

# A comparison of visual observation and DNA metabarcoding to assess the diet of juvenile sea turtle *Caretta caretta* in the French Mediterranean sea

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## ABSTRACT

The contents of the digestive tracts of 21 loggerhead turtles (20 juveniles and 1 adult) collected along the French Mediterranean coast were extracted during necropsies. Hard and soft items were collected and identified by visual observation. A 15-mL sample of the liquid from the rinsed contents of each digestive tract was collected and dietary DNA (dDNA) was extracted and amplified with a 18S universal eukaryotic primers. The obtained reads were compared with taxonomic DNA reference database. Ten taxonomic groups (annelids, cnidarians, crustaceans, echinoderms, fish, insects, molluscs, plants, poriferans and tunicates) were identified with visual observation and eight (ctenophores, cnidarians, crustaceans, fish, molluscs, poriferans, tunicates and plants) with dDNA metabarcoding. Annelids, echinoderms and insects were detected only with visual observation, whereas ctenophores were detected only with dDNA. The two methods were complementary to cover the range of prey, with more soft-bodied organisms detected by dDNA and more hard-shelled organisms detected by visual observation. The increased use of the dDNA metabarcoding method will help compare sea turtle diets of the different stages such as juveniles and adults, or specimens living in different areas, despite the limitations and complexity associated with its use.

**Keywords:** *Caretta caretta*, diet, DNA metabarcoding, French Mediterranean sea, loggerhead turtle, marine turtle, methodology, trophic ecology.

## Introduction

A species' diet is a key element of its ecology. Sea turtles spend most of their lifetime at sea, often making them very inaccessible to study, and their diet is still poorly understood at a global scale. In the Mediterranean Sea, the loggerhead turtle (*Caretta caretta* L.) can frequent epipelagic or benthic feeding habitats throughout its life and its diet has been described as carnivorous (e.g. Plotkin *et al.* 1993; Houghton *et al.* 2000; Lazar *et al.* 2001; Seney and Musick 2007; Casale *et al.* 2008; Frick *et al.* 2009). The standard methods used to infer its diet is the visual identification of ingested items from the gastric lavage of live turtles (e.g. Forbes and Limpus 1993) or from the digestive tracts of dead turtles (Plotkin *et al.* 1993; Laurent and Lescure 1994; Godley *et al.* 1997; Seney and Musick 2007; Frick *et al.* 2009). Some prey remains, e.g. shell debris, can also be found in faeces (Frick *et al.* 2009). However, prey identification at the species level is a time-consuming practice that relies on taxonomic expertise, which is made even more challenging by the mastication and digestive processes of the predator. The ingested elements are exposed to the digestion processes, leading to degradation and fragmentation; this is especially the case for soft-bodied organisms, which can thus be under-represented in diet identification. The analysis of stable isotopes in tissue, applicable for either dead or live turtles (McClellan *et al.* 2010), reflects the individual's level in the food web and the location of foraging grounds (e.g. Blasi *et al.* 2018; Haywood *et al.* 2020). However, it does not allow

identification of the ingested taxa, but rather broader groups of organisms with the same isotopic signature. Other methods of interest are direct in-water observation (Houghton *et al.* 2000; Schofield *et al.* 2006) or video cameras attached to sea turtles, which can also provide valuable information on their foraging ecology (Heithaus *et al.* 2002; Patel *et al.* 2016).

A relatively recent method to identify ingested prey is the use of dietary DNA (dDNA), which has allowed the description of the diet of terrestrial and marine species such as bears, rodents, bats, passerines, whales, dolphins, penguins, amphipods and bivalves (review in Valentini *et al.* 2009; Matley *et al.* 2018). In this method, the extracted and amplified DNA fragments of ingested organisms are compared with sequences in reference databases for taxa identification. This shows high promise for assessing the diet of animals that are difficult to observe in their natural environment or that have complex diets (De Barba *et al.* 2014).

The objective of this study was to test the dDNA metabarcoding method for the first time on sea turtle diet (Plotkin *et al.* 1993; Houghton *et al.* 2000; Seney and Musick 2007; Frick *et al.* 2009). We used a universal marker for all eukaryotic species located in the nuclear 18S region. This universal primer was used to access the turtle diet without any a priori assumptions and to avoid missing potential prey group. The dietary analysis was performed using the gut contents of dead loggerheads collected along the French Mediterranean coast. We compared the diet composition obtained with dDNA metabarcoding with the diet profiles revealed by the conventional method of visual observation, and then assessed the pros and cons of each method.

## Materials and methods

### Sample collection

The samples for the study were gathered from 21 dead loggerhead turtles (*Caretta caretta*) found stranded on the beach ( $n = 2$ ), floating at sea ( $n = 3$ ) or caught as bycatch (trawler:  $n = 2$ ; longline:  $n = 13$ ; unidentified gear:  $n = 1$ ) collected between 2014 and 2017. The turtles were frozen ( $-20^{\circ}\text{C}$ ) to preserve them until necropsy. Each turtle's weight ( $\pm 1$  g) and carapace length ( $\pm 1$  mm; Bolten and Carr 1999) were recorded. Only turtles with a decomposition state 'fresh' (i.e. recently dead, with internal organs in good conditions, without autolysis and swollen) were necropsied following INDICIT protocol (INDICIT Consortium 2018). First, the whole gastrointestinal tract (GI) was removed in three parts separated by clamps (the oesophagus, stomach and intestine). Then the three parts of the GI were opened, with the contents sliding directly onto a 1-mm mesh sieve, where they were rinsed with tap water above a 10-L stainless steel bucket. When the entire interior of the GI had been

rinsed, the resulting liquid was stirred with an aluminium stick to put the particles in suspension. Then a 15-mL sample was extracted with a disposable pipette and stored in a sterile 50-mL Falcon tube filled with 33 mL of absolute ethanol and 1.5 mL of buffer solution consisting of 3-molar mass acetate (Ficetola *et al.* 2008). The tubes were stored at  $-20^{\circ}\text{C}$ . All the materials (the sieves, the stick and the bucket) were decontaminated with commercial bleach (diluted to a 50% sodium hypochlorite solution) before and after each sampling (Prince and Andrus 1992). The GI contents collected in the sieve were stored in individual zipped bags (for each of the three sections of each GI) at  $-20^{\circ}\text{C}$  for later visual identification.

### Visual observation

The GI contents previously collected in the sieve were defrosted. The remains were separated into two categories, hard and soft items, and were identified to the lowest taxonomic level possible by eye or stereomicroscope ( $40\times$ ). For each GI, the presence of items (e.g. parts of shell, exoskeleton) was identified and counted (presence or absence) for each section of the GI tract (oesophagus, stomach and intestine). The scientific names of the prey were obtained from the World Register of Marine Species (<http://www.marinespecies.org/>). The prey items in the three sections were then pooled for comparison with the dDNA results, as the dDNA samples came from the liquid obtained from rinsing the whole GI. As the taxa assignment from dDNA metabarcoding were limited to the order level, the results from visual identification that had higher taxonomic resolution were adjusted to the same level for comparison.

### Blocking primer design and validation

Before the DNA amplification a blocking primer was designed following the strategy described in Vestheim and Jarman (2008). A tissue sample of *C. caretta* (blood) was extracted using DNeasy Blood & Tissue Extraction Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. DNA amplifications were performed in a final volume of 25  $\mu\text{L}$ , using 3  $\mu\text{L}$  of DNA extract as a template. The amplification mixture contained 1 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems; [www.appliedbiosystems.com](http://www.appliedbiosystems.com)), 10 mM of Tris-HCl, 50 mM of KCl, 2.5 mM of  $\text{MgCl}_2$ , 0.2 mM of each deoxynucleotide triphosphate (dNTP), 0.2  $\mu\text{M}$  of Euka02 primers (universal eukaryotic primers amplifying a  $\sim 123$ -bp region of the 18S rDNA (V7), Guardiola *et al.* 2015; Taberlet *et al.* 2018) and 0.2  $\mu\text{g}$   $\mu\text{L}^{-1}$  of bovine serum albumin (BSA; Roche Diagnostic; [www.roche.com](http://www.roche.com)). The PCR products were purified and sequenced (forward and reverse) using Sanger technology at the Eurofins MWG Operon sequencing facilities (Ebersberg, Germany). Sequences were aligned and primers were trimmed using GENEIOUS (ver. 6.0, Biomatters,

Auckland, New Zealand, see <http://www.geneious.com/>). The areas of the DNA fragment immediately following the sequence of the universal primer were selected for the design of a blocking primer. The identified blocking primers, either in position 5' or in position 3' have different lengths, increasingly longer, so as to include as many mismatches as possible between the blocking primer and the sequence of potential prey. Each new blocking primer design was validated by virtual PCR by using ecoPCR software (Bellemain *et al.* 2010; Ficetola *et al.* 2010) on release 138 of the European Molecular Biology Laboratory (EMBL) genetic database. Following this analysis, a blocking primer was thus selected. This blocking primer is located next to the forward primer and is made up of 43 bp and contains a 3' spacer (5'-TCCGATAA CGAACGAGACTCTGGCATGCTAACTAGTTATGCGA-SPC3-3'). The results of the test for this blocking primer show a potential risk of binding to species other than *C. caretta* (*Chrysemys* sp. JM-2004 and *Alligator mississippiensis*).

## DNA analysis

The DNA extraction was performed. First, the tubes were centrifuged at 15 000g for 15 min at 6°C, and the supernatants were discarded. After this step, 720 µL of ATL buffer from the DNeasy Blood & Tissue Extraction Kit (Qiagen, Hilden, Germany) was added. The tubes were then vortexed, and the supernatants were transferred to 2-mL tubes containing 20 µL of proteinase K. The tubes were then incubated at 56°C for 2 h. Subsequently, DNA extraction was performed using NucleoSpin Soil (MACHEREY-NAGEL GmbH & Co., Düren, Germany), starting from Step 6 and following the manufacturer's instructions. The elution was performed by adding 100 µL of an SE-buffer twice. A negative extraction control, a tube containing only extraction buffers, was extracted in parallel to monitor for possible contamination.

After the extraction, all the samples were tested for inhibition by quantitative PCR (qPCR; Biggs *et al.* 2015). The qPCR was performed in a final volume of 25 µL, using 3 µL of template DNA, 3 µL of  $3.53 \times 10^5$  copies µL<sup>-1</sup> of DNA of a synthetic gene, 12.5 µL of TaqMan Environmental Master Mix 2.0 (Life Technologies), 3.5 µL of ddH<sub>2</sub>O, 1 µL of each specific primer for the synthetic gene (10 µM) and 1 µL of probe (2.5 µM) under thermal cycling at 50°C for 5 min and 95°C for 10 min, followed by 55 cycles of 95°C for 30 s and 52°C for 1 min. All samples were analysed in duplicate. If at least one of the replicates showed at least two cycles of difference from the positive control run in parallel, the sample was considered inhibited and diluted five-fold before the library preparation.

After testing samples for inhibition, metabarcoding library preparation was performed using the universal eukaryotic primer pair in a final volume of 25 µL, using 3 µL of DNA extract as a template. The amplification mixture contained 1 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems; [www.appliedbiosystems.com](http://www.appliedbiosystems.com)), 10 mM

of Tris–HCl, 50 mM of KCl, 2.5 mM of MgCl<sub>2</sub>, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 0.2 µM of Euka02 primers (universal eukaryotic primers amplifying a ~123-bp region of the 18S rDNA (V7), [Guardiola \*et al.\* 2015](#); [Taberlet \*et al.\* 2018](#)), 0.2 µg µL<sup>-1</sup> of bovine serum albumin (BSA; Roche Diagnostic; [www.roche.com](http://www.roche.com)) and 4 µM of *C. caretta* blocking primer. The primers were 5'-labelled with a unique eight-nucleotide tag (with at least three differences between tags), allowing the assignment of sequences to the respective sample during the DNA sequence analysis. The PCR mixture was denatured at 95°C for 10 min, followed by 40 cycles of 30 s at 95°C, 30 s at 45°C and 1 min at 72°C, followed by a final elongation at 72°C for 7 min in a room dedicated to amplified DNA with negative air pressure and physical separation from the DNA extraction rooms (with positive air pressure). Four PCR replicates were performed per sample. Four replicates of the negative extraction control and four PCR control (ddH<sub>2</sub>O) were amplified and sequenced in parallel. After amplification, the samples were titrated using capillary electrophoresis (QIAxcel; Qiagen GmbH) and purified using a MinElute PCR purification kit (Qiagen GmbH). Before sequencing, purified DNA was titrated again using capillary electrophoresis. The purified PCR products were pooled in equal volumes to achieve an expected sequencing depth of 50 000 reads per sample. Library preparation and sequencing were performed at the Fasteris facility (Geneva, Switzerland). The library was prepared using the Metafast protocol (<https://www.fasteris.com/>). The library was sequenced using Illumina MiSeq (2 × 125 bp; Illumina, San Diego, CA, USA) and the MiSeq Reagent Kit (ver. 3, Illumina), following the manufacturer's instructions.

The sequence reads were analysed using programs implemented in the OBITools package (<http://metabarcoding.org/obitools>), following a protocol already described in [Valentini \*et al.\* \(2016\)](#). The forward and reverse reads were assembled using the illumina paired end program, by using a minimum score of 40 and by retrieving only joined sequence. The reads were then assigned to each sample by using the ngsfilter program. A separate data set was created for each sample by splitting the original data set in several files using obisplit. After this step, each PCR replicate was analysed individually before merging the taxon list for final ecological analysis. Strictly identical sequences were clustered together by using obiuniq. Sequences shorter than 20 bp, or with occurrence lower than 10 reads, were excluded using the obigrep program. The obiclean program was then run within a PCR replicate. All sequences labelled internal, that most likely correspond to PCR substitutions and indel errors, were discarded. Taxonomic assignment of the molecular operational taxonomic units (MOTUs) was performed using the *ecotag* program, with the sequences extracted from ENA Release 134 (standard sequences) of the European Bioinformatics Institute's EMBL database using the ECOPCR program ([Bellemain \*et al.\* 2010](#); [Ficetola \*et al.\* 2010](#)). Only MOTUs showing a similarity higher than 90% to the

EMBL reference database were retained for the analysis. The sequence of *C. caretta* was identified at 99% similarity with the sequences with the accession number AY859627 that correspond to *Chrysemys* sp. JM-2004 (Mallatt and Winchell 2007), which belongs to the same order of *C. caretta*. This demonstrated that the Euka2 marker cannot discriminate between two species belonging to the same order. As a consequence, all taxa detected were assigned at the order level. Finally, to take into account any incorrect assignments of sequences to the wrong sample linked to tag-jump (Schnell et al. 2015), we discarded all MOTUs with an occurrence frequency below 0.001 per library (Pont et al. 2018).

### Statistical analysis

Richness (number of taxa) and relative abundance (number of time the taxon is observed) with each method (dDNA and visual) were used to calculate the Shannon–Wiener diversity index ( $H$ ), with:

$$H = -\sum p_i \log(p_i)$$

where  $p_i$  is the proportional abundance of taxa  $i$  (R package Vegan, Function diversity). The measures of similarity between the two methods were evaluated by the Jaccard index, as the size of the intersection between the two data sets (i.e. number of taxa recorded by the two methods) divided by the size of the union of the two samples. Finally, so as to take into account the individual diets, we used a permutational linear model with the number of times the taxa was detected per individual diets as a response variable and the method (Visual, dDNA) as an explanatory variable. We consider the individual diets as random effects so as to consider the error caused by inter-individual variations (lmer library in R).

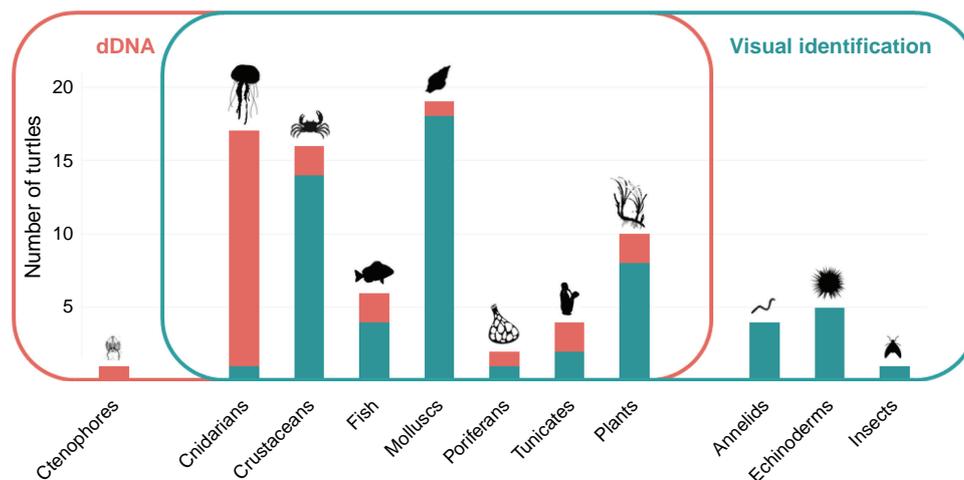
## Results

The 21 loggerhead turtles analysed ranged from 29.0 to 76.0 cm in curved carapace length (CCL; average 53.2 cm) and weighed between 3.3 and 58.4 kg (average 22.4 kg). One individual was identified as an adult (male); the remaining 20 specimens were juveniles. Of the 21 digestive tracts examined, all contained food remains, with both identifiable and unidentifiable items.

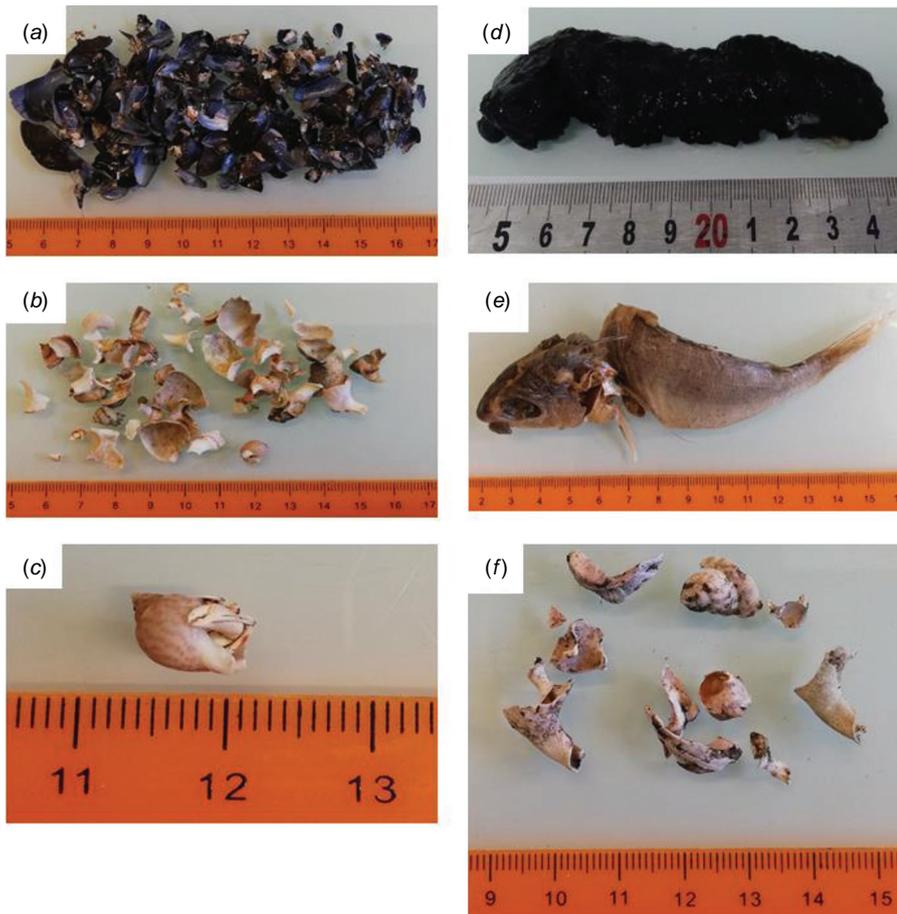
### Visual identification of prey

In total, 10 taxonomic groups (annelids, cnidarians, crustaceans, echinoderms, fish, insects, molluscs, plants, poriferans and tunicates) were identified with visual observation (Fig. 1). The main hard items collected were from annelids (e.g. tubes), crustaceans (e.g. parts of exoskeleton, carapace), insects (parts of exoskeleton and wings), echinoderms (e.g. calcareous plates), fish (e.g. scales, bones), molluscs (e.g. shells of bivalves, operculum of gastropods, beak of cephalopods) and plants (wood). Soft items mainly consisted of body parts of crustaceans (from the entire organism to fragments), echinoderms (gonads or entire organism for *Holothuroidea*), fish (from skin to entire organism), molluscs (e.g. partly digested cephalopods), poriferans and tunicates (from the entire specimen to identified fragments; details in Supplementary Table S1).

For one specimen, no items could be identified by visual observation of the gut contents. For the remaining 20 individuals, the number of observed items ranged from one to seven (average 3.5) per individual diet. The most frequently detected prey were crustaceans (in 14 diets), gastropods (in 13 diets) and bivalves (in 12 diets). Concerning gastropods, the specimen was probably eaten alive when the entire organism was observed (in 3 diets), or when operculum



**Fig. 1.** Diet composition of 21 loggerheads turtles found in the Gulf of Lion (French Mediterranean coast) between 2014 and 2017. Venn diagram showing the occurrence of the taxa identified up to the order level by dDNA metabarcoding (red), visual identification (blue) or both methods.



**Fig. 2.** A selection of items collected in loggerhead digestive tracts. (a) Bivalvia: *Mytilus galloprovincialis* (freshly broken shell). (b) Gastropoda: *Tritia mutabilis* (freshly broken shell). (c) Decapoda: *Pagurus* sp. (entire organism inside a *Tritia mutabilis* shell). (d) Echinoderm: *Holothuria mammata*. (e) Fish: *Pagellus acarne*. (f) Ancient shell of gastropod *Tritia mutabilis* (for a comparison, see the recent shell in b).

was observed (in 2 diets). In four diets, the shell was ancient (or shell hash, i.e. pieces of old shell that were not part of a living animal), with clear tracks of wear indicating that the living organism was not there; in three of these four diets, a crustacean (Paguridae) was identified.

In the analysis of the food remains in the separate parts of the GI, the number of taxa progressively increased from the oesophagus ( $N = 6$ ), to the stomach ( $N = 7$ ), and to the intestine ( $N = 10$ ). The respective proportion of hard and soft parts for each taxon was similar in the three sections of the digestive tract (Supplementary Fig. S1).

Several groups were identified up to specific taxonomic levels (details in Supplementary Table S2). The abrasion of the shell or the presence of operculum can provide information about the status (alive or dead) of bivalves (Fig. 2a) and gastropods (Fig. 2b) when ingested. The shells of *Tritia mutabilis* were strongly abraded, indicating that the specimen died many years ago (Fig. 2f), whereas the presence of a smooth broken shell without tracks of abrasion argue for their recent ingestion as live prey (Fig. 2b). Crustaceans including decapods and hermit crabs (Paguridae) were also identified (Fig. 2c). The presence of parts of Paguridae exoskeletons (carapace and claws) and abraded shells (or fragments) of gastropods (e.g. *Tritia mutabilis* and

*Euspira catena*) indicated that the directly ingested prey was probably the crustacean (which use the gastropod shell as shelter), and not the gastropod. Echinoderms such as several species of echinoids (e.g. *Sphaerechinus granularis*) were identified from small parts of calcareous plates (1–5 mm), and holothuroids (e.g. *Holothuria tubulosa*, *H. mammata*) were also observed (Fig. 2d). Fish identified through visual observation included a partly digested *Pagellus acarne* (Fig. 2e).

### dDNA identification of prey

Microorganisms from the Chromista and Fungi kingdoms, detected with dDNA metabarcoding, were removed from the analysis, because they were most certainly present in the water or on other ingested items and do not make up part of the intentional diet of loggerheads. The DNA of Testudines was also detected in the GI contents of 12 turtles, for one of which, it was the only genetic material detected. These results were also removed from the diet composition analysis, as it is likely that the detected DNA belonged to the individuals themselves and was not the result of predation on other turtles (although see ‘Discussion’). Of the remaining organisms detected by dDNA metabarcoding, eight taxonomic groups (cnidarians, ctenophores, crustaceans, fish,

molluscs, plants, poriferans and tunicates) were identified (Fig. 1). No annelids, echinoderms or insects were detected with dDNA metabarcoding. In three individuals, no genetic material that might belong to a loggerhead's diet was detected (Supplementary Fig. S2). For the remaining 18 turtles, the collected samples (15 mL of liquid from the rinsed GI contents) allowed the identification of the taxonomic group and the number of detected taxa ranged from one to seven (average 2.1) per individual. The most frequently detected type of prey using dDNA metabarcoding was cnidarians (in 15 diets). More specifically, scyphozoans were detected in 13 diets, hydrozoans in 6 diets, and anthozoans in 5 diets (Supplementary Table S2). In total, 3 290 239 genetic sequences assigned to loggerhead dietary taxa were retrieved, corresponding to an average of  $99\,951 \pm 96\,862$  reads per samples. The groups with the highest number of reads were cnidarians (960 337; average 185 021), fish (89 021; average 44 510.5) and crustaceans (56 697; average 28 348.5).

In total, 11 taxonomic groups were detected with either dDNA metabarcoding or visual identification (in the entire gastrointestinal tract; Fig. 1). Three groups were detected only visually, including annelids (in four diets), echinoderms (in five diets) and insects (in one diet). One taxonomic group was detected only with dDNA metabarcoding, namely Ctenophores (in one diet). The occurrence rates (number of diets with the item/total number of diets) varied between the visual and dDNA methods (Fig. 1): cnidarians were detected in 88.9% (16/18) of diets by dDNA and 10.0% (2/20) of diets by visual observation. Crustaceans were detected in 11.1% (2/18) of diets by dDNA and 70.0% of diets (14/20) by visual observation. Molluscs were detected in 5.6% (1/18) of diets by dDNA and 90.0% (18/20) of diets by visual observation. Fish were detected in 11.1% (2/18) of diets by dDNA and 20.0% (4/20) of diets by visual observation. Poriferans were detected in 11.1% (2/18) of diets by dDNA and 10.0% (2/20) by visual observation. Tunicates were detected in 22.2% (4/18) of diets by dDNA and 20.0% (2/20) by visual observation. Finally, plants were detected in 22.2% (4/18) of diets by dDNA and 35.0% (7/20) of diets by visual observation.

The Shannon–Wiener diversity index was  $H = 1.255$  for dDNA and  $H = 1.522$  for visual observation, indicating that both richness and evenness were similarly evaluated. This similarity is confirmed by the high value of the Jaccard similarity index ( $=0.69$ ). Finally, the comparison of the methods taking into account the potential differences among individual diets with a permutational linear model confirmed the similarity of the diets described at the taxonomic level used for these comparisons (adjusted  $R^2 = -0.002071$ ;  $F$ -statistic: 0.8161, d.f. = 88,  $P$ -value: 0.3688).

## Discussion

The 21 loggerhead turtles used for sampling were collected in the French coastal environment of the Mediterranean Sea,

a region used by the species as a feeding area, especially for juveniles (Laurent et al. 1998; Darmon et al. 2017). The observed ingested items confirmed the carnivorous diet of this species (Plotkin et al. 1993; Seney and Musick 2007; Frick et al. 2009). Studies have shown that benthic organisms, hard-shelled and soft prey, sessile as well as small moving prey are all consumed by loggerheads, with crustaceans (e.g. Casale et al. 2008) and molluscs (e.g. Larzar et al. 2001) being an important part of the diet (Plotkin et al. 1993; Laurent and Lescure 1994; Godley et al. 1997; Houghton et al. 2000; Wallace et al. 2009). The observation of fish in the digestive tract confirms that loggerheads can also catch more mobile prey (Hirama and Witherington 2012). However, feeding of entrails of fish discarded by fishermen (Houghton et al. 2000), fish caught in nets (Tomas et al. 2006) or used as bait (Revelles et al. 2007; Seney and Musick 2007) has been described.

We found that some taxa were detected only by either the visual or the dDNA metabarcoding method. In our study, ctenophores were detected only by dDNA metabarcoding and cnidarians were detected in 16 of 21 diets by dDNA metabarcoding and in 2 of 21 diets by visual observation. The absence of an exoskeleton or hard shell makes these organisms highly digestible and hard to detect visually once ingested, but their remaining DNA in the digestive tract was successfully detected. This suggests that it is highly probable that soft organisms are under-represented in sea turtle diets characterised by visual identification.

By contrast, annelids, echinoderms and insects were detected only by visual observation. The non-detection of taxa in dDNA methods can result from missing information at several steps of the procedure, including DNA degradation (the diet was studied from dead stranded specimens), sample collection (if the DNA is absent from the 15-mL sample of the liquid from the rinsed digestive contents), laboratory procedural steps (from issues with DNA extraction, PCR amplification or sequencing) and data analysis (because of incorrect taxa assignment and lack of resolution of the universal primer chosen; Valentini et al. 2009; Pompanon et al. 2012; De Barba et al. 2014). It should also be noted that, because the diet of *C. caretta* is very diverse, it was not possible to test the blocking primer *in vitro* and, thus, it was not possible to evaluate whether it will reduce the amplification of some of the key target species. Increasing the volume of samples (e.g. > 15 mL) may reduce the possibility of missing DNA present at a low density. Increasing technical replicates (e.g. multiple extractions or PCR amplifications from the same sample) can decrease the risk of false negatives (Ficetola et al. 2015; Grey et al. 2018), as well as increasing the sequencing depth per sample. The development of specific primers for some key groups (e.g. as in Decapoda, Komai et al. 2019) in genetic regions with higher resolutive power (e.g. *16S* or *COI*) and increasing the contents of the reference database will also improve the detectability and quality of taxa identification with DNA metabarcoding. However, limitations remain,

because, even for the most commonly used barcoding gene (*COI*), currently only 40% of mollusc species are represented in DNA barcode reference libraries (Weigand *et al.* 2019).

Another typical bias in diet analysis is the identification of an organism in the gut contents that was not a selected prey. For instance, considering the signs of wear, we found that the remains of most shelled molluscs seemed to originate from ancient or recently dead organisms; it is likely that, given the large number of hermit crabs found in the same samples, these creatures inhabited the shells and were the selected prey. Turtles may also ingest some items accidentally, such as shells or echinoderm fragments, when feeding on the seabed. Similarly, dDNA metabarcoding can detect the presence of DNA of secondary prey (e.g. present in the stomach of fish prey), or of epibionts (e.g. annelids or cnidarians on the shells of molluscs).

Our dDNA metabarcoding analysis also detected DNA from the order testudines in 12 samples. The presence of a high quantity of an organism's own DNA in its gut content suggests that the blocking primer most likely failed in these 12 samples. However, Shehzad *et al.* (2012) found that the technique of blocking oligonucleotides inhibited the amplification of the predator's DNA (which may represent more than 90% of retrieved sequences), but uncovered more prey taxa in the diet that had not been amplified previously without the blocking oligonucleotide. Given that we used this blocking technique, the possibility of cannibalism cannot be excluded, a rare but not unknown phenomenon when considering the diet of loggerheads; an adult was found to have consumed a hatchling in South Africa, and another specimen (56 cm CCL) had remains of a juvenile (10 cm long) in its gut contents in the Azores (Frick *et al.* 2009).

## Conclusions

A comparison of visual identification and dDNA metabarcoding methods to infer sea turtle diets suggests that the two methods are complementary. Both have certain drawbacks; visual identification can be time consuming and somewhat subjective, whereas prey can be undetected with dDNA metabarcoding if their DNA sequences are not available in reference databases. Nonetheless, as dDNA metabarcoding methods are now being successfully used for marine biota inventories (e.g. Yamamoto *et al.* 2017), the taxonomic coverage of the databases is improving, even if there is not yet consensus on the genomic region used. These advances make dDNA metabarcoding a promising tool for studying trophic interactions and many other ecological topics for a wide range of terrestrial and marine biota, despite the limitations and complexity associated with its use (Alberdi *et al.* 2019).

## Supplementary material

Supplementary material is available [online](#).

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**Data availability.** The data sets collected and analysed during the current study are available from the corresponding author on reasonable request. All Illumina raw sequences data are available on FigShare doi:10.6084/m9.figshare.13386314.

**Conflicts of interest.** A. Valentini and T. Dejean are research scientists in a private company that specialises on the use of eDNA for species detection.

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