hotspots and population groups at risk (see Chapter 3).

Sub-lethal adverse effects: While we have extensive knowledge of the physical impacts caused by plastic ingestion in marine turtles, our understanding of the sub-lethal effects associated with plastic ingestion remains unclear. It has been observed that ingested plastic displacing dietary items reduces stomach capacity and turtles' feeding stimulus, which can lead to dietary dilution and malnutrition (McCauley & Bjorndal, 1999; Santos et al., 2020; Tourinho et al., 2010). Another related sub-lethal effect results from the chemical leaching of ingested plastics. This is a particular concern in marine turtles due to their long gastrointestinal transit times (González-Paredes et al., 2021b; see Chapter 2), making them highly susceptible to chronic exposure to toxic substances leached from ingested plastic. It has been speculated that such additives or derivatives of plastics can lead to malfunctions in metabolic and endocrine systems and disorders in somatic growth rates and reproductive outputs (Andersson et al., 2016; Clukey et al., 2018; Marn et al., 2020; Nelms et al., 2016; Ryan et al., 2016). Further research should address the impacts of plasticisers on the turtles' health to extend our understanding of these sub-lethal effects of plastic ingestion.

Standardisation of methods: The threat of plastic pollution to marine turtles is being addressed from various perspectives, involving scientists and conservationists in research institutions or NGOs, as

well as the general public and stakeholders through citizen science initiatives (Borrelle et al., 2017; Nelms et al., 2016). However, there is limited consensus regarding adopting consistent procedures and protocols, leading to disparities in reporting. A consensus in the use of methods, along with the sharing of data in open-access repositories, will facilitate the comparison of results across studies, contributing significantly to a comprehensive assessment of this threat to marine turtles. The best practice framework, developed in Chapter 5, provides a series of standardised and established methodologies for assessing plastic ingestion in marine turtles.

Systematic studies: Plastic pollution represents an escalating threat to marine turtles. However, most assessments of this threat are based on a relatively small number of turtles obtained opportunistically or as a by-product of studies where evaluating plastic ingestion was not a primary research goal (Casale et al., 2016; Fuentes et al., 2023; Senko et al., 2020). Shifting towards well-structured and systematic studies, including representative samples of dead and live animals (see Chapters 3 and 4), would generate statistically robust sample sizes to enable broader impact assessments over time.

Population-level assessments: While our understanding of the individual-level consequences of plastic ingestion is extensive, significant gaps persist in evaluating the broader population-scale impacts (Fuentes et al., 2023; Nelms et al., 2016; Senko et al., 2020). Assessing the effects of plastic ingestion on marine turtle populations is particularly challenging due to their complex life history and extensive distributions. Promoting collaborative research efforts is crucial for comprehensively assessing these impacts across populations and identifying hotspots for marine turtles within the regional management units (RMUs) (Wallace et al., 2023). Furthermore, broader assessments of plastic ingestion would contribute to more accurate and realistic evaluations of population conservation status (see section 6.2).

Multiple stressors: Marine turtles confront various threats across their life stages, including plastic pollution, climate change, habitat destruction, and fisheries bycatch among others. The cumulative

impacts of these threats are often intensified by synergistic interactions between them (Fuentes et al. 2023). However, current assessments focus on isolated stressors, constraining broader analysis of spatial and interconnected effects. Research efforts should prioritize assessing the cumulative and synergistic interactions of the multiple threats, including plastic pollution, affecting marine turtle populations.

To conclude, implementing these recommendations would contribute to advancing our understanding of the diverse and complex impacts of plastic ingestion on marine turtles and play a crucial role in developing and implementing effective conservation strategies. By conducting further research and increasing monitoring efforts, we will improve our knowledge of this threat, which is essential for informing targeted conservation actions and management measures to mitigate the pervasive impact of plastic pollution. Furthermore, by integrating these findings into broader conservation frameworks and collaborating with stakeholders, policymakers, and the general public, we can promote greater awareness, engagement, and collective responsibility in reducing plastic pollution and safeguarding the long-term survival of marine turtle populations.

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SUPPLEMENTARY MATERIAL

Chapter 3 Appendixes

Appendix 3A

Classification of ingested plastic based on its morphology (modified from Van Franeker et al. (2011) and the MSFD Technical Subgroup on Marine Litter (2013)).

Plastic category	Type	Code	Description
Industrial plastics (IND)	Pellets	IND	Industrial plastic pellets. Small, cylindrically shaped granules of ± 4 mm diameter.
Domestic use plastics (USE)	Sheet like	SHE	Laminar soft items like plastic bags, foils, etc., usually broken up in smaller pieces.
	Thread like	THR	Plastic threads or fibres, like pieces of rope, nets, nylon monofilaments, packaging straps, fishing lines, etc.
	Foam like	FOAM	Pieces of foamed polystyrene cups or packaging, foamed polyurethane in mattresses, or construction foams.
	Fragments	FRAG	Hard plastic, pieces of bottles, boxes, toys, tools, equipment housing, toothbrushes, lighters, etc.
	Others	POTH	Cigarette filters, rubber, elastics, balloons, etc., i.e. items that are 'plastic-like' or do not fit into a clear category.

Appendix 3B

Formula modified from Provencher et al. (2015) and Lavers et al. (2021) for assessing plastic ingestion in marine turtles. n is the sample size required, z is the t value, α is the Type I error (false positive) rate, π is the Type II error (false negative) rate, μ_I is the difference to detect (where μ_I = 105 indicates a 5% difference, 110 is a 10% difference, etc.), and CV is the coefficient of variation (SD/mean * 100) in the time series of %FO and quantities of plastic ingested. We set α = 0.05 and π = 0.90, meaning that $z_{\alpha/2} - z_{\pi}$ = 3.242 (van Franeker and Meijboom 2002).

$$n = 2 \times \left\{ \frac{\left[z_{\alpha/2} - z_{\pi} \right] \times \frac{CV}{100} \times \mu_I}{\mu_I - 100} \right\}^2$$

Appendix 3C

Logistic regressions (GLMs) to examine trends in the annual incidence of plastic ingestion across the sources of specimens.

A] Stranded Turtles

```
incidence_stranded <- glm(annual_incidence ~ year, data = stranded, family =
binomial(link = logit))

Deviance Residuals:
       1         2         3         4         5         6
-0.27742  -0.02100   0.42295  -0.05685   0.34131  -0.36364

Coefficients:
             Estimate Std. Error z value Pr(>|z|)
(Intercept)  436.3023  1063.3020    0.41    0.682
year          -0.2160     0.5272   -0.41    0.682

(Dispersion parameter for binomial family taken to be 1)

    Null deviance: 0.68128  on 5  degrees of freedom
Residual deviance: 0.50825  on 4  degrees of freedom
AIC: 9.21

Number of Fisher Scoring iterations: 4
```

B] Bycaught Turtles

```
incidence_bycaught <- glm(annual_incidence ~ year, data = bycaught, family =
binomial(link = logit))

Deviance Residuals:
       1         2         3         4         5
 0.17069  -0.34612   0.33496   0.15240   0.06873

Coefficients:
             Estimate Std. Error z value Pr(>|z|)
(Intercept) -3217.353   3635.599  -0.885    0.376
year            1.597      1.805   0.885    0.376

(Dispersion parameter for binomial family taken to be 1)

    Null deviance: 1.92784  on 4  degrees of freedom
Residual deviance: 0.28908  on 3  degrees of freedom
AIC: 5.715

Number of Fisher Scoring iterations: 7
```

C] Captured Turtles

```
incidence_captured <- glm(annual_incidence ~ year, data = captured, family =
binomial(link = logit))
```

```
Deviance Residuals:
       1         2         3         4         5         6
-0.60174   0.57181   0.57107   0.28603   0.01941  -0.38149

Coefficients:
             Estimate Std. Error z value Pr(>|z|)
(Intercept)   -3.95761 1349.57981  -0.003    0.998
year           0.00282    0.66894   0.004    0.997

(Dispersion parameter for binomial family taken to be 1)

    Null deviance: 1.2429  on 5  degrees of freedom
Residual deviance: 1.2429  on 4  degrees of freedom
AIC: 5.9542

Number of Fisher Scoring iterations: 4
```

D] Rescued Turtles

```
incidence_rescued <- glm(annual_incidence ~ year, data = rescued, family =
binomial(link = logit))
```

```
Deviance Residuals:
      1        2        3
 0.4559  -0.4663   0.2241

Coefficients:
             Estimate Std. Error z value Pr(>|z|)
(Intercept) 1372.7139  3917.0074    0.35    0.726
year          -0.6791     1.9398   -0.35    0.726

(Dispersion parameter for binomial family taken to be 1)

    Null deviance: 0.60785  on 2  degrees of freedom
Residual deviance: 0.47549  on 1  degrees of freedom
AIC: 5.3085

Number of Fisher Scoring iterations: 5
```

Appendix 3D

Summary of the analysis on characteristics of plastic ingested by necropsied turtles.

	Stranded turtles			Bycaught turtles		
Incidence of plastic ingestion		**Turtles**	**%FO**		**Turtles**	**%FO**
		89/124	71.8%		14/18	77.8%
	Mean ± SD	**Range**	**Total / %FO**	**Mean ± SD**	**Range**	**Total / %FO**
Ingested plastic [a,c]	260.4 ± 343.7	0 - 1580	18,464 / 100%	42.6 ± 67.8	0 - 165	270 / 100%
Ingested plastic distribution [a,c]						
Oesophagus	6.6 ± 20.7	0 - 155	472 / 2.6%	0.3 ± 0.7	0 - 2	3 / 1.1%
Stomach	114.4 ± 183.2	0 - 911	8124 / 44.0%	5.6 ± 11.8	0 - 38	56 / 20.7%
Intestines	139.0 ± 229.7	0 - 1244	9868 / 53.4%	21.1 ± 48.1	0 - 154	211 / 78.1%
Ingested plastic categories [a,c]						
IND	0.6 ± 1.4	0 - 7	41 / 0.2%	0.0 ± 0.0	0 - 0	0 / 0.0%
SHE	104.5 ± 137.8	0 - 592	7418 / 40.2%	14.6 ± 31.0	0 - 90	146 / 54.1%
THR	79.8 ± 105.6	0 – 926	5668 / 30.7%	8.0 ± 15.6	0 - 49	80 / 29.6%
FRAG	63.1 ± 110.7	0 – 628	4480 / 24.3%	3.4 ± 6.9	0 - 18	34 / 12.6%
FOAM	8.0 ± 16.3	0 – 95	569 / 3.1%	0.2 ± 0.4	0 - 1	2 / 0.7%
POTH	4.1 ± 7.3	0 – 36	288 / 1.6%	0.8 ± 2.2	0 - 7	8 / 3.0%
Ingested plastic colour [b,c]						
Clear/Transp.	69.4 ± 72.2	0 – 255	1803 / 21.1%	8.9 ± 16.1	0 - 40	89 / 33.0%
White	120.1 ± 118.6	0 – 417	3123 / 36.6%	8.3 ± 20.3	0 - 65	83 / 30.7%
Pink/Purple	2.9 ± 3.8	0 – 15	76 / 0.9%	0.1 ± 0.3	0 - 1	1 / 0.4%
Red	3.5 ± 4.1	0 – 13	92 / 1.1%	0.3 ± 0.9	0 - 3	3 / 1.1%
Orange	5.3 ± 6.4	0 – 22	139 / 1.6%	1.9 ± 5.3	0 - 17	19 / 7.0%
Yellow	17.8 ± 36.6	0 - 178	463 / 5.4%	0.9 ± 1.7	0 - 5	9 / 3.3%
Green	16.0 ± 23.6	0 – 88	416 / 4.9%	2.7 ± 7.5	0 - 24	27 / 10.0%
Blue	29.5 ± 32.0	0 – 113	766 / 9.0%	1.5 ± 2.8	0 - 8	15 / 5.6%
Brown	31.0 ± 70.9	0 – 368	807 / 9.4%	1.4 ± 2.9	0 - 9	14 / 5.2%
Grey	8.5 ± 9.6	0 – 32	221 / 2.6%	0.1 ± 0.3	0 - 1	1 / 0.4%
Black	24.5 ± 22.3	0 – 86	637 / 7.5%	0.9 ± 1.4	0 - 4	9 / 3.3%
Plastic class sizes [b,c]						
Micro (< 5mm)	43.2 ± 47.0	0 – 176	1123 / 13.1%	2.2 ± 4.3	0 – 12	22 / 8.1%
Meso (5 - 25mm)	183.5 ± 182.2	0 – 768	4771 / 55.8%	11.4 ± 23.1	0 – 70	114 / 42.2%
Macro (> 25mm)	101.9 ± 123.8	0 – 580	2649 / 31.1%	13.4 ± 27.5	0 – 87	134 / 49.6%
Flexibility & Sharpness Index [b,c]						
FSI 2	227.2 ± 265.0	0 – 1277	5907 / 69.1%	15.7 ± 28.9	0 - 88	157 / 58.1%
FSI 3	29.4 ± 32.0	0 – 110	764 / 8.9%	7.4 ± 17.0	0 – 53	74 / 27.4%
FSI 4	11.6 ± 13.5	0 – 56	301 / 3.5%	1.0 ± 2.5	0 – 8	10 / 3.7%
FSI 5	13.5 ± 26.2	0 – 131	351 / 4.1%	0.1 ± 0.3	0 – 1	1 / 0.4%
FSI 6	46.9 ± 69.5	0 – 320	1220 / 14.3%	2.8 ± 5.9	0 - 15	28 / 10.4%

Acronyms: %FO (frequency of occurrence), SD (standard deviation), IND (industrial plastics), SHE (plastics sheet-like), THR (plastics thread-like), FRAG (rigid plastic fragments), FOAM (foam), POTH (other plastics), FSI (flexibility & sharpness index)
[a] Stranded turtles - Subsample A (n=71); [b] Stranded turtles - Subsample B (n=26); [c] Bycaught turtles - Subsample C (n=10)

Appendix 3E

Summary of the analysis on characteristics of plastic ingested by turtles examined through faecal matter monitoring.

	Captured turtles			Rescued turtles		
Incidence of plastic ingestion		**Turtles** 51/59	**%FO** 86.4%		**Turtles** 27/37	**%FO** 73.0%
	Mean ± SD	**Range**	**Total / %FO**	**Mean ± SD**	**Range**	**Total / %FO**
Ingested plastic	19.3 ± 26.2	0 – 152	1141 / 100%	10.2 ± 25.0	0 – 143	376 / 100%
Ingested plastic categories						
IND	0.0 ± 0.1	0 – 1	1 / 0.1%	0.0 ± 0.0	0 – 0	0 / 0.0%
SHE	9.7 ± 19.6	0 – 135	570 / 50.0%	3.0 ± 3.6	0 – 13	112 / 29.8%
THR	7.8 ± 12.3	0 – 58	463 / 40.6%	6.3 ± 22.7	0 – 133	233 / 62.0%
FRAG	1.6 ± 3.2	0 – 13	92 / 8.1%	0.5 ± 1.7	0 – 8	19 / 5.1%
FOAM	0.2 ± 1.0	0 – 6	13 / 1.1%	0.2 ± 1.0	0 – 6	8 / 2.1%
POTH	0.0 ± 0.1	0 – 1	1 / 0.1%	0.1 ± 0.4	0 – 2	4 / 1.1%
Ingested plastic colour						
Clear/Transp.	5.7 ± 10.0	0 – 66	337 / 29.6%	2.2 ± 3.1	0 – 10	81 / 21.5%
White	7.0 ± 11.6	0 – 69	408 / 35.8%	3.0 ± 8.3	0 – 47	111 / 29.5%
Pink/Purple	0.0 ± 0.2	0 – 1	2 / 0.2%	0.1 ± 0.5	0 – 3	3 / 0.8%
Red	0.1 ± 0.4	0 – 2	7 / 0.6%	0.2 ± 0.7	0 – 3	8 / 2.1%
Orange	0.2 ± 0.7	0 – 5	11 / 1.0%	0.2 ± 0.7	0 – 4	8 / 2.1%
Yellow	0.1 ± 0.3	0 – 2	5 / 0.4%	0.1 ± 0.5	0 – 3	4 / 1.1%
Green	0.8 ± 1.3	0 – 5	48 / 4.2%	0.7 ± 2.5	0 – 14	28 / 7.4%
Blue	1.8 ± 2.3	0 – 10	104 / 9.1%	2.1 ± 7.6	0 – 46	77 / 20.5%
Brown	0.7 ± 2.1	0 – 13	41 / 3.6%	0.2 ± 0.7	0 – 4	6 / 1.6%
Grey	0.1 ± 0.4	0 – 2	6 / 0.5%	0.4 ± 1.7	0 – 10	16 / 4.3%
Black	2.9 ± 5.1	0 – 22	171 / 15.0%	0.9 ± 2.0	0 – 8	34 / 9.0%
Plastic class sizes						
Micro (< 5mm)	2.1 ± 3.7	0 – 15	124 / 10.9%	0.5 ± 1.0	0 – 3	20 / 5.3%
Meso (5 – 25mm)	8.1 ± 12.3	0 – 71	478 / 41.9%	4.7 ± 12.7	0 – 72	175 / 46.5%
Macro (> 25mm)	9.2 ± 12.1	0 – 66	538 / 47.2%	4.9 ± 12.0	0 – 69	181 / 48.1%
Flexibility & Sharpness Index						
FSI 2	6.3 ± 15.4	0 – 108	374 / 32.8%	5.5 ± 17.0	0 – 99	203 / 54.0%
FSI 3	11.0 ± 14.9	0 – 62	651 / 57.1%	3.2 ± 7.3	0 – 43	118 / 31.4%
FSI 4	0.4 ± 1.0	0 – 5	26 / 2.3%	0.1 ± 4.6	0 – 28	35 / 9.3%
FSI 5	0.3 ± 0.9	0 – 4	20 / 1.8%	0.3 ± 1.0	0 – 5	11 / 2.9%
FSI 6	1.2 ± 2.6	0 – 12	69 / 6.1%	0.2 ± 0.9	0 – 5	9 / 2.4%

Acronyms: %FO (frequency of occurrence), SD (standard deviation), IND (industrial plastics), SHE (plastics sheet-like), THR (plastics threat like), FRAG (rigid plastic fragments), FOAM (foam), POTH (other plastics), FSI (flexibility & sharpness index)

Appendix 3F

Logistic regressions (GLMs) to examine trends in in the mean quantities of plastic ingested by captured turtles.

```
model_quantities <- glm(annual_mean ingestion ~ year, data = captured turtles,
family = poisson(link = "log"))

Deviance Residuals:
      1       2       3       4       5       6
-0.8537  1.1941 -1.5206  1.0363  0.9650 -1.1374

Coefficients:
             Estimate Std. Error z value Pr(>|z|)
(Intercept) -115.28258  115.89728  -0.995    0.320
year           0.05856    0.05744   1.019    0.308

(Dispersion parameter for poisson family taken to be 1)

    Null deviance: 8.8082  on 5  degrees of freedom
Residual deviance: 7.7658  on 4  degrees of freedom
AIC: Inf

Number of Fisher Scoring iterations: 4
```

Appendix 3G

[A] Table for the comparison of models based on their Akaike Information Criterion (AIC) values
[B] Linear mixed effects regression model (LMER) to analyse the correlation between turtle size (CCL) and quantities of ingested plastics (number of pieces), adding specimen source of specimens and year of turtle encounter as random effects (best-fitted model).

A] AICs model comparison

```
Models:
ccl_mixed1: lmer(number of pieces ~ ccl + (1 | source of specimen), data = ccl)
ccl_mixed2: lmer(number of pieces ~ ccl + (1 | date), data = ccl)
ccl_mixed3: lmer(number of pieces ~ ccl + (1 | source of specimen) + (1 | year),
data = ccl)

           npar  AIC     BIC   logLik  deviance  Chisq  Df  Pr(>Chisq)
ccl_mixed1   4  2496.0  2508.8 -1244.0   2488.0
ccl_mixed2   4  2506.0  2518.8 -1249.0   2498.0   0.000  0
ccl_mixed    5  2492.2  2508.2 -1241.1   2482.2  15.837  1  6.904e-05 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

B] Best-fitted model

```
ccl_mixed3 = lmer(number of pieces ~ ccl + (1 | source of specimen) + (1 | year),
data = ccl)

REML criterion at convergence: 2482.2
```

```
Scaled residuals:
    Min      1Q  Median      3Q     Max
-1.7770 -0.3782 -0.0787  0.1167  3.6711

Random effects:
 Groups   Name        Variance Std.Dev.
 date     (Intercept) 32087    179.13
 specimen (Intercept)  7912     88.95
 Residual             20873    144.47
Number of obs: 183, groups:  date, 146; specimen, 4

Fixed effects:
            Estimate Std. Error      df t value Pr(>|t|)
(Intercept)  333.003    120.792  65.985   2.757  0.00754 **
ccl           -6.194      2.818 134.400  -2.198  0.02967 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:
    (Intr)
ccl -0.909
```

Appendix 4A

Summary of necropsy reports of the examined turtles.

Turtle_ID	Database	Source	Date	Hour	Latitude	Longitude	Sex	CCL (cm)	Weight(kg)	BCI	Plastic	IPS
4	Necropsied	stranded	25/02/2014	18:00	-34.404	-53.787	female	33.1	3.5	1.1	1	3
34	Necropsied	stranded	16/07/2015	10:00	-34.821	-55.322	male	37.7	3.3	0.7	1	3
57	Necropsied	stranded	5/02/2016	15:30	-33.849	-53.477	female	34.8	3.24	0.9	1	3
60	Necropsied	bycaught	18/02/2016	13:43	-33.942	-53.511	female	36	4.16	1.0	1	3
65	Necropsied	stranded	15/03/2016	8:22	-33.849	-53.477	ind	40.2	7.15	1.3	1	3
81	Necropsied	stranded	8/12/2017	6:20	-33.885	-53.502	female	31	1.5	0.6	1	3
106	Necropsied	stranded	12/02/2018	13:00	-34.918	-54.976	male	31	2.4	0.9	1	3
111	Necropsied	stranded	3/05/2018	12:00	-34.277	-53.778	female	37.4	3.47	0.8	1	3
114	Necropsied	stranded	16/06/2018	15:00	-34.879	-55.136	female	32.8	3	1.0	1	3
119	Necropsied	stranded	22/02/2018	18:00	-34.420	-53.840	male	34.2	3.3	0.9	1	3
7	Necropsied	stranded	5/03/2014	11:30	-34.065	-53.552	female	34.6	4.1	1.1	1	2
9	Necropsied	stranded	29/06/2014	16:00	-34.841	-54.633	female	40	6.3	1.1	1	2
25	Necropsied	stranded	24/02/2015	8:00	-33.981	-53.532	female	32.1	2.85	1.0	1	2
31	Necropsied	stranded	20/05/2015	14:00	-33.901	-53.509	female	37.9	5	1.1	1	2
45	Necropsied	bycaught	27/12/2015	13:05	-34.344	-53.785	female	40.8			1	2
67	Necropsied	stranded	27/03/2016	11:15	-33.895	-53.507	female	37.9	5.7	1.2	1	2
77	Necropsied	stranded	2/12/2017	18:00	-33.992	-53.534	female	35.3	4.38	1.1	1	2
78	Necropsied	stranded	7/12/2017	16:50	-33.849	-53.477	indet	32.5	4.16	1.4	1	2
110	Necropsied	stranded	1/04/2018	15:02	-33.981	-53.532	female	36.9	4.28	1.0	1	2
113	Necropsied	stranded	16/06/2018	13:49	-34.404	-53.787	female	34	3.9	1.1	1	2
1	Necropsied	stranded	17/02/2014	15:00	-33.942	-53.511	female	41.5	7	1.1	1	1
38	Necropsied	bycaught	27/12/2015	13:00	-34.344	-53.785	female	41.6	6.88	1.1	1	1
55	Necropsied	stranded	13/01/2016	16:00	-34.040	-53.539	male	45	10.35	1.3	1	1
85	Necropsied	stranded	18/12/2017	19:29	-33.895	-53.507	female	34.8	5	1.4	1	1
99	Necropsied	bycaught	6/01/2018	17:25	-33.901	-53.509	female	37.7	5.52	1.2	1	1
104	Necropsied	bycaught	6/02/2018	12:35	-33.942	-53.511	male	51	14.92	1.3	1	1
146	Necropsied	stranded	2/02/2019	11:20	-33.922	-53.512	female	38.8	6	1.2	1	1
149	Necropsied	stranded	21/01/2019	16:30	-33.921	-53.512	female	33	3.3	1.1	1	1

Turtle_ID	Total pieces	Volume (mm3)	Weight (gr)	Body burden	Carapace	Plastron	Muscle atrophy	Body fat	Mouth	Eyes	Rehabilitation
4	1407	86593.55	99.94	28.55	clean	clean	no	low-med	clean	normal	1
34	368	24354.89	6.54	1.98	barnacles	barnacles	no	low	clean	suken	1
57	318	45171.08	8.62	2.66	barnacles	clean	no	low	clean	suken	0
60	75	2821.11	1.35	0.32	clean	clean	no	low	clean	suken	0
65	435	50639.64	12.05	1.69	barnacles	lesson (larvaes)	moderate	low	clean	suken	1
81	676	51350.93	22.45	14.97	clean	clean	moderate	low	clean	suken	0
106	592	27451.36	13.39	5.58	clean	clean	no	low	clean	suken	1
111	347	26935.59	8.28	2.39	clean	barnacles	no	low	clean	normal	1
114	248	27394.40	7.51	2.50	clean	clean	no	low	clean	suken	0
119	602	35391.99	7.80	2.36	injure	clean	moderate	low	clean	normal	1
7	208	20417.06	5.12	1.25	clean	clean	no	low	clean	normal	1
9	474	49568.42	13.98	2.22	clean	clean	no	low	clean	normal	0
25	18	16154.31	2.94	0.10	barnacles	clean	no	low-med	clean	suken	1
31	779	90750.46	27.49	5.50	clean	clean	moderate	low	clean	normal	1
45	165	16121.68	5.89		clean	clean	no	low	clean	normal	0
67	355	64088.23	9.62	1.69	barnacles	barnacles	no	low	clean	suken	1
77	319	1187.45	0.40	0.09	clean	clean	no	low	clean	normal	0
78	272	1409.74	0.46	0.11	clean	clean	no	low-med	clean	normal	0
110	582	28305.49	9.55	2.23	barnacles	barnacles	moderate	low	clean	suken	1
113	461	31565.99	6.82	1.75	clean	clean	no	low	clean	normal	1
1	2	2.03	0.01	0.00	clean	clean	no	medium	clean	suken	0
38	1	1307.26	0.05	0.01	clean	clean	no	medium	foam	normal	0
55	3	4.39	0.01	0.00	clean	clean	moderate	low-med	clean	suken	0
85	16	370.00	0.13	0.03	clean	clean	no	medium	clean	normal	0
99	9	120.59	0.07	0.01	clean	clean	no	medium	foam	normal	0
104	1	233.58	0.06	0.00	clean	clean	no	medium	foam	normal	0
146	41	1669.68	0.02	0.00	clean	clean	no	medium	foam	normal	0
149	6	229.10	0.02	0.01	clean	clean	no	low-med	clean	normal	0

Turtle_ID	Date of death	Cuase of death	Diagnosis
4	2/19/2014	GIT blockage by plastic / DTS / Starvation	Presence of fecalisths in the intestines / Morbidity / fishing line in the anus
34	3/28/2014	GIT blockage by plastic / DTS / Starvation	Presence of fecalisths in the intestines / Morbidity
57	7/25/2014	GIT blockage by plastic / DTS / Starvation	Presence of fecalisths in the intestines / Morbidity / injuries in the stomach / infection in the bowel
60	7/01/2014	GIT blockage by plastic / DTS / Starvation	Presence of fecalisths in the intestines/ Morbidity associated to PI
65	5/05/2015	GIT blockage by plastic / DTS / Starvation	Lung infection / Presence of fecalisths in the intestines/ Morbidity associated to PI
81	6/17/2015	GIT blockage by plastic / DTS / Starvation	Presence of fecalisths in the intestines / Morbidity associated to PI / cysts in the lungs and intestines
106	10/16/2015	GIT blockage by plastic / DTS / Starvation	Septicemia / Presence of fecalisths in the intestines/ Morbidity associated to PI
111	12/27/2015	GIT blockage by plastic / DTS / Starvation	Presence of fecalisths in the intestines/ Morbidity associated to PI
114	12/27/2015	GIT blockage by plastic / DTS / Starvation	Presence of fecalisths in the intestines/ Morbidity associated to PI
119	1/14/2016	GIT blockage by plastic / DTS / Starvation	Presence of fecalisths in the intestines/ Morbidity associated to PI
7	2/06/2016	Euthanasia / DTS	Morbidity associated to PI / massive amount of plastic in the intestines
9	2/21/2016	Debilitated Turtle Syndrome (DTS)	Presence of fecalisths / Morbidity associated to PI
25	7/08/2016	Debilitated Turtle Syndrome (DTS)	Morbidity associated to PI / massive amount of plastic in the intestines
31	4/14/2016	Debilitated Turtle Syndrome (DTS)	Presence of fecalisths / Morbidity associated to PI
45	12/03/2017	Drowning by bycatch	Moderate morbidity/massive amount of plastic in the intestines
67	indet	Debilitated Turtle Syndrome (DTS)	Morbidity associated to PI / massive amount of plastic in the intestines
77	12/09/2017	Debilitated Turtle Syndrome (DTS)	Morbidity associated to PI / massive amount of plastic in the intestines
78	12/19/2017	Debilitated Turtle Syndrome (DTS)	Fibropapillomatosis (FP) / Morbidity associated to PI / massive amount of plastic in the intestines
110	indet	Coelomitis / DTS	Morbidity associated to PI / massive amount of plastic in the intestines
113	indet	Electrocution / DTS	Morbidity associated to PI / massive amount of plastic in the intestines
1	5/10/2018	Chronic lung infection	DTS / Morbidity
38	5/10/2018	Drowning by bycatch	Fishing bycatch
55	6/12/2018	Septicemia	DTS / Morbidity
85	6/28/2018	Pneumonia	DTS / Morbidity
99	6/16/2018	Drowning by bycatch	Fishing bycatch
104	3/05/2018	Drowning by bycatch	Fishing bycatch (frontal flippers amputated)
146	indet	Drowning by bycatch	Fishing bycatch
149	1/23/2019	indet	DTS

Appendix 4B

[A] Multinomial analysis using the generalized linear regression model to examine the relationship between the total volumes of plastic ingested and impact severity (IPS groups) [B] The Tukey pairwise comparison test for assessing differences in the means of total volumes ingested by turtles grouped according to their Impact Severity Index (IPS).

```
A] Multinomial Logistic Regression model

model_ips <- multinom(ips ~ total_vol, data = data_vol)

summary(model)
```

```
Coefficients:
  (Intercept)    total_vol
2   -3.280337 0.0002328716 **
3   -4.288734 0.0002547050 **

Std. Errors:
   (Intercept)    total_vol
2 3.994172e-09 4.750688e-05 ***
3 2.437279e-09 4.786482e-05 ***

Residual Deviance: 79.92318


AIC: 87.92318

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

```
B] Post hoc analysis (Tuckey pairwise comparison)
```

```
contrast    estimate     SE df t.ratio p.value
 ips1 - ips2    0.569 0.1208  4   4.709  0.0201 *
 ips1 - ips3    0.669 0.0926  4   7.225  0.0043 **
 ips2 - ips3    0.100 0.0297  4   3.382  0.0587

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Appendix 4C

Logistic regression to examine the relationship between the total volume of each particle size class and impact severity (IPS groups).

```
model_particle-size <- glm(impact ~ micro_vol + meso_vol + macro_vol, family =
binomial(), data = data_vol)
Anova(model_partitioned)

summary(model_partitioned)
```

```
Coefficients: Estimate  Std. Error z value  Pr(>|z|)
(Intercept)  -5.403e+00  1.335e+00  -4.049  5.15e-05 ***
micro_vol     8.334e-03  2.973e-03   2.804  0.00505 **
meso_vol     -3.746e-04  4.095e-04  -0.915  0.36038
macro_vol    -1.637e-05  1.703e-04  -0.096  0.92339
Null deviance: 113.401  on 105  degrees of freedom
Residual deviance:  25.788  on 102  degrees of freedom
```

```
Analysis of Deviance Table (Type II tests)

Response: impact
           LR Chisq Df Pr(>Chisq)
micro_vol   20.1339 1  7.221e-06 ***
meso_vol    0.9070  1  0.3409
macro_vol   0.0095  1  0.9222

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Appendix 4D

Analysis of deviance to test the relationship between total volume of each particle size class and location along the digestive tract for the three IPS groups.

```
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: proptype1
                        Chisq Df Pr(>Chisq)
Particle               0.3324  2     0.8469
Location              71.0731  2  3.687e-16 ***
ips1                   0.0000  1     0.9977
ccl                    0.1350  1     0.7133
Particle:Location      6.2018  4     0.1846
Particle:ips1          0.0475  2     0.9765
Location:ips1          0.2157  2     0.8978
Particle:Location:ips1 2.0238  4     0.7314

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Appendix 4E

Logistic regression to examine the relationship between the total ingested volumes for each plastic type and the severity of the impact in impacted turtles (IPS 2 and IPS 3 groups), also considering the effect of turtle size (CCL).

```
adjusted_model_ips23 <- glm(impact ~ she + thr + frag + foam + poth + ind + ccl,
data = ips23, family = binomial(link = logit))

summary(adjusted_model_ips23)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-1.6088  -0.3497  -0.1464   0.5037   2.0288

Coefficients:
             Estimate Std. Error z value Pr(>|z|)
(Intercept)  7.888e+00  7.962e+00   0.991   0.3219
she          2.737e-04  1.385e-04   1.976   0.0481 *
thr         -1.349e-03  6.762e-04  -1.995   0.0460 *
frag         8.433e-05  1.261e-04   0.669   0.5036
foam         1.649e-06  1.044e-04   0.016   0.9874
poth         2.045e-04  2.326e-04   0.879   0.3792
ind          1.069e-02  1.880e-02   0.569   0.5695
```

```
ccl         -2.884e-01  2.323e-01  -1.241   0.2145

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for binomial family taken to be 1)

    Null deviance: 32.601  on 23  degrees of freedom
Residual deviance: 15.569  on 16  degrees of freedom
AIC: 31.569

Number of Fisher Scoring iterations: 6
```

Appendix 4F

Logistic regression to examine the relationship between turtle size, reflected as curved carapace length (CCL), and impact severity (IPS groups).

```
impact_ccl <- glm(impact ~ ccl , data = vol_data, family = binomial(link = logit))

summary(impact_ccl)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-1.1854  -0.7565  -0.5489  -0.1972   2.0401

Coefficients:
            Estimate Std. Error z value Pr(>|z|)
(Intercept)  5.78755    2.50195   2.313  0.02071 *
ccl         -0.18729    0.06808  -2.751  0.00594 **
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1


(Dispersion parameter for binomial family taken to be 1)

    Null deviance: 113.40  on 105  degrees of freedom
Residual deviance: 103.31  on 104  degrees of freedom
AIC: 107.31

Number of Fisher Scoring iterations: 5
```