




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

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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

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(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,

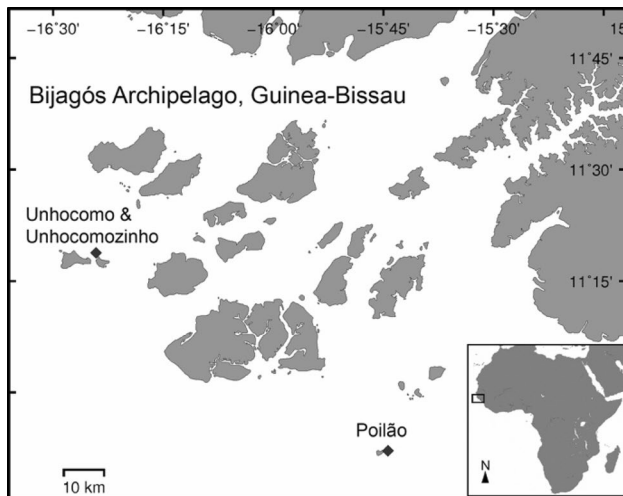


Fig. 1 Map of the study site: Poilão and Unhocomo islands in Guinea-Bissau, West Africa

with the plastron on a flat surface and front flippers held close to the body. Cloacal and esophageal swab sampling were performed as follows: for the esophageal swab, the mouth of the turtle was gently kept open and the esophageal entrance sampled as deep as possible using a 20 cm long sterile swab (making a gentle swirl 2–3 times) not touching any other parts of the turtle; for the cloacal swab, the opening of the cloaca was cleaned with a chlorhexidine wet gauze before inserting a smaller sterile swab 2 cm into the cloaca (2–3 swirls as before). Gloves were used at all times and changed between individual turtles and also between collection of esophageal and cloacal samples within the same individual. Handling of turtles was kept to a minimum (max. 15 min per individual), to reduce their stress, and turtles were kept covered with a wet towel while onboard the research vessel. Swabs were stored in individual centrifuge tubes (esophagus swabs in 50 mL and cloaca swabs in 2 mL tubes) filled with 96% EtOH and kept at room temperature until stored at 2–5 °C for a week and then at –20 °C at the CCMAR laboratory.

Additionally, digestive tract samples (intestines) from hatchlings were collected to be used as methodological control, since newly emerged hatchlings have not yet fed (Smith and Daniel 1946; Hughes 1974) and should thus be free of any foreign DNA indicative of food ingestion. Dissection of digestive tracts was only performed on recently dead hatchlings found on the beach in Poilão Island (10° 52' N; 15° 43' W, Fig. 1), a major rookery for green turtles in the Bijagós archipelago (Patrício et al. 2019). Swabbing was performed in intestines and swabs kept in 2 mL microcentrifuge tubes with 96% EtOH.

DNA extraction and analysis of metabarcoding data

Nucleic acid extraction was performed with the QIAGEN DNeasy PowerBiofilm kit, following manufacturer's instructions plus a few customized steps. A small portion of the cotton tip of the sampling swab (30–100 mg) was initially rinsed in sterile water to remove EtOH, then centrifuged at maximum speed and transferred to the bead tube. Bead tubes were incubated at 65 °C for 30 min and then homogenized in a TissueLyser for 3 min at 18,000 rpm.

DNA extracts were sent on dry-ice to MRDNA (Texas, USA) and a 133-bp fragment of the 18S rRNA gene was amplified using the primer set 18S_Euka02(V7) (forward: 5'-TTTGTCTGTTAATTSCG-3'; reverse: 5'-CACAGA CCTGTTATTGC-3') as recommended by Ortega et al. (2020) and following standard conditions (Guardiola et al. 2015). Sequencing took place on an Illumina MiSeq platform using 2 × 300-bp paired-end technology. Demultiplexed reads were analyzed as single nucleotide variants using QIIME2 as described in Bolyen et al. (2019; Version 2019.7 <https://qiime2.org>). Forward and reverse primers were removed using FASTqProcessor (<http://www.mrdnabio.com/mrdnafreesoftware/fastq-processor.html>) and chimeric sequences were removed using DADA2 (phred > 35; trimming = 10) (Callahan et al. 2016). Sequences were grouped using DADA2 (Callahan et al. 2016) based on 100% sequence similarity, generating representative sequences subsequently referred to as amplicon sequence variants (ASVs). For taxonomic assignment, a naïve-Bayes classifier was trained on the SILVA v132 99% database (Quast et al. 2013), with sequences limited to the V7 region of the 18S rRNA genes. Plastid-derived sequence reads and singletons were removed from the dataset, which was then rarefied to an even sequencing depth of 250 reads (allowing to keep most samples in the analyses). Alpha diversity was analyzed with Shannon and Chao1 diversity indexes along with richness (observed number of ASVs) (Jones et al. 2018). Multivariate statistics for the community composition were performed in R (R Development Core Team 2008) and included: analysis of similarity (ANOSIM, 'vegan package' Oksanen et al. 2013; Holloway-Adkins and Hanisak 2017), permutational multivariate analysis of variance (PERMANOVA, 'vegan package' (Oksanen et al. 2013; adonis2; Jones et al. 2018), multivariate homogeneity of group dispersion (PERMDISP; 'vegan package' Oksanen et al. 2013; betadisper; Jones et al. 2018) and non-metric multidimensional scaling (NMDS, 'phyloseq package' McMurdie and Holmes 2013; Holloway-Adkins and Hanisak 2017), all based on Bray–Curtis dissimilarities. Graphs were created in R using ggplot2 (Wickham 2009) and phyloseq (McMurdie and Holmes 2013) packages. Statistical analyses were performed using rarefied data. Filtered data without ASVs that did

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, $F_{(1,9)} = 0.757$, $p = 0.4069$).

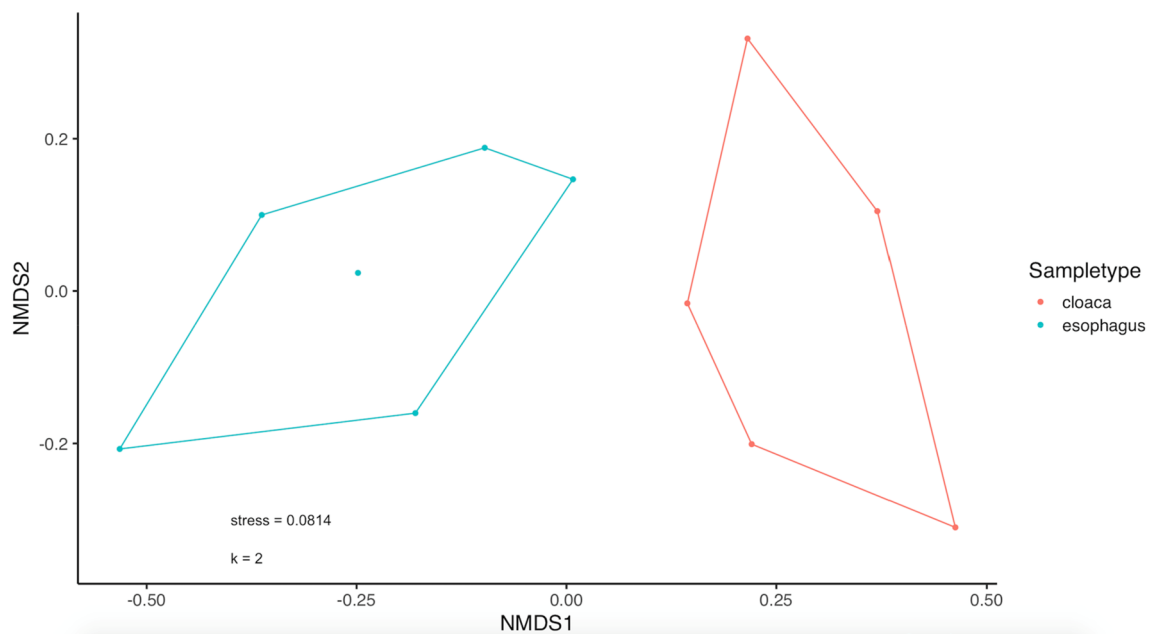


Fig. 2 Community structure of green turtle diet items for cloaca ($n=5$) and esophagus ($n=6$) samples. NMDS plot based on Bray–Curtis similarity

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

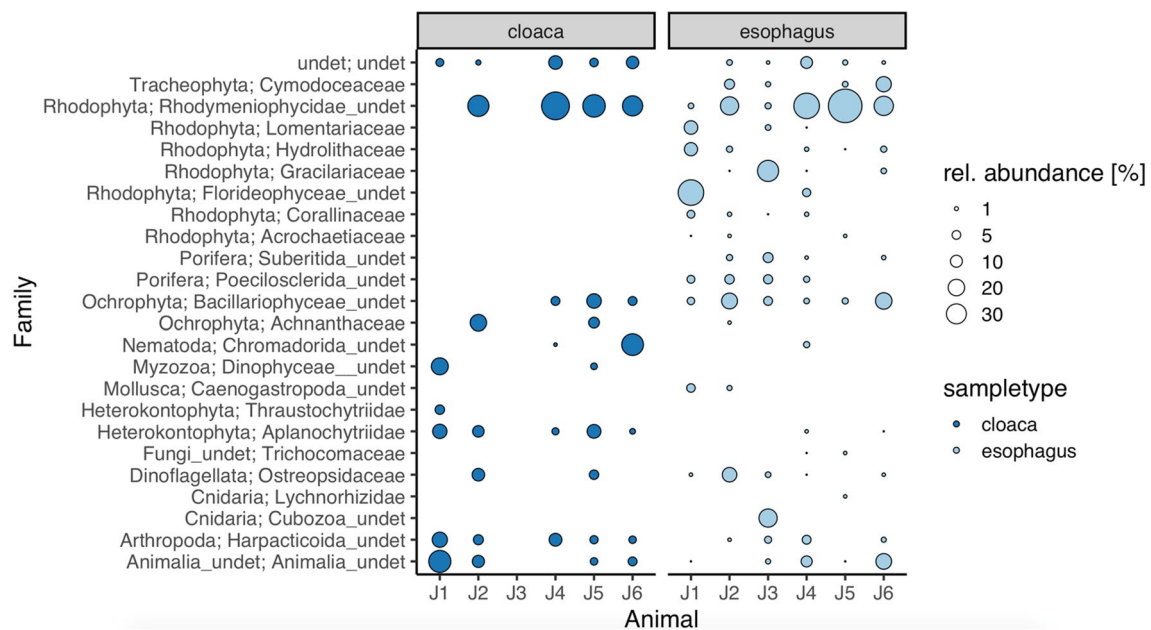


Fig. 3 Diet community composition for cloaca and esophagus of green turtles. Size of bubble plots represents relative abundance of 18S rRNA gene amplicon sequence variants (%) and diet items are given at the taxonomic levels of phylum and family. When family affiliation was not possible to determine,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

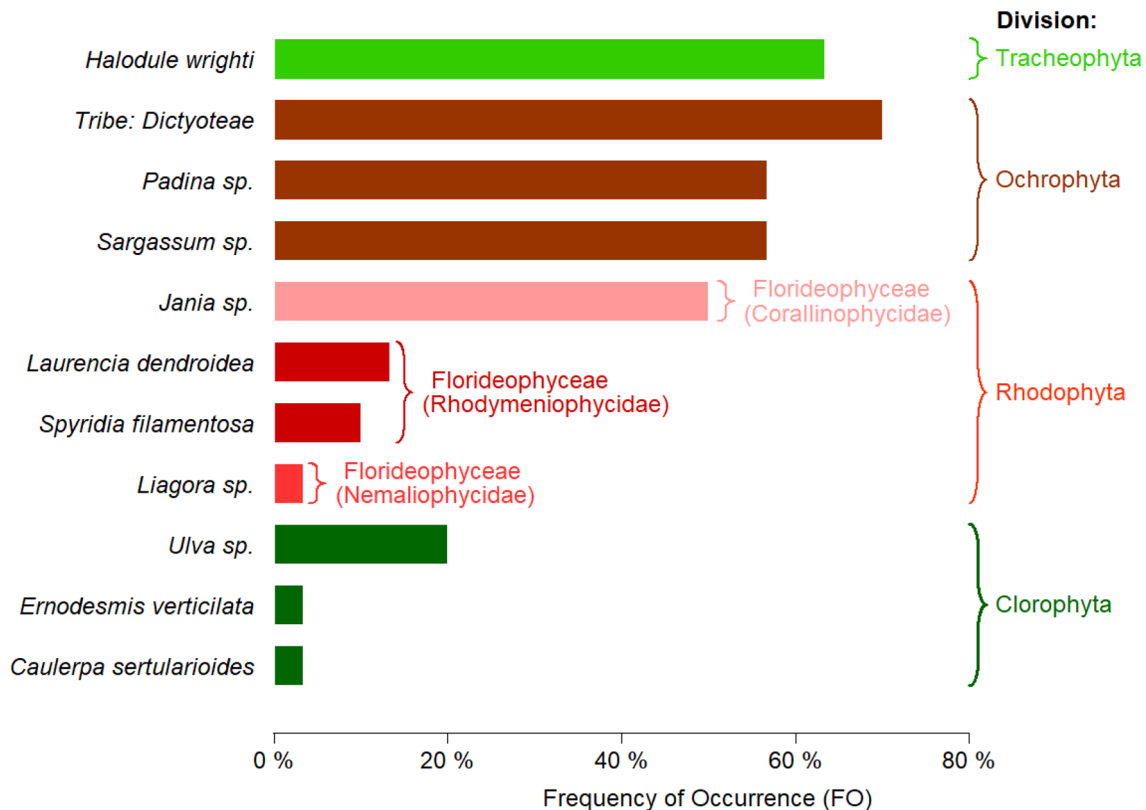


Fig. 4 Frequency of occurrence FO, (in percent) of main macroalgae and seagrass groups found in the sampling area, listed by division. Rhodophyta are further subdivided in Classes (Subclasses). Data

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

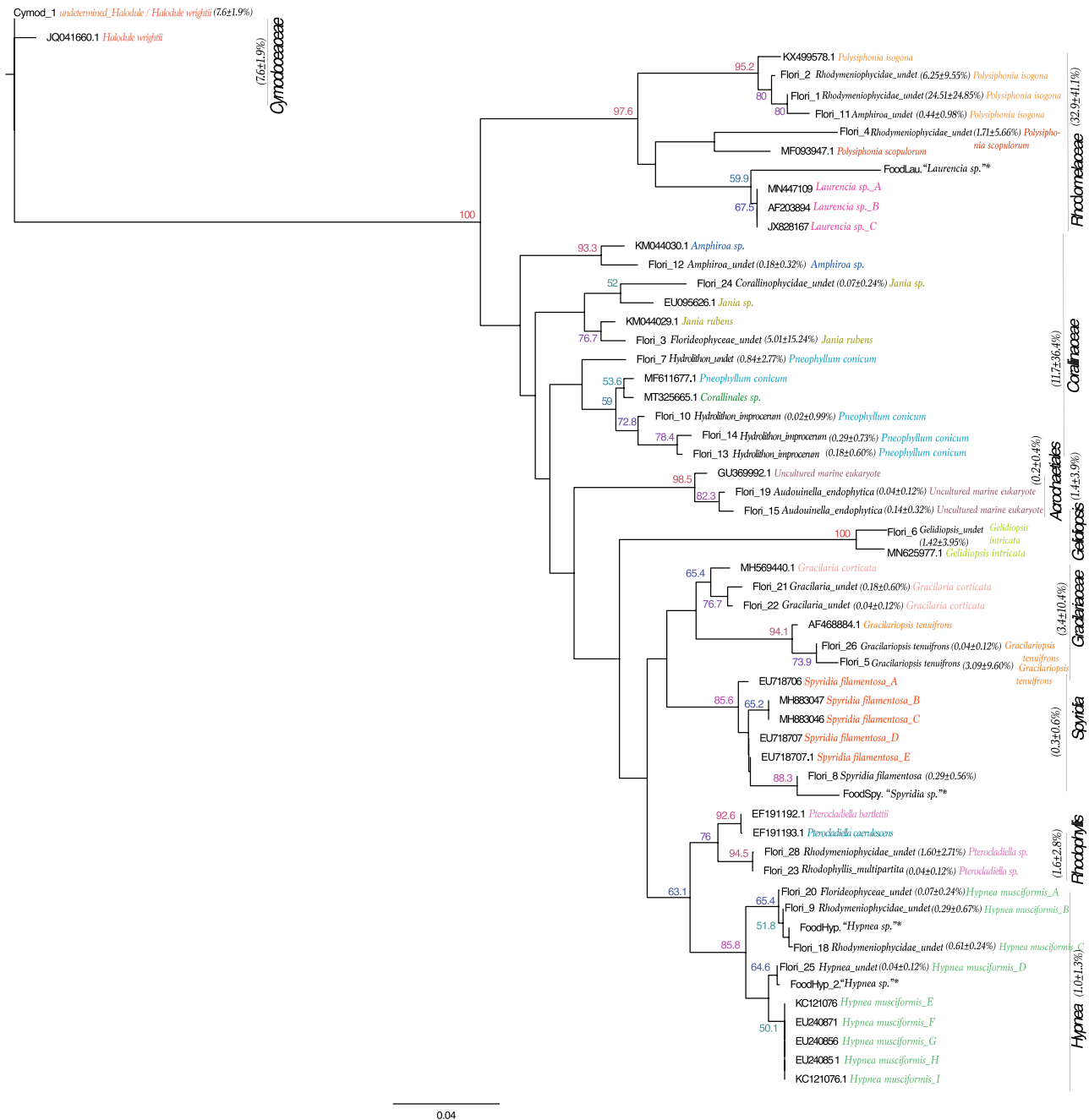


Fig. 5 Phylogenetic tree generated from the 18S rRNA gene metabarcoding of the local macrophytes. Esophageal reads belonging to the dominant red algae Rhodmeniophycidae subclass ASVs are represented as (*Flori_X*) and named following the results of the phylogenetic analysis down to species level (black 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.

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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.

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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.

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