




eDNA metabarcoding for diet analyses of green sea turtles (*Chelonia mydas*)

Lucía Díaz-Abad^{1,8} · Natassia Bacco-Mannina¹ · Fernando Miguel Madeira² · João Neiva¹ · Tania Aires¹ · Ester A. Serrao^{1,3} · Aissa Regalla⁴ · Ana R. Patrício^{5,6} · Pedro R. Frade^{1,7} 

Received: 20 May 2021 / Accepted: 24 November 2021
© The Author(s) 2021

Abstract

Understanding sea turtle diets can help conservation planning, but their trophic ecology is complex due to life history characteristics such as ontogenetic shifts and large foraging ranges. Studying sea turtle diet is challenging, particularly where ecological foraging observations are not possible. Here, we test a new minimally invasive method for the identification of diet items in sea turtles. We fingerprinted diet content using DNA from esophageal and cloacal swab samples by metabarcoding the 18S rRNA gene. This approach was tested on samples collected from green turtles (*Chelonia mydas*) from a juvenile foraging aggregation in the Bijagós archipelago in Guinea-Bissau. Esophagus samples ($n=6$) exhibited a higher dietary richness (11 ± 5 amplicon sequence variants (ASVs) per sample; average \pm SD) than cloacal ones ($n=5$; 8 ± 2 ASVs). Overall, the diet was dominated by red macroalgae (Rhodophyta; $48.2 \pm 16.3\%$ of all ASVs), with the main food item in the esophagus and cloaca being a red alga belonging to the Rhodymeniophycidae subclass ($35.1 \pm 27.2\%$), followed by diatoms (Bacillariophyceae; $7.5 \pm 7.3\%$), which were presumably consumed incidentally. Seagrass and some invertebrates were also present. Feeding on red algae was corroborated by field observations and barcoding of food items available in the benthic habitat, validating the approach for identifying diet content. We conclude that identification of food items using metabarcoding of esophageal swabs is useful for a better understanding of the relationships between the feeding behavior of sea turtles and their environment.

Keywords DNA barcoding · Guinea-Bissau · Macrophytes · Green turtle · Feeding ecology · Marine biodiversity

Introduction

Effective planning of biodiversity conservation depends on a good understanding of the relationships between species or populations of interest and their environment (Bremner

2008). Identifying diet items of threatened species is important to understand food preference, the availability and quality of food in their habitats, and to account for trophic interactions when designing habitat protection (Hamann et al. 2010). Understanding the foraging ecology and diet composition of species that go through ontogenetic diet shifts (Carr 1987) and/or that have large foraging ranges

Responsible Editor: L. Avens.

✉ Pedro R. Frade
pedro.frade@nhm-wien.ac.at

¹ CCMAR-Centre of Marine Sciences, CIMAR, University of Algarve, Faro, Portugal

² cE3c Centre for Ecology, Evolution and Environmental Changes, Faculdade de Ciências da Universidade de Lisboa, Lisbon, Portugal

³ CIBIO/InBIO-Centro de Investigação em Biodiversidade e Recursos Genéticos, Vairão, Portugal

⁴ IBAP-Instituto da Biodiversidade e das Áreas Protegidas, Dr. Alfredo Simão da Silva, Bissau, Guinea-Bissau

⁵ MARE-Marine and Environmental Sciences Centre, ISPA-Instituto Universitário, Lisbon, Portugal

⁶ Centre for Ecology and Conservation, College of Life and Environmental Sciences, University of Exeter, Exeter, UK

⁷ Natural History Museum Vienna, Vienna, Austria

⁸ IMBRSea, International Master of Science in Marine Biological Resources, IMBRSea Universities Consortium, Ghent, Belgium

(Hazel et al. 2013) is challenging, often requiring invasive sampling. These challenges are very relevant for sea turtles, which are charismatic species of conservation concern (Arthur et al. 2008). The seven species of sea turtles are threatened by human exploitation and environmental change and are included in the IUCN Red list as critically endangered, endangered, vulnerable or data deficient. Despite the recovery of some major populations in the last decade following conservation efforts (Mazaris et al. 2017), many populations continue to face large-scale anthropogenic threats, namely degradation and loss of nesting and feeding habitats, incidental capture in fisheries, direct harvesting, plastic ingestion and climate change (Santidrián-Tomillo et al. 2015; Casale and Heppell 2016; Nelms et al. 2016; Summers et al. 2018; Patrício et al. 2021). Accumulated evidence emphasizes the importance of adopting conservation actions to protect key habitats such as nesting beaches and feeding grounds (Wallace et al. 2011; Godley et al. 2020).

Since it is very difficult to observe sea turtles feeding under completely natural conditions (Legler 1976), diet studies have traditionally relied on the direct observation of stomach contents (Williams et al. 2013; Brandis et al. 2014) and, in some cases, of what remains in fecal material (Bjorndal 1997). To assess green turtle (*Chelonia mydas*) diet using stomach contents, several studies have used stranded or bycaught (dead) turtles (Mortimer 1981; André et al. 2005; Howell et al. 2016; Howell and Shaver 2021). Although useful, this approach limits the sampling to a group of animals that may not be representative of the population (Levey and Martinez del Rio 2001). Another traditional approach is to conduct esophageal lavage, flushing out recently ingested food items (Holloway-Adkins and Hanisak 2017). This procedure requires appropriate training and is invasive, which can stress and harm the turtles (Legler 1977; Amarocho and Reina 2007). For coastal life stages, lavage is typically only appropriate for the herbivorous green turtle whose diet is dominated by soft food items. Stable isotope analysis has also been used as an indirect, less invasive method to infer the diet of sea turtles (e.g., Seminoff et al. 2006; Arthur et al. 2008; Shimada et al. 2014), but with low resolution, not allowing direct food species identification and quantification, and requiring information on the isotopic composition of putative diet items (Gannes et al. 1997; Post 2002; Burkholder et al. 2011). Some techniques used to assess the diet of sea turtles are summarized in Table S1.

Recently, feeding studies have been revolutionized by the onset of DNA metabarcoding (Taberlet et al. 2012). This approach has been used, for example, to characterize the gut microbiome of loggerhead sea turtles (Arizza et al. 2019) and to characterize the diet of a diverse range of other marine taxa (e.g., Albaina et al. 2016). The combination of DNA metabarcoding with less invasive wildlife sampling techniques, such as the use of swabs for digestive tract sample

collection, enables species-level identification of diet items. In other organisms such as birds, cloacal swabs have already been used to successfully estimate species-specific diet composition (Williams and Athrey 2020; Bodawatta et al. 2020). Such specificity requires the use of genetic markers for which a reference databank of relevant sequences of potential diet items is available. The 18S rRNA gene was shown to be particularly useful in barcoding marine macrophytes (Ortega et al. 2020), fish (Albaina et al. 2016) and marine invertebrates (Zhang et al. 2018), which are within the typical diet items of sea turtles (Bjorndal 1997).

Here, we aimed to tackle the difficulties in assessing sea turtle diets in the field by testing a new method for the identification of food items in the diet of sea turtles, based on esophageal and cloacal swab samples analyzed by metabarcoding of the 18S rRNA gene. To the best of our knowledge, no study has used metabarcoding of diet content on *C. mydas*, and this study contributes to a better understanding of the feeding behavior of the sea turtles. With this new method, we aim to identify eukaryotic DNA sequences from food items present in esophageal and cloacal samples. The method was tested using a juvenile foraging aggregation of green turtles from the western coast of Africa. We address two main objectives: (1) determining whether DNA metabarcoding with the 18S rRNA gene is useful to identify food items of sea turtles; and (2) comparing the information between esophageal and cloacal samples, to determine which sample type is better for a more complete diet assessment of green turtles (*C. mydas*).

Materials and methods

Study sites and sampling

Esophageal and cloacal swab samples were collected from juvenile green turtles (*Chelonia mydas*; sizes ranged from 36.5 to 48.1 cm of curved carapace length, with an average of 40.6 cm \pm 4.5 cm SD) foraging around Unhocomo and Unhocomozinho islands (11° 31' N; 16° 40' W, Fig. 1), located at the westernmost limit of the Bijagós Archipelago, Guinea-Bissau, West Africa. Sampling took place on 27 October 2019. Sea floor was mostly sandy with some rocky areas, covered by patches of green, red and brown algae, and sparse seagrass (*Halodule* sp., see description of benthic community below).

Green turtles were caught using one entanglement net (800 m long, 4 m deep, 20 cm knot to knot mesh size), deployed from a pirogue (canoe), operated by local Bijagós fishermen, enclosing foraging sites for periods of 1 h, under constant monitoring for entangled turtles. Captured turtles were brought onboard another vessel (anchored next to the net) and positioned comfortably,

not occur at least one time in 10% of the samples were used for the multivariate analyses. Estimates are given as average \pm SD unless otherwise specified.

Overall, three different sample types were collected and successfully sequenced for identification of diet items: cloaca ($n=6$) and esophagus ($n=6$) swabs from six juvenile green turtles, and intestine samples ($n=3$) from three dead green turtle hatchlings (see Table S2 for overview). After rarefaction, one sample was discarded (due to low number of reads) leading to a total of $n=5$ cloaca and $n=6$ esophagus samples originating from six juvenile green turtles. This is considered a sufficient sample size for analyses of ecological communities (Anderson et al. 2006). Because there were only three samples for the category “intestines”, these were excluded from the statistical analyses.

Benthic community characterization and barcoding of local food items

To describe local benthic macrophyte composition and compare with the results from the swab samples, we deployed quadrats (50×50 cm) placed at 30 random points inside the netted area. At each location, all identifiable algae and seagrass taxa inside the quadrat were noted. This procedure was repeated during each sampling session.

To increase the resolution of taxonomic assignments of metabarcoding sequences, locally available organisms putatively used as food items by green turtles (according to their availability found during the benthic community characterization) were obtained during fieldwork and preserved as described for the swab samples. The 18S rRNA gene of a subset of these items was sequenced at CCMAR by Sanger sequencing using the same primer used for metabarcoding. This local database was used to assign the dominant metabarcoding reads obtained from the sea turtle samples to regionally common genus-level taxa macrophytes potentially consumed as food. The sequences were compared against nucleotide databases using blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch), and best matches were ranked based on sequence coverage and % identity. 18S rRNA gene sequences and best blastn hits were aligned using the MUSCLE implementation built into Geneious (v 5.6.5), allowing phylogenetic comparisons using maximum likelihood phylogenetic trees built with PHYML and using the HKY85 substitution model and 1000 bootstraps for determining branch support. Moreover, to increase the resolution of the identification, some of the key local food items (*Spyridia* sp., *Laurencia* sp. and *Hypnea* sp.) were further sequenced for the standard barcoding marker *cox1* using GAZ primers (Saunders and McDevit 2012)

and blasted against nucleotide databases as for the 18S rRNA gene.

Data availability

Demultiplexed sequences and metadata are available from the NCBI Sequence Read Archives (SRA) under BioProject Accession number PRJNA781615.

Results

Sampling

Six juvenile green turtles were sampled in four capture sessions, with body sizes ranging from 36.5 to 48.1 cm of curved carapace length, with an average of $40.4 \text{ cm} \pm 4.0 \text{ cm}$ SD. For more information on the sampled individuals, see Table S2. Many swabs presented small pieces of seagrass after esophageal swabbing took place, and nearly all swabs led to a change in the fixative color (from clear to brown, orange, gray or yellow green) when plunged into it.

Analysis of metabarcoding data

A total of 699,971 good-quality reads (i.e., phred > 35 , non-chimeric) were generated from the 15 samples sequenced (Table S3), corresponding to ca. 48% of the initial number of reads. The samples had an average of $51,128 \pm 12,823$ reads, which clustered into 306 unique ASVs across all samples combined. As expected, due to the eukaryotic-universal coverage of the 18S rRNA gene primers, a large proportion of reads corresponded to turtle DNA ($99.8 \pm 0.2\%$ for intestine samples, $98.7 \pm 1.8\%$ for cloaca and $59.6 \pm 23.6\%$ for esophagus, see Fig. S1). These reads were removed from the analyses to focus on the diet.

Overall, alpha diversity differed among the sample types analyzed (i.e., esophagus vs cloaca vs intestine; see Fig. S2). The esophagus sequences contained more diet items (11 ± 5 ASVs per sample) than cloacal ones (8 ± 2 ASVs; $F_{(1,9)} = 9.252$, $p = 0.014$). As expected, intestine samples from hatchlings, used as a methodological control yielded the lowest number of ASVs (5 ± 6 , Fig. S2), with a negligible number of reads (0.2%) assigned to diet (i.e., non-turtle). These samples were thus not further analyzed for diet content. The food items assemblage identified was differently structured according to the compartment of origin (i.e., esophagus vs cloaca samples) (Fig. 2; PERMANOVA, $F_{(1,9)} = 2.709$, $p = 0.0019$; ANOSIM, $R = 0.596$, $p = 0.0032$), despite not differing in multivariate dispersion (PERMDISP, $F_{(1,9)} = 0.757$, $p = 0.4069$).

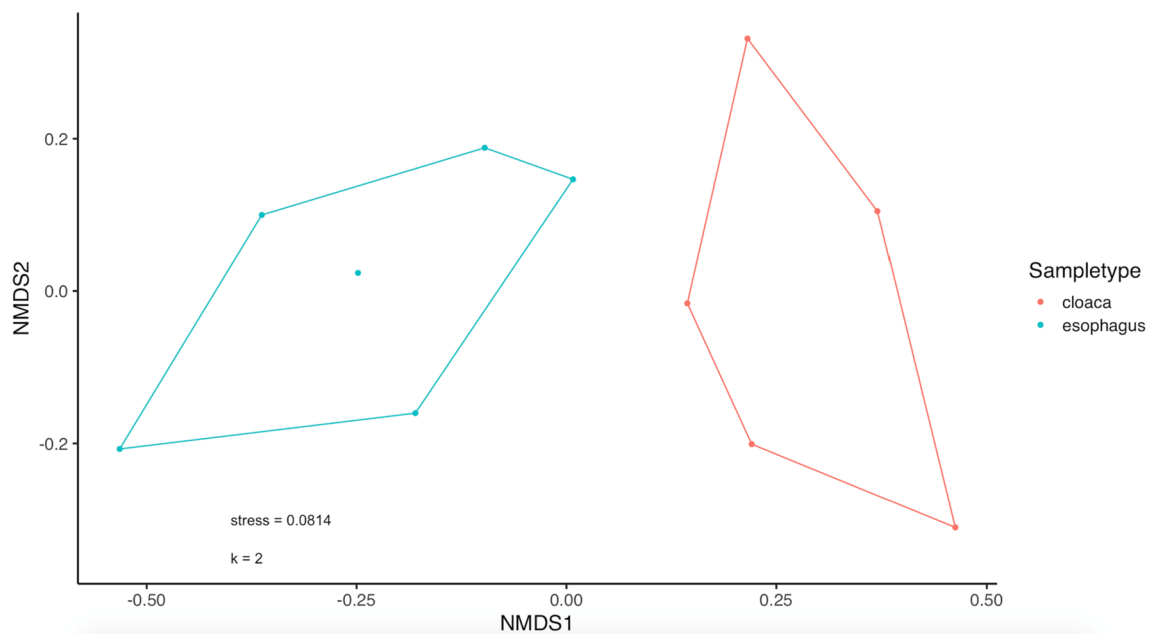


Fig. 2 Community structure of green turtle diet items for cloaca ($n=5$) and esophagus ($n=6$) samples. NMDS plot based on Bray–Curtis similarity

Diet characterization

The main non-chelonian DNA sequences found were red macroalgae, seagrasses, different animal groups and

unicellular eukaryotes (Fig. 3). Rhodophyta (red algae) were the predominant food items for both sample types combined ($48.2 \pm 16.3\%$ of total ASVs), followed by Bacillariophyceae (diatoms) ($7.5 \pm 7.3\%$), Cymodoceaceae (seagrass

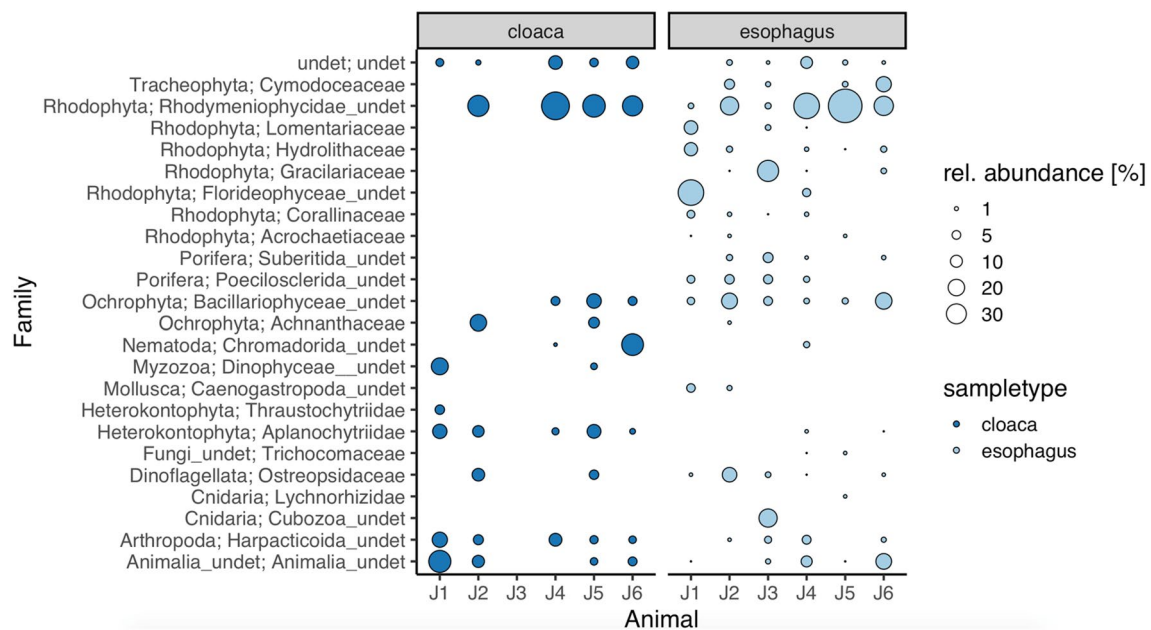


Fig. 3 Diet community composition for cloaca and esophagus of green turtles. Size of bubble plots represents relative abundance of 18S rRNA gene amplicon sequence variants (%) and diet items are given at the taxonomic levels of phylum and family. When family affiliation was not possible to determine, the lowest identified taxo-

nomic rank is shown (followed by “undet”; i.e., undetermined). Only the most abundant diet items are shown. Note that for one cloaca sample (Animal J3), diet data are not shown due to low number of reads and exclusion during rarefaction

family of which the only local species is *Halodule wrightii*) ($2.6 \pm 6.8\%$) and undetermined groups of animals (undetermined Animalia). The esophagus samples presented higher content of red algae ($32.3 \pm 18.2\%$) and seagrasses ($2.6 \pm 6.5\%$) than the cloaca, which had a higher content of groups of organisms other than macrophytes, such as protists ($9.8 \pm 6.5\%$), crustaceans ($4.2 \pm 5.5\%$) and nematodes ($3.6 \pm 16.0\%$).

There were differences in specific taxa identified between esophagus and cloaca (see Fig. 3), but also some overlap. For instance, an undetermined family in the Rhodymeniophycidae red algae subclass (Rhodophyta, class Florideophyceae) was the most abundant family ($35.1 \pm 27.2\%$) of diet items in both cloacal and esophageal samples, followed by diatoms (Bacillariophyceae; $7.5 \pm 7.3\%$). Another red algal family (undetermined Florideophyceae; Fig. 3) was the second most abundant ($5.4 \pm 15.2\%$) food item present in the esophagus, but was absent in the cloaca. The seagrass family Cymodoceaceae (locally *Halodule wrightii*) was also present in esophageal samples ($2.6 \pm 6.8\%$), but missing from the cloacal ones of the same sea turtle individuals. In contrast, an undetermined family in the nematode order Chromadorida was the second most abundant taxa in

cloacal samples ($3.6 \pm 16.0\%$), and almost absent from the esophagus ($0.2 \pm 1.0\%$). Less than 1% of reads in esophagus and cloaca samples combined corresponded to green ($0.02 \pm 6.51\%$) and brown macroalgae ($0.01 \pm 0.00\%$).

Benthic community characterization and barcoding of local food items

Regional benthic macrophyte communities at the turtle sampling site were characterized by a dominance of the seagrass *Halodule wrightii* (Tracheophyta), and the brown algae (Phaeophyceae), *Padina* sp. and *Sargassum* sp., which were present in at least 50% of the random quadrat points (see Table S4; Fig. 4), followed by red algae. Of these, two species of Rhodymeniophycidae were particularly abundant (frequency of occurrence > 10% each), *Laurencia dendroidea* and *Spyridia filamentosa* (as identified by *cox1* barcoding analyses; results not shown). Grazed patches of seagrass (*Halodule* sp.) were observed.

The dominant red algae reads found in the esophagus samples clustered closely with 18S rRNA gene barcodes generated from local macrophytes (seagrasses and seaweeds) collected in the study site. Matching esophageal

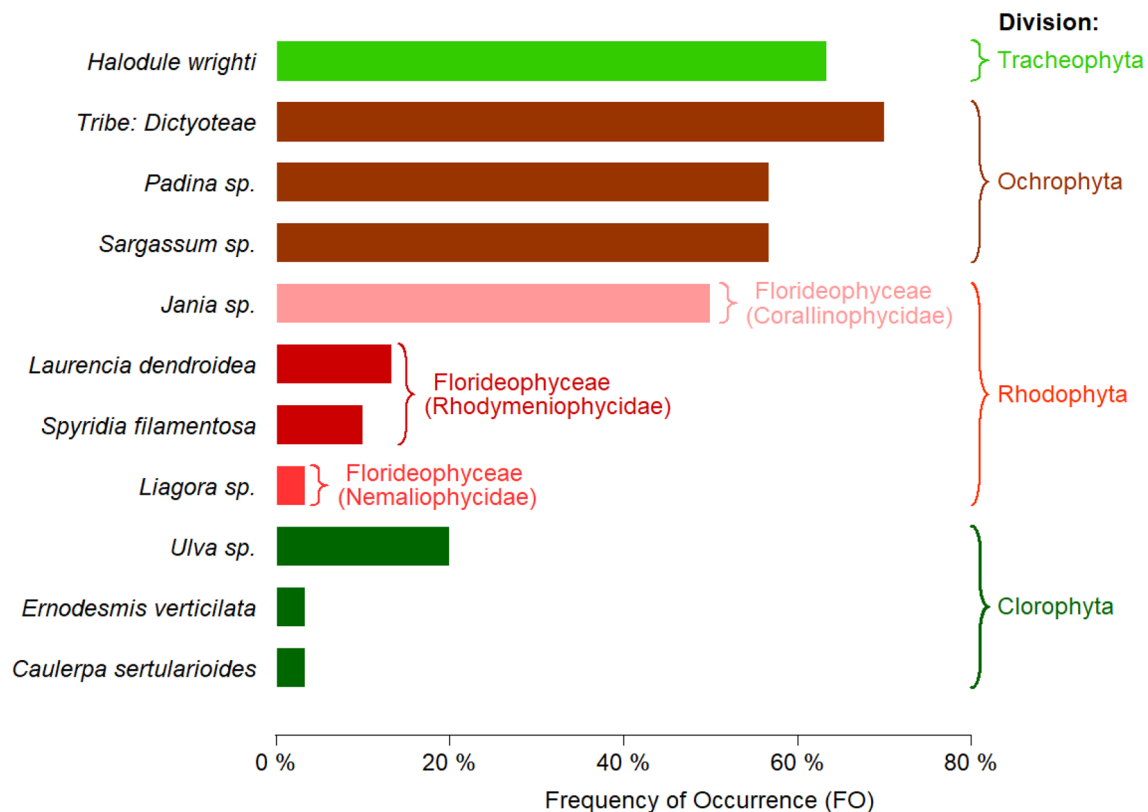


Fig. 4 Frequency of occurrence FO, (in percent) of main macroalgae and seagrass groups found in the sampling area, listed by division. Rhodophyta are further subdivided in Classes (Subclasses). Data

were obtained by 30 random 50×50 cm quadrats per session (two sessions). FO represents the percentage of quadrats for which the taxon was found to be present

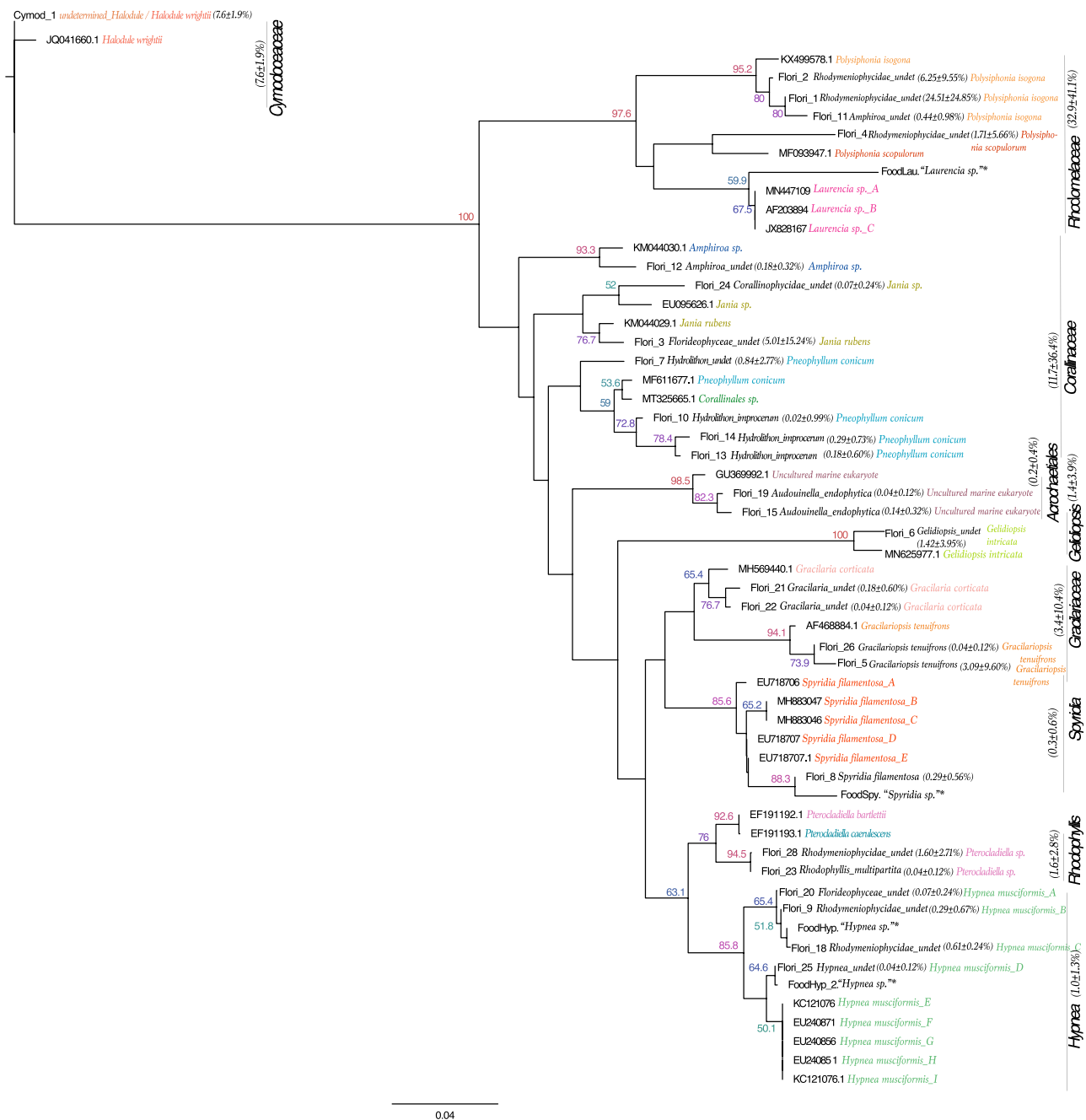


Fig. 5 Phylogenetic tree generated from the 18S rRNA gene metabarcoding of the local macrophytes. Esophageal reads belonging to the dominant red algae Rhodmeniophycidae subclass ASVs are represented as (*Flori_X*) and named following the results of the phylogenetic analysis down to species level (black labels); next to them, the relative abundance in the esophagus is shown as mean percentage and standard deviation. Close relatives of the (*Flori_X*) sequences according to NCBI are displayed as accession numbers. The ID given by Blast (color labels) to the lowest taxonomic level appears next to the

accession numbers and after the relative abundances for each ASV (*Flori_X*). Items with an asterisk (*) correspond to the barcoded local prey items found during the surveys (name between quotes as given by the collector in the field). Vertical labels give higher taxonomic levels and relative abundances. The outgroup corresponds to the only seagrass ASV in the analysis, identified as *Halodule_undet* (i.e., undetermined) (Tracheophyta: Cymodoceaceae in Fig. 3). Bootstrap values < 50% not shown

reads (named as *FloriX* in Fig. 5) were closely related to *Polysiphonia* sp., *Laurencia* sp. and *Hypnea* sp. (Fig. 5). Nevertheless, calcareous coralline algae (order Corallinales; such as *Jania* sp.) were also commonly ingested, as many of the esophageal reads clustered within this group of algae (Fig. 5). Most abundant ASVs closely matched *Polysiphonia* sp. (undetermined Rhodymeniophycidae, $24.5 \pm 24.8\%$; undetermined Rhodymeniophycidae, $6.2 \pm 9.6\%$) and *Jania rubens* (undetermined Florideophyceae, $5.0 \pm 15.2\%$).

Discussion

The diet identification method here tested, based on metabarcoding of esophagus and cloaca swabs of green turtles with the 18S rRNA gene, allowed the identification, at acceptable taxonomic resolution, of food items of green turtle diets. We consider that this method can be widely applied for sea turtle diet assessments, with higher resolution and lower invasiveness than previously available methods. Red algae, seagrasses and diatoms were the most dominant sequenced taxa in samples from six juvenile green turtles captured in Guinea-Bissau. Ingestion of red algae and seagrasses was corroborated by field observations. First, three turtles were observed locally with pieces of seagrass (*Halodule* sp.) in their mouth and three others exhibited red saliva, likely related to the ingestion of red algae. Second, grazed patches of seagrass (*Halodule* sp.) were observed (Madeira, pers. obs.). Finally, a necropsy of a juvenile individual showed that most of its stomach contents were red algae, identified as *Laurencia dendroidea*; nevertheless, small quantities of *Halodule* sp., *Dictyota* sp. and *Caulerpa* sp. were also present. These observations support the metabarcoding results suggesting the ingestion of red algae and seagrasses and highlight the efficacy of the method used in this study.

Rhodophyta were the most abundant items in turtle diet, particularly red algae in the Rhodymeniophycidae subclass. Our attempts to narrow down the taxonomic identification of the red algae by using a barcoding approach of local items (using 18S, complemented by *cox1*) allowed to identify tissue samples of *Polysiphonia isogona*, *Laurencia* sp. and *Hypnea* sp. as the main diet items of green sea turtles in these sampling locations. Although in low abundance, *Halodule* sp. was also found in the metabarcoding analysis, exclusively in esophagus samples. The presence of the red algae *Polysiphonia isogona* as the most abundant diet item (most abundant ASV, $24.5 \pm 24.8\%$) corroborates previous published information, as *Polysiphonia* spp. have been shown to be components of green turtle diet, along with a variety of red and green algae (Spotila 2004).

Despite field observations of dominance of brown algae in the benthos, particularly Dictyotales and *Sargassum* sp., these taxa were barely identified in the analyses of the turtle digestive tracts (less than 1% of reads corresponded to brown macroalgae). This result suggests the hypothesis of feeding preferences of green turtles for the less abundant red algae over the locally dominant brown algae. An additional hypothesis is that the turtles might have been feeding on other grounds more dominated by red algae, which still support the hypothesis of food preference for red algae. Two red algal species of Rhodymeniophycidae were nevertheless particularly frequent (> 10% each) at the sampling site, *Laurencia dendroidea* and *Spyridia filamentosa*. These are the two dominant red algal species of the marine forests of most islands of Bijagós, and in other surveyed sites of this archipelago they reach > 90% cover (unpublished data).

The high abundance of diatoms in both the cloaca and esophagus likely reflects passive ingestion explained by their previously described epiphytic lifestyle on seagrasses such as *Halodule* sp. (Morgan and Kitting 1984; Dawes and Walter 2010). This is also likely the case for the calcareous coralline algae found in the diet (James et al. 2009; Martin et al. 2008; Perry and Beavington-Penney 2005), despite previous reports that epiphytic coralline algae may prevent overgrazing of seagrass (Leemans et al. 2020). Moreover, other organisms such as foraminifera, ciliates, and other benthic animals found in the analysis are likely present in the diet because they are also epiphytes of seagrass and seaweed (Langer 1993; Larkum et al. 2006), and not necessarily because of targeted consumption.

Differences observed between cloacal- and esophageal-derived diet composition are most likely to be due to digestion. The higher content of red algae and seagrass in the esophagus and not in the cloaca suggests that green turtles digest and absorb effectively these food resources (Bjorndal 1980). On the contrary, the presence of items different from algae and seagrass in the cloaca, such as crustaceans and protists, could be interpreted as items harder to digest or as parasites or symbionts of the cloaca, a body region more prone to such colonization by external taxa than the esophagus. Finally, elements present in both the esophagus and cloaca could hypothetically represent non-digestible items. Overall, these findings indicate that the esophagus is likely the preferred compartment for the diet characterization of sea turtles. However, to fully validate this conclusion, future studies will be needed including a larger number of samples as well as a broader time window that is able to cover diel variation of ingested items.

The intestine samples from hatchlings proved a good control of the method. The samples yielded a very low number of non-chelonian 18S rRNA gene reads, as expected from the absence of feeding by sea turtle hatchlings in their first

days of life (Smith and Daniel 1946; Hughes 1974). Notably, one of the intestine samples showed a scarce presence of the pathogenic yeast *Malassezia pachydermatis*, which is likely the result of post-mortem infection. The high number of turtle reads obtained from cloaca and esophagus samples indicates that the protocol could still be enhanced to obtain a larger relative proportion of sequences from the diet items, for instance, using turtle DNA blocking primers.

A diet composition similar to the one we describe here was found (via esophageal lavages) in green turtles from southern Brazil (Reisser et al. 2013), dominated by red macroalgae *Pterocladia capillacea*. Dominance of red algae in green turtle diets has also been described for the East Coast of Florida (Holloway-Adkins 2001) and in Bahía de los Angeles in the Gulf of California, in Mexico, with *Gracilariopsis lemaneiformis* being the most abundant species (Seminoff et al. 2002). *Gracilariopsis* sp. was also the main food item of green turtles from Flathead Gutter, Moreton Bay in south-east Queensland, Australia (Brand-Gardner et al. 1999). Taken together, these studies suggest that red algae are a recurrent food item despite large spatial variability regarding the most locally consumed species (probably related to availability).

Although we found the diet to be almost exclusively herbivorous, therefore corroborating previous evidence (Seminoff et al. 2002; Fuentes et al. 2006; Stokes et al. 2019), we also identified other diet components such as nematodes, sponges, corals, molluscs, annelids and even crustaceans. These might be invertebrates found in the seaweed and seagrass eaten by the turtles. A similar mixture of diverse diet items of green turtles has been previously found in Eastern Pacific green turtles in Colombia with presence of tunicates (Salpidae and Doliolidae), red mangrove fruits, algae (Rhodophyta, Chlorophyta, Cyanophyta), small crustaceans (shrimp larvae) and plant leaves (Amarocho and Reina 2007). This mixture of diverse prey, especially invertebrates, could be beneficial as nitrogen supplement (Forbes 1996).

Taxonomic assignment of turtle diet items, as assessed with 18S rRNA gene metabarcoding, is limited by the absence of a local database for turtle food items from Guinea-Bissau. We used the best database available (SILVA, see methods section), but the capacity to identify diet items could still be improved with a larger local database than the one created here. Barcoding of tissue samples of local *Caulerpa* sp. and *Jania* sp. yielded foraminifera and ciliate sequences, respectively, which indicates that some of these animals are likely present as epibionts of targeted food items. Importantly, the diet results presented in this manuscript only provide a single-site snapshot of what the animals had eaten in recent feeding events. For comprehensive assessments of the diet of foraging aggregations, samples should be collected at different periods in time, encompassing any existing seasonal variations.

We conclude that our minimally invasive metabarcoding method, preferentially based on esophagus swabs (rather than cloacal swabs), is suitable and a promising alternative to other more intrusive techniques. A direct comparison to other techniques is warranted to evaluate the performance of eDNA metabarcoding approach with respect to other available methods. Control studies with experimentally fed animals and/or targeting multiple foraging grounds dominated by distinct macrophyte communities (seagrass, brown or red algae) are also natural next steps to further evaluate the strengths of this promising approach. Additionally, we have contributed to the scarce information about green turtle feeding preferences in Guinea-Bissau, by establishing a baseline for the diet of this foraging aggregation. Diet characterization combined with foraging ecology studies can help conservation managers to identify critical habitats in need of long-term protection and enable a better understanding of foraging hotspots and regional connectivity (Stokes et al. 2019). Our findings, corroborated by field observations, validate esophagus swab metabarcoding to accurately characterize the diet of green turtles. This method is less intrusive compared to traditional esophageal lavage, reduces animal handling, and is also easily reproducible. Thus, we consider it to be of general application to assess the diet of green turtles and we propose that future studies should explore the utility of this technique with other sea turtle species.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00227-021-04002-x>.

Acknowledgements Research permits and logistic support in Guinea-Bissau were provided by the Instituto da Biodiversidade e das Áreas Protegidas Dr. Alfredo Simão da Silva (IBAP). Fieldwork was conducted in collaboration with IBAP staff members and with the local communities of the Bijagós, to whom we are deeply thankful. Also supported by MAVA (PNBA-site emblématique pour les tortues) and PRCM/STM P000A4/OA9.

Author contributions NBM, ARP, EAS and PRF conceived and designed the experiments; NBM and FM collected turtle swabs; NBM and PRF performed molecular work; TA, JN and FM barcoded diet groups. LDA and PRF analyzed the data and prepared figures and tables; LDA wrote a first draft of the manuscript. All authors contributed to and approved the final version of manuscript.

Funding Metabarcoding analyses were funded by the FCT-Science and Technology Foundation of Portugal (UIDB/04326/2020) and a Pew Marine Fellowship to EAS and Tropibio Era Chair (EU grant 854248 and NORTE-01-0145-FEDER-000046). FCT funded fellowships to PRF (SFRH/BDP/110285/2015), EAS (SFRH/BSAB/150485/2019), TA (SFRH/BPD/116774/2016) and contract DL57/2016/CP1361/CT0010 to JN. ARP was funded by Fundación La Caixa, project ‘TurtleResC—sea turtle resilience to climate change impacts’ and FCT (UIDB/04292/2020 and UIDP/04292/2020) awarded to MARE. Fieldwork was funded by the project ‘Consolidation of sea turtle conservation at the Bijagós Archipelago, Guinea-Bissau’, awarded to IBAP by

the MAVA Foundation. The funders had no role in the study designing, data collection and analysis, decision to publish, or preparation of the manuscript.

Data availability Demultiplexed sequences and metadata are available from the NCBI Sequence Read Archives (SRA) under BioProject Accession number PRJNA781615.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval Research permits were provided by the National Governmental Institute for Biodiversity Conservation, the 'Instituto da Biodiversidade e das Áreas Protegidas, Dr. Alfredo Simão da Silva (IBAP—Guinea-Bissau)'. Sampling procedures were carried out by trained personnel following recommended guidelines (NMFS-SFC 2008), to reduce stress to the animals and improve their welfare.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Albaina A, Aguirre M, Abad D, Santos M, Estonba A (2016) 18S rRNA V9 metabarcoding for diet characterization: a critical evaluation with two sympatric zooplanktivorous fish species. *Ecol Evol* 6(6):1809–1824. <https://doi.org/10.1002/ece3.1986>
- Amarocho DF, Reina RD (2007) Feeding ecology of the East Pacific green sea turtle *Chelonia mydas agassizii* at Gorgona National Park, Colombia. *Endanger Spec Res* 3:43–51. <https://doi.org/10.3354/esr003043>
- Anderson MJ, Ellingsen KE, McArdle BH (2006) Multivariate dispersion as a measure of beta diversity. *Ecol Lett* 9(6):683–693
- André J, Gyuris E, Lawler IR (2005) Comparison of the diets of sympatric dugongs and green turtles on the Orman Reefs, Torres Strait, Australia. *Wildl Res* 32:53–62. <https://doi.org/10.1071/WR04015>
- Arizza V, Vecchioni L, Caraccappa S, Sciarba G, Berlinghieri F, Gentile A et al (2019) New insights into the gut microbiome in loggerhead sea turtles *Caretta caretta* stranded on the Mediterranean coast. *PLoS ONE* 14(8):e0220329
- Arthur KE, Boyle MC, Limpus CJ (2008) Ontogenetic changes in diet and habitat use in green sea turtle (*Chelonia mydas*) life history. *Mar Ecol Prog Ser* 362:303311. <https://doi.org/10.3354/meps07440>
- Bjorndal KA (1980) Nutrition and grazing behaviour of the green turtle, *Chelonia mydas*. *Mar Biol* 56:147–154
- Bjorndal KA (1997) Foraging ecology and nutritional ecology of sea turtles. In: Lutz P, Musick JA (eds) *Biology of the sea turtles*. CRC Press, Boca Raton, pp 237–283
- Bodawatta KH, Puzejova K, Sam K et al (2020) Cloacal swabs and alcohol bird specimens are good proxies for compositional analyses of gut microbial communities of Great tits (*Parus major*). *Anim Microb*. <https://doi.org/10.1186/s42523-020-00026-8>
- Bolyen E, Rideout JR, Dillon MR, Caporaso JG (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* 37:852–857. <https://doi.org/10.1038/s41587-019-0209-9>
- Brand-Gardner SJ, Limpus CJ, Lanyon JM (1999) Diet selection by immature green turtles, *Chelonia mydas*, in subtropical Moreton Bay, south-east Queensland. *Austral J Zool* 47:181–191
- Brandis R, Mortimer J, Reilly B, van Soest R, Branch G (2014) Diet Composition of hawksbill turtles (*Eretmochelys imbricata*) in the Republic of Seychelles. *West Indian Ocean J Mar Sci* 13:81–91
- Bremner J (2008) Species' traits and ecological functioning in marine conservation and management. *J Exp Mar Biol Ecol* 366(1–2):37–47
- Burkholder DA, Heithaus MR, Thomson JA, Fourqurean JW (2011) Diversity in trophic interactions of green sea turtles *Chelonia mydas* on a relatively pristine coastal foraging ground. *Mar Ecol Prog Ser* 439:277–293. <https://doi.org/10.3354/meps09313>
- Callahan BJ et al (2016) DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods* 13:581
- Carr A (1987) New perspectives on the pelagic stage of sea turtle development. *Conserv Biol* 1:103–121. <https://doi.org/10.1111/j.1523-1739.1987.tb00020.x>
- Casale P, Heppell SS (2016) How much sea turtle bycatch is too much? A stationary age distribution model for simulating population abundance and potential biological removal in the Mediterranean. *Endanger Spec Res* 29(3):239–254
- Dawes C, Walter A (2010) Epiphytes of the seagrass *Halodule wrightii* in Hillsborough Bay, Florida; a 14-year study in an estuary recovering from eutrophication. *Fla Sci* 73:185–195
- Forbes GA (1996) The diet and feeding ecology of the green sea turtle (*Chelonia mydas*) in an algal-based coral reef community. PhD thesis, James Cook University
- Fuentes MPB, Lawler IR, Gyuris E (2006) Dietary preferences of juvenile green turtles (*Chelonia mydas*) on a tropical reef flat. *Wildl Res* 33:671–678
- Gannes LZ, Martínez del Rio C, Koch P (1997) Stable isotopes in animal ecology: assumptions, caveats, and a call for more laboratory experiments. *Ecology* 78:1271–1276
- Godley BJ, Broderick AC, Colman LP, Formia A, Godfrey MH, Hamann M, Shanker K (2020) Reflections on sea turtle conservation. *Oryx* 54(3):287–289
- Guardiola M, Uriz MJ, Taberlet P, Coissac E, Wangenstein OS, Turon X (2015) Deep-sea, deep-sequencing: metabarcoding extracellular DNA from sediments of marine canyons. *PLoS ONE* 10(10):e0139633. <https://doi.org/10.1371/journal.pone.0139633>
- Hamann M, Godfrey MH, Seminoff JA, Arthur K, Barata PCR, Bjorndal KA, Bolten AB, Broderick AC, Campbell LM, Carreras C, Casale P, Chaloupka M, Chan SKF, Coyne MS, Crowder LB, Diez CE, Dutton PH, Epperly SP, Fitzsimmons NN, Formia A, Girondot M, Hays GC, Cheng IJ, Kaska Y, Lewinson R, Mortimer JA, Nichols WJ, Reina RD, Shanker K, Spotila JR, Tomás J, Wallace BP, Work TM, Zbinden J, Godley BJ (2010) Global research priorities for sea turtles: informing management and conservation in the 21st Century. *Endanger Spec Res* 11:245–269
- Hazel J, Hamann M, Lawler IR (2013) Home range of immature green turtles tracked at an offshore tropical reef using automated passive acoustic technology. *Mar Biol* 160:617–627. <https://doi.org/10.1007/s00227-012-2117-0>
- Holloway-Adkins KG (2001) A comparative study of the feeding ecology of *Chelonia mydas* (green turtle) and the incidental ingestion of *Prorocentrum* sp. Retrospective Theses and Dissertations. p. 1207

- Holloway-Adkins KG, Hanisak MD (2017) Macroalgal foraging preferences of juvenile green turtles (*Chelonia mydas*) in a warm temperate/subtropical transition zone. *Mar Biol* 164:161. <https://doi.org/10.1007/s00227-017-3191-0>
- Howell LN, Shaver DJ (2021) Foraging Habits of Green Sea Turtles (*Chelonia mydas*) in the Northwestern Gulf of Mexico. *Front Mar Sci* 8:658368. <https://doi.org/10.3389/fmars.2021.658368>
- Howell LN, Reich KJ, Shaver DJ, Landry AM Jr, Gorga CC (2016) Ontogenetic shifts in diet and habitat of juvenile green sea turtles in the northwestern Gulf of Mexico. *Mar Ecol Prog Ser* 559:217–229. <https://doi.org/10.3354/meps11897>
- Hughes GR (1974) The sea turtles of south-east Africa. II. The biology of the Tongaland loggerhead turtle *Caretta caretta* L. with comments on the leatherback turtle *Dermochelys coriacea* L. and the green turtle *Chelonia mydas* L. in the study region. *Oceanogr Res Inst Invest Rep* 36:96
- James NP, Bone Y, Brown KM, Cheshire A (2009) Calcareous epiphyte production in cool-water carbonate seagrass depositional environments—southern Australia. In: Swart PK, Eberli GP, McKenzie JA, Jarvis I, Stevens T (eds) Perspectives in carbonate geology: a tribute to the career of Robert Nathan Ginsburg, vol 41. IAS Special Publication, Kolkata, pp 123–148
- Jones J, DiBattista JD, Stat M, Bunce M, Boyce MC, Fairclough DV, Travers MJ, Huggett MJ (2018) The microbiome of the gastrointestinal tract of a range-shifting marine herbivorous. *Fish Front Microbiol* 9:2000. <https://doi.org/10.3389/fmicb.2018.02000>
- Langer M (1993) Epiphytic Foraminifera. *Mar Micropaleontol* 20:235–265. [https://doi.org/10.1016/0377-8398\(93\)90035-V](https://doi.org/10.1016/0377-8398(93)90035-V)
- Larkum AWD, Orth RJ, Duarte CM (2006) Seagrasses: biology, ecology and conservation. Springer, Berlin
- Leemans L, Martínez I, van der Heide T et al (2020) A mutualism between unattached coralline algae and seagrasses prevents overgrazing by sea turtles. *Ecosystm* 23:1631–1642. <https://doi.org/10.1007/s10021-020-00492-w>
- Legler JM (1976) Feeding habits of some Australian short-necked tortoises. *Vic Nat* 93:40–43
- Legler JM (1977) Stomach flushing: a technique for chelonian dietary studies. *Herpetology* 33(3):281–284
- Levey DJ, Martinez del Rio C (2001) It takes guts (and more) to eat fruit: lessons from avian nutritional ecology. *Auk* 118:819–831
- Martin S, Rodolfo-Metalpa R, Ransome E, Rowley S, Buia MC, Gattuso JP, Hall-Spencer J (2008) Effects of naturally acidified seawater on seagrass calcareous epibionts. *Biol Lett* 4:689–692. <https://doi.org/10.1098/rsbl.2008.0412>
- Mazaris AD, Schofield G, Gkazinou C, Almpandou V, Hays GC (2017) Global sea turtle conservation successes. *Sci Adv* 3(9):e1600730
- McMurdie PJ, Holmes S (2013) phyloseq: a R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* 8:e61217
- Morgan MD, Kitting CL (1984) Productivity and utilization of the seagrass *Halodule wrightii* and its attached epiphytes 1. *Limnol Oceanogr* 29(5):066–107
- Mortimer JA (1981) The feeding ecology of the west Caribbean green turtle (*Chelonia mydas*) in Nicaragua. *Biotropica* 13:49–58. <https://doi.org/10.2307/2387870>
- National Marine Fisheries Service Southeast Fisheries Center (2008) Sea Turtle Research Techniques Manual. NOAA Technical Memorandum NMFS-SEFSC-579, p. 92
- Nelms SE, Duncan EM, Broderick AC, Galloway TS, Godfrey MH, Hamann M, Godley BJ (2016) Plastic and marine turtles: a review and call for research. *ICES J Mar Sci* 73(2):165–181
- Oksanen J et al (2013) vegan: community ecology package. R package version 2:0–9
- Ortega A, Geraldi NR, Díaz-Rúa R, Ørberg SB, Wesselmann M, Krause-Jensen D, Duarte CM (2020) A DNA mini-barcode for marine macrophytes. *Mol Ecol Resour* 20(4):920–935. <https://doi.org/10.1111/1755-0998.13164>
- Patrício AR, Varela MR, Barbosa C, Broderick AC, Cattray P, Hawkes LA, Regalla A, Godley BJ (2019) Climate change resilience of a globally important sea turtle nesting population. *Glob Change Biol* 25(2):522–535
- Patrício AR, Hawkes LA, Monsinjon JR, Godley BJ, Fuentes MM (2021) Climate change and marine turtles: recent advances and future directions. *Endanger Spec Res* 44:363–395
- Perry CT, Beavington-Penney SJ (2005) Epiphytic calcium carbonate production and facies development within sub-tropical seagrass beds, Inhaca Island. *Mozamb Sediment Geol* 174:161–176. <https://doi.org/10.1016/j.sedgeo.2004.12.003>
- Post DM (2002) Using stable isotopes to estimate trophic position: models, methods and assumptions. *Ecology* 83:703–718
- Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucl Acids Res* 41(D1):D590–D596
- R Development Core Team (2008) R: a language and environment for statistical computing. R Foundation for Statistical Computing
- Reisser J, Proietti M, Sazima I et al (2013) Feeding ecology of the green turtle (*Chelonia mydas*) at rocky reefs in western South Atlantic. *Mar Biol* 160:3169–3179. <https://doi.org/10.1007/s00227-013-2304-7>
- Santidrián-Tomillo P, Roberts SA, Hernández R, Spotila JR, Paladino FV (2015) Nesting ecology of East Pacific green turtles at Playa Cabuyal, Gulf of Papagayo, Costa Rica. *Mar Ecol* 36(3):506–516
- Saunders GW, McDevitt DC (2012) Methods for DNA barcoding photosynthetic protists emphasizing the macroalgae and diatoms. *Methods Mol Biol* 858:207–222. https://doi.org/10.1007/978-1-61779-591-6_10
- Seminoff JA, Resendiz A, Nichols WJ (2002) Diet of East Pacific green turtles (*Chelonia mydas*) in the central Gulf of California, Mexico. *J Herpetol* 36:447–453
- Seminoff JA, Jones TT, Eguchi T, Jones DR, Dutton PH (2006) Stable isotope discrimination ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) between soft tissues of the green sea turtle *Chelonia mydas* and its diet. *Mar Ecol Prog Ser* 308:271–278
- Shimada T, Aoki S, Kameda K, Hazel J, Reich K, Kamezaki N (2014) Site fidelity, ontogenetic shift and diet composition of green turtles *Chelonia mydas* in Japan inferred from stable isotope analysis. *Endang Spec Res* 25:151–164. <https://doi.org/10.3354/esr00616>
- Smith KU, Daniel RS (1946) Observations of behavioural development in the loggerhead turtle (*Caretta caretta*). *Sci* (Washington DC) 104:154–156
- Spotila J (2004) Sea turtles: a complete guide to their biology, behavior, and conservation. Johns Hopkins University Press, Baltimore
- Stokes HJ, Mortimer JA, Hays GC et al (2019) Green turtle diet is dominated by seagrass in the Western Indian Ocean except amongst gravid females. *Mar Biol* 166:135. <https://doi.org/10.1007/s00227-019-3584-3>
- Summers TM, Martin SL, Hapdei JR, Ruak JK, Jones TT (2018) Endangered green turtles (*Chelonia mydas*) of the Northern Mariana Islands: nesting ecology, poaching, and climate concerns. *Front Mar Sci* 4:428
- Taberlet P, Coissac E, Pompanon F, Brochmann C, Willerslev E (2012) Towards next-generation biodiversity assessment using DNA metabarcoding. *Mol Ecol* 21:2045–2050. <https://doi.org/10.1111/j.1365-294X.2012.05470.x>
- Wallace BP, DiMatteo AD, Bolten AB, Chaloupka MY, Hutchinson BJ, Abreu-Grobois FA, Mortimer JA, Seminoff JA, Amorocho D, Bjørndal KA, Bourjea J (2011) Global conservation priorities for marine turtles. *PLoS ONE* 6(9):e24510
- Wickham H (2009) ggplot2: elegant graphics for data analysis. Springer, New York

- Williams T, Athrey G (2020) Cloacal swabs are unreliable sources for estimating lower gastro-intestinal tract microbiota membership and structure in broiler chickens. *Microorganism* 8:718
- Williams N, Bjørndal K, Lamont M, Carthy R (2013) Winter diets of immature green turtles (*Chelonia mydas*) on a northern feeding ground: integrating stomach contents and stable isotope analyses. *Estuar Coast*. <https://doi.org/10.1007/s12237-013-9741-x>
- Zhang G, Chain F, Abbott C, Cristescu M (2018) Metabarcoding using multiplexed markers increases species detection in complex

zooplankton communities. *Evol Appl* 11:1901–1914. <https://doi.org/10.1111/eva.12694>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.