

DNA metabarcoding in diet studies: Unveiling ecological aspects in aquatic and terrestrial ecosystems

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Funding information

Fundação para a Ciência e a Tecnologia,
Grant/Award Number: (IF-FCT
contract IF/00359/2015, PTDC/
BIA-MIC/27995/2017-POCI-01-
0145-FEDER-027995 and PTDC/
MAR-BIO/0902/2014-POCI-01-0145-
FEDER-016550;

Abstract

Effective conservation of species and ecosystems requires the understanding of important ecological traits, such as dietary habits, food webs, and trophic niches. In diet studies, the visual identification of partially digested prey has been enhanced with the recent more powerful and accurate technique, DNA barcoding. Here, we summarize the contribution of this recent methodology to the investigation of both terrestrial and aquatic taxa diet, and compare the level of novelty uncovered through the use of this technique regarding species' ecology. From a total of 150 studies analyzed, focusing on more than 250 vertebrate wild species, seven domesticated taxa, and humans, we suggest that barcoding has led to more significant findings for aquatic taxa and ecosystems, where direct observations of feeding events and consequent trophic niche understanding are typically limited. Finally, we introduce the term dietary DNA (dDNA) to describe environmental approaches that use DNA extracted from gut, stomach, or fecal contents, aiming to assess both species dietary habits and describe local biodiversity. Particularly, we highlight the complementarity of environmental DNA (eDNA) and dDNA as a new tool for biodiversity assessments in remote areas, including most of the aquatic realm.

KEYWORDS

dietary DNA, dietary sampling, environmental DNA, food webs, high-throughput sequencing, trophic niche

1 | DIFFERENT METHODOLOGIES FOR THE ASSESSMENT OF TROPHIC INTERACTIONS

The description of trophic interactions and food webs is a fundamental process in understanding ecosystems functionality (Krahn et al., 2007; Symondson, 2002). Multiple ecological-informed management decisions rely on dietary studies, such as the trophic position of species and their demographic regulation, based on the importance of feeding resources, or the impact of species interactions

(e.g., predation) on populations and communities (e.g., Alonso et al., 2014). Moreover, predator–prey interactions define most of the food web dynamics (Carreon-Martinez & Heath 2010; Jo, Gim, Jeong, Kim, & Joo, 2014), and given the current environmental changing scenario, their better knowledge is necessary to design effective management and conservation plans (Bradley et al., 2007; Roslin & Majaneva, 2016).

Traditionally, species' diets have been mostly identified through direct observation of predatory events or by visual morphological analysis of stomach, gut, or fecal contents (Caryl et al., 2012; Reñones, Polunin, & Goni, 2002). The accuracy of such

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identifications relies on portions of undigested prey items and on considerable taxonomic expertise (Sheppard & Harwood, 2005). Additionally, the morphological identification of such undigested items depends on the existence of complete regional reference collections of potential prey, besides being considerably time-consuming (Packer, Gibbs, Sheffield, & Hanner, 2009). On the other hand, these traditional methods require almost no equipment and are relatively inexpensive if one does not take into account the costs of person-hours. Other methods, such as stable isotopes and quantitative fatty acid analysis, do not require such taxonomic knowledge and allow for long-term data on dietary habits. However, these alternatives are invasive and are not prey-specific (Bradshaw et al., 2003; Iverson, Field, Don Bowen, & Blanchard, 2004). Although not without controversy (see, e.g., de Carvalho et al., 2007; Wägele et al., 2011), a current shortage of taxonomists has led to the so-called "taxonomic impediment" (Wheeler, Raven, & Wilson, 2004) and the rise of molecular barcoding as a widely used tool for the identification of species (Hebert, Ratnasingham, & Waard, 2003). Indeed, DNA barcoding is now acknowledged to address many of the problems inherent to morphological taxonomy, such as identifying cryptic species or linking different life stages (e.g., larvae) to a single species (Packer et al., 2009). Importantly, unlike morphological examination or stable isotopes, DNA barcoding can provide taxonomic resolution to the species level, allowing a more accurate description of the diversity of prey items (e.g., Granquist, Esparza-Salas, Hauksson, Karlsson, & Angerbjörn, 2018; Jeanniard-du-Dot, Thomas, Cherel, Trites, & Guinet, 2017).

The first studies using DNA barcoding in the assessment of wild-life dietary habits, with particular emphasis on invertebrates, were reviewed by Symondson (2002). Briefly, the first molecular assay developed to assess a species' diet dates from 1992 and was conducted to determine whether plant DNA could survive digestion in the European brown bear *Ursus arctos* (Höss, Kohn, Pääbo, Knauer, & Schröder, 1992). These authors successfully amplified via polymerase chain reaction (PCR) a portion of the chloroplast *rbcl* gene from feces, suggesting that barcoding approaches could be used to study the diet of endangered species, using noninvasive sampling. Later, Taberlet & Fumagalli (1996) used general primers developed for the amplification of mammalian DNA extracted from bones retrieved from owl pellets (*Strix aluco* and *Tyto alba*). Two years later, another groundbreaking study successfully sequenced the *rbcl* retrieved from coprolites (ancient feces) of the extinct ground sloth *Nothrotheriops shastensis*, allowing the identification of several plants to order and family level (Poinar et al., 1998). At the same time, Scribner & Bowman (1998) developed a strategy to identify and distinguish waterfowl species in the stomach contents of the gulls *Larus hyperboreus* using microsatellites. These earlier studies led ecologists to realize the versatility of molecular barcoding approaches to identify prey items from various taxonomic groups, which explains the rise in the number of studies using these techniques (see Figure 1a and Table S1).

The investigation of the diet of aquatic organisms poses particular challenges: (a) the difficulty to use noninvasive methods, such as collecting feces or pellets; (b) the rarity of an opportunistic encounter of a recently deceased animal to access its gut content; and (c) the less likelihood of a direct observation of feeding interactions than in the terrestrial environment. This might explain why the total number of metabarcoding studies focusing on aquatic taxa is lagging well behind those focusing on terrestrial vertebrates (Figure 1a). The first studies using molecular approaches to study the diets of aquatic animals aimed at identifying specific groups of prey (krill and fish taxa) from the guts or feces of the sand shrimp *Crangon affinis* (Asahida et al., 1997); the giant squid *Architeuthis* sp. (Jarman, Deagle, & Gales, 2004); whales *Balaenoptera musculus* (Jarman, Gales, Tierney, Gill, & Elliott, 2002; Jarman & Wilson, 2004); penguins (*Pygoscelis adeliae* and *Eudyptes chrysolophus*) (Deagle et al., 2007; Jarman et al., 2002; Jarman & Wilson, 2004); seals (*Halichoerus grypus* and *Phoca vitulina*) (Kvitrud, Riemer, Brown, Bellinger, & Banks, 2005; Parsons, Piernney, Middlemas, Hammond, & Armstrong, 2005); and sea lions *Eumetopias jubatus* (Deagle et al., 2005). Over the last decade, but particularly in the last couple of years, the number of diet studies on aquatic species using molecular strategies significantly increased and presently they are almost as frequent as the studies on terrestrial organisms (Figure 1a).

In this review, we compiled published studies using barcoding techniques to study the diets of terrestrial and aquatic vertebrate species to (a) assess the main methodological differences used in studies conducted in these two environments; (b) highlight most relevant outcomes; and (c) discuss the use of metabarcoding dietary studies to unravel ecosystem diversity, especially in the more inaccessible aquatic environment. We argue that metabarcoding has been, and will remain, a valuable tool to unveil new trophic interactions, dietary compositions, and foraging behaviors, besides assisting in a better taxonomic understanding, particularly of aquatic ecosystems. Lastly, we suggest and highlight the potential of using a dietary DNA (or dDNA) approach (i.e., environmental DNA approaches that use DNA extracted from gut, stomach, or fecal contents), assisted or not by other sampling schemes, with the purposes of describing local aquatic biodiversity.

1.1 | The advent of high-throughput sequencing and the expansion of metabarcoding as a tool for studying diets

Although the results from the first seminal studies using molecular barcodes highlighted the potential of this technique to (a) identify highly digestible and fully digested diet items, impossible to classify through morphologic analyses (Höss et al., 1992; Kasper, Reeson, Cooper, Perry, & Austin, 2004), and (b) even disclose diet items present in ancient fecal samples (Poinar et al., 1998), most of these studies required a priori knowledge regarding consumed prey (but see Riemann et al., 2010). Taxa-specific primers were often designed to assess particular items on the diet of the target organism, such as salmonid-specific (Parsons et al., 2005) or krill-specific primers

(Jarman et al., 2002), and frequently required previous methodological validation (Deagle et al., 2005; Purcell, Mackey, LaHood, Huber, & Park, 2004).

Recent methodological advances have set aside most of these constraints. A major breakthrough was the advent of high-throughput sequencing (HTS) allowing the parallel sequencing of millions of DNA fragments. Through the combination of high-throughput sequencing (Margulies et al., 2005), the use of "universal" PCR primers to maximize DNA detection from the widest possible range of prey species (Jusino et al., 2019; Valentini, Pompanon, & Taberlet, 2009), and new bioinformatic tools for the selection of appropriate molecular barcodes and data curation (Brown et al., 2012), the metabarcoding of mixed samples (feces, stomach, and gut contents) is now viable and widely used. Importantly, the diet of high numbers of hosts (potentially many hundreds) can be assessed in a single sequencing run, at a relatively low cost per sample, and without a considerable

a priori knowledge regarding the diet composition. Due to the high number of samples that can be processed simultaneously, studies focusing simultaneously in different predator species, with similar (e.g., Pansu et al., 2019) or distinct feeding ecologies (e.g., Gordon et al., 2019), are increasing (Figure 1b).

Briefly, the implementation of this methodology involves (a) DNA extraction from a bulk sample (of either partially or fully digested items) (Aguilar et al., 2017; Harms-Tuohy, Schizas, & Appeldoorn, 2016); (b) a PCR targeting small fragments of DNA (to reduce biases caused from DNA degradation) from all the organisms present; and (c) HTS. Posterior data analysis, which allows taxonomic inferences of the prey items, requires comparing the obtained sequences with public databases (e.g., GenBank from the National Center for Biotechnology Information, or the Barcode of Life Data System, BOLD) (Ratnasingham & Hebert, 2007; <http://www.boldsystems.org/>). Therefore, it is only at this

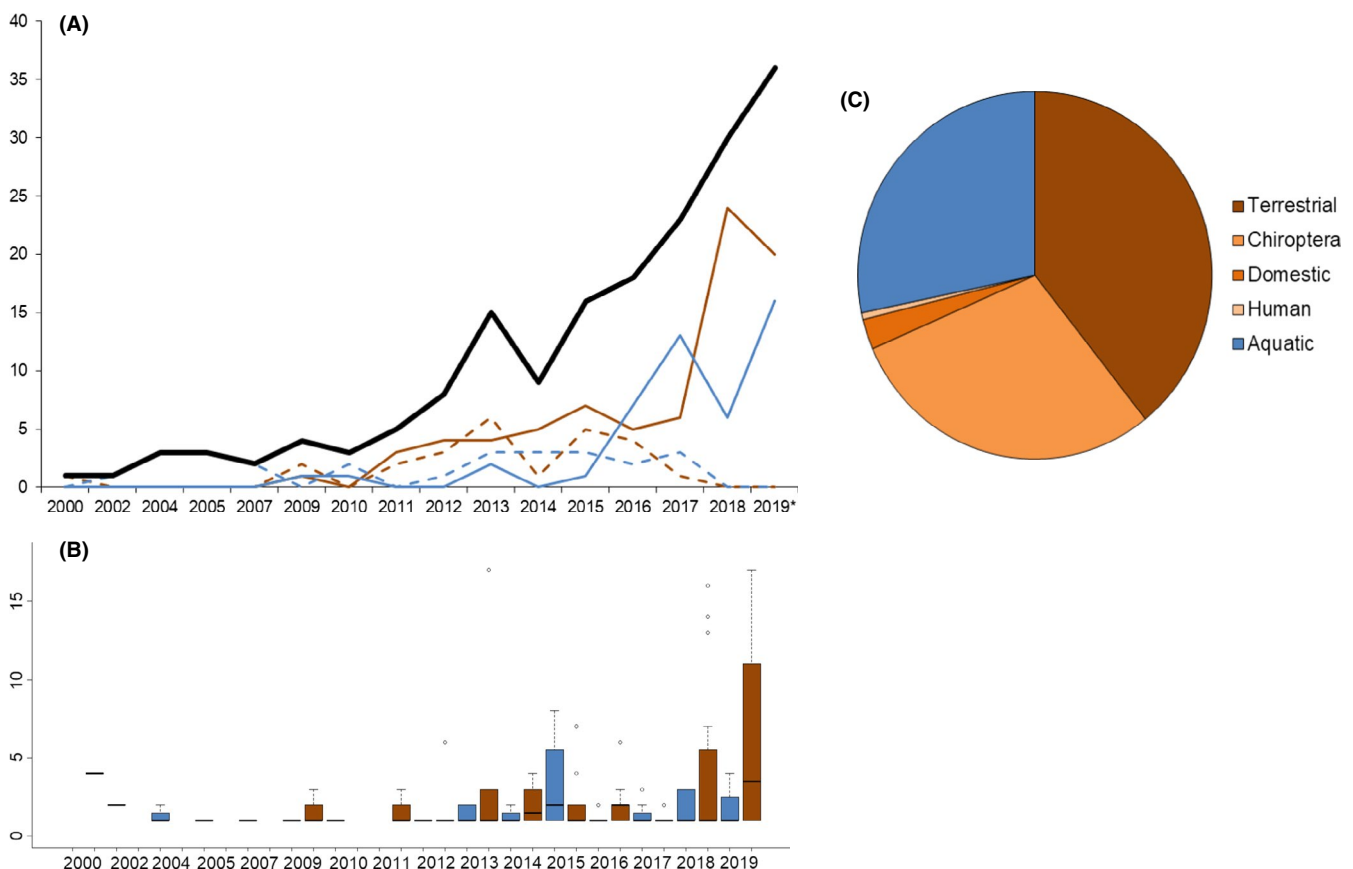


FIGURE 1 Number of studies on diet of both terrestrial and aquatic vertebrate species recovered from a search on Google Scholar using the words "metabarcoding," "vertebrates," and "diet," for the period between 2000 and 31 March 2019. (a) Total number of studies per year (bold black line), sequencing platform (thin straight lines: high-throughput next-generation sequencing techniques; thin dashed lines: Sanger sequencing method or other traditional molecular technique, e.g., RFLP), and habitat (brown–dark gray lines: terrestrial; blue–light gray lines: aquatic and interface). * Counts for 2019 are estimates for the whole year, considering the same rate of publication as in the first trimester of that year. (b) Boxplots for the number of species analyzed simultaneously per year and ecosystem. Coghlan et al. (2013) and Corse et al. (2019) were included in the terrestrial dataset, because the number of terrestrial species included was largely superior (15 out of 17 and eight out of 11, respectively). (c) Relative numbers of species from different habitats, whose diet was assessed through barcoding techniques. Some species were studied more than once, in a total of 261 different wild species, two unidentified (pipit, Coghlan et al., 2013; and *Bison* sp., Willerslev et al., 2014), seven domesticated taxa, two studies focused on humans, and one taxa described only by a superior taxonomic rank (i.e., Muridade; Shirako et al., 2015)

stage that some previous knowledge might still be needed, particularly for more accurate taxonomic identification of the ingested items (Bohmann et al., 2011). Currently, we are on the verge of acquiring important ecological insights in terms of species dietary clarification, and consequently of trophic niches and species interactions, since we can now more readily identify prey items to genus and even species levels (e.g., Crisol-Martínez et al., 2016; Jo et al., 2014). Despite being broadly consensual that DNA-based methodologies allow a more accurate taxonomic understanding of prey items in digested material (Pickett, Bergey, & Di Fiore, 2012; Zeale, Butlin, Barker, Lees, & Jones, 2011), several authors still advocate the use of both nonmolecular (morphology) and molecular (barcoding) techniques to describe vertebrates' diets, particularly in terrestrial ecosystems (e.g., Groom, White, Mitchell, Roberts, & Mawson, 2017; Pereira, Xavier, Perera, Salvi, & Harris, 2019; Yang, Zhan, Cao, Meng, & Xu, 2016) and to study ancient dietary habits in humans (Sawafuji, Saso, Suda, Hattori, & Ueda, 2018). Stable isotope analysis was also recommended by some authors to complement the study of diet habits of aquatic organisms (Sylväranta, Harrod, Kubicek, Cappanera, & Houghton, 2012; Tverin et al., 2019). Ultimately, however, DNA metabarcoding is foreseen as the most powerful tool to reconstruct both trophic networks (Roslin & Majaneva, 2016) and networks of ecological interactions (Berry et al., 2019; Bohan et al., 2017; Evans, Kitson, Lunt, Straw, & Pocock, 2016). It is not difficult then to envisage how ecosystem management could benefit from a wide metabarcoding application to diet studies, especially since species adjustments due to environmental perturbation could then readily be assessed.

1.2 | Marker choice for metabarcoding of diets: Differences between terrestrial versus aquatic species

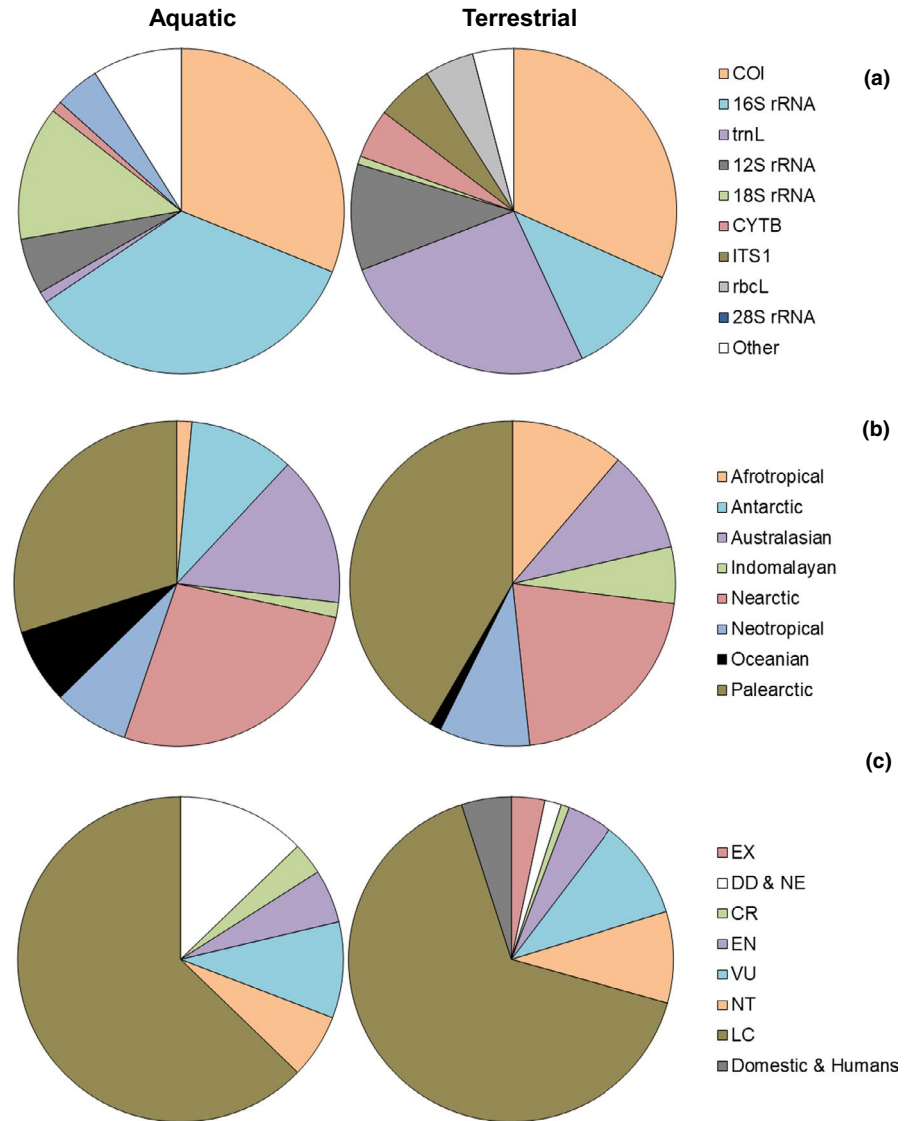
Traditional DNA barcoding approaches are dependent on specifically designed primers that target the genomes under investigation (Fišer Pečnikar & Buzan, 2014). In this regard, the use of the cytochrome c oxidase subunit 1 (COI) as a suitable molecular marker for the barcode for animal life is well established (Coissac et al., 2016; Hebert et al., 2003; Lobo et al., 2013), and this was the most widely used marker in the dietary studies surveyed herein (Figure 2a; 67 out of 213 of markers and types of markers, i.e., genomic approaches and microsatellites; Table S1). Currently, more than 35,000 COI sequences from vertebrate species are deposited in GenBank (last accessed on April 13 2019), and BOLD contains more than 500,000 barcodes for Chordata (last accessed on April 15 2019). However, the use of COI as a molecular barcode is not consensual. While some authors question the use of a single molecular barcode marker, and specifically the use of COI (reviewed by Taylor & Harris, 2012), others suggest the use of a combination of different primer sets to amplify this marker in multiple taxonomic groups (Corse et al., 2019). For these reasons, the use of other molecular markers has been implemented in many diet studies with success at recovering a broad taxonomic range of prey items. In this regard, 16S rRNA is

increasingly being preferred over COI, since this is a more conserved locus across taxonomic ranks and the primers employed can be more "universal" while attaining the same approximate taxonomic resolution (Dunshea, 2009), and is the second most widely used marker in the dietary studies published since the early 2000s (Figure 2a). In the case of diet studies of aquatic species, this gene has been applied in the same proportion as COI (Figure 2a). Ultimately, the approach that will warrant best results in terms of taxonomic coverage is the combination of multiple markers (Chen et al., 2009; McFadden et al., 2011; Shearer & Coffroth, 2008), and this has been applied in several dietary studies (Berry et al., 2017; Pereira et al., 2019; Xavier et al., 2018), albeit only about one third of the studies surveyed herein used a multiloci approach (Table S1). Finally, genomic approaches, describing complete organelle genomes and nuclear ribosomal DNA, encompassing many potential barcoding loci, have been suggested as a more accurate and powerful alternative (Coissac et al., 2016). Notwithstanding, this has been applied only to study the diet of two terrestrial mammalian model species: in a methodological study focusing on the house rat *Rattus rattus* (Pearman et al., 2018) and for disclosing diets of humans (Søe et al., 2018).

2 | CONFIRMATION OF TERRESTRIAL DIETARY PREFERENCES AND A PARADIGM SHIFTING TOOL FOR AQUATIC SPECIES

Generally, the most common result from comparisons between traditional morphological analyses and molecular-based methodologies is a higher taxonomic resolution and, consequently, the disclosure of higher prey diversity (e.g., Berry et al., 2015; Zeale et al., 2011). In the case of terrestrial species, the use of molecular-based methodologies for diet assessments has contributed to a better understanding of trophic niche and/or to detect seasonal variation in dietary preferences. This was the case of the European free-tailed bat *Tadarida teniotis* for which a significant dietary difference was found to be gender-linked (Mata et al., 2016) or that of the little brown bat *Myotis loncifugus* whose diet was found to be driven by both seasonal and geographical changes in insect diversity (Clare et al., 2014). In fact, this seasonal signature in diet variability is present throughout the terrestrial ecosystems, where a continuous adjustment of diet to seasonal fluctuation of resource availability is observed (from lizards to larger mammals; e.g., Bergmann et al., 2015; Kartzinell & Pringle, 2015). DNA metabarcoding has also been able to clarify the feeding ecology of terrestrial elusive species, for example, in the case of the snow leopard *Panthera uncia* (Shehzad, McCarthy, et al., 2012; Weiskopf, Kachel, & McCarthy, 2016) and that of large African mammalian herbivores, for which the diet composition was found to strongly differ across newly identified cryptic species, irrespective of feeding guild (Kartzinell et al., 2015). Ultimately, there was also an increased value of metabarcoding approaches to study the feeding ecology of endangered species, where the accurate description of dietary habits is crucial to the design and enforcement

FIGURE 2 Relative counts of (a) molecular markers used, (b) realms assessed and (c) species per conservation status (IUCN, 2019) analyzed in the 150 surveyed studies, per ecosystem. (b) and (c) Some species were studied more than once. Molecular marker: COI, cytochrome c oxidase I; 16S rRNA, 16S ribosomal ribonucleic acid; *trnL* P6, loop of the *trnL* (UAA) intron; 12S rRNA, 12S ribosomal ribonucleic acid; 18S rRNA, 18S ribosomal ribonucleic acid; CYTB, cytochrome b; ITS1, internal transcribed spacer 1; *rbcL*, ribulose biphosphate carboxylase large chain; and 28S rRNA, 28S ribosomal ribonucleic acid and other: ATPase6 ATP synthase membrane subunit 6, cytochrome c oxidase III, displacement loop, growth hormone type-2, internal transcribed spacer 2, rRNA large subunit ribosomal ribonucleic acid, NADH ubiquinone oxidoreductase core subunit 3, rRNA small subunit ribosomal ribonucleic acid. Threatened status: EX, extinct; DD, data deficient; NE, not evaluated; CR, critically endangered; EN, endangered; VU, vulnerable; NT, near threatened; and LC, least concern



of conservation and management strategies. For example, introduced plant species in an oceanic island in Japan are an important food source for the critically endangered red-headed wood pigeon *Columba janthina nitens*; hence, rapid elimination of these invasives could compromise the survival of that species (Ando et al., 2013). Albeit achieving higher taxonomic resolution relatively to traditional visual inspection of prey remains, most of the dietary studies for terrestrial species mainly fostered the confirmation of a species' dietary preferences, with few studies revealing previously unknown prey items, ecological traits, or trophic interactions (e.g., Gordon et al., 2019; Vesterinen et al., 2016).

In comparison, the use of metabarcoding techniques to inspect aquatic trophic niches (including marine birds) has led to a noticeably higher number of unexpected new ecological findings (Granquist et al., 2018; Leray, Meyer, & Mills, 2015; Riemann et al., 2010; Table S1). Many of the studies reviewed herein allowed the repositioning of species in aquatic trophic webs and uncovered previously unknown species interactions. The difference in relevance of these new findings for aquatic organisms is presumably linked with the fact that the

observation of feeding events or collection of feces is generally less feasible in the aquatic ecosystems, and therefore, previous knowledge was more limited (e.g., Waap et al., 2017). Examples of such relevant novelties include the surprising finding of cartilaginous prey in the diet of the Australian fur seals *Arctocephalus pusillus doriferus* (Deagle, Kirkwood, & Jarman, 2009); the cannibalistic predation of the red lionfish *Pterois volitans* and its ecological meaning (Valdez-Moreno, Quintal-Lizama, Gómez-Lozano, & García-Rivas, 2012, which led to the subsequent works of Côté, Green, Morris Jr, Akins, & Steinke, 2013; Dahl et al., 2018; Maji, Bhattacharyya, & Pal, 2016); the confirmation of the occurrence of ontogenetic shifts in the dietary preferences in the largemouth sea bass *Micropterus salmoides* and sunfish *Mola mola* (Jo et al., 2014; Sousa et al., 2016); the detection of a much more diverse prey range than expected for the Adelie penguins *P. adeliae* and the sunfish revealing a much more generalist feeding behavior for these species (Jarman et al., 2013; Sousa et al., 2016); or more recently the high diversity and frequency of bony fishes in the diet of Mobula rays (Bessey et al., 2019). From new diet compositions to the revelation of novel feeding strategies, we

expect that similar novelties will likely continue in future studies on aquatic taxa. The increased taxonomic resolution achieved by molecular approaches almost always added new prey to the “menus” of aquatic species, even when prey items could be easily detected morphologically. For example, an additional 17 taxa were detected in the diet of Cory's shearwater *Calonectris diomedea* (Alonso et al., 2014), highlighting the intrapopulation trophic segregation of this species. Moreover, fine-scale behavioral patterns of predator–prey interactions and feeding ecology have also been uncovered for the nocturnal Bulwer's petrel *Bulweria bulwerii* dietary habits in relation to the lunar cycle, where no shift was noted, while revealing a more specialized predation, not related to ambient light (Waap et al., 2017). Lastly, important conservation implications and management decisions may be informed by DNA metabarcoding dietary studies. As an example, the absence of salmonids in the diet of Icelandic harbor seal *P. vitulina* populations calls for the discontinuation of the seal culling in the region preys since predation on salmonids has been the reason proposed to support such culls (see Granquist et al., 2018). Interestingly, despite the significantly lower number of studies focusing on aquatic species (only 61 from 150), a higher proportion of studies in this ecosystem tested new methodologies (about 27% in studies focusing aquatic taxa vs. 13% on terrestrial species) highly contributing to the current success of metabarcoding approaches in determining diets (e.g., Jakubavičiūtė, Bergström, Eklöf, Hanel, & Bourlat, 2017; Leray et al., 2015).

3 | TAXONOMIC AND GEOGRAPHICAL BIAS IN TERRESTRIAL AND AQUATIC METABARCODING STUDIES

Upon reviewing the available literature, besides a noticeably smaller number of studies on aquatic vertebrates relatively to their terrestrial counterparts, we found two other trends: some species are preferred over others (Figure 1c) and some regions have been, so far, better sampled than others (Figure 2b). More than one third of the studies on terrestrial species, compiled herein, were focused on bat species (Figure 1c), which may not be surprising since most of the species of this group prey upon soft preys, difficult to identify through morphological analysis. Yet, the diet of several species was investigated more than twice (Table S1). For instance, after an initial description of the dietary habits of the little brown bat *Myotis lucifugus* based on metabarcoding approaches (Clare et al., 2011; Clare et al., 2014), this species was used in comparative dietary studies assessing human influence on feeding habits of bats (Cravens et al., 2018) and to improve the identification of arthropods in DNA-based diet studies (Jusino et al., 2019). Favoring their place as good biological models in the assessment of vertebrates' diets, bat species have distinct feeding and foraging habits, and different ecologic requirements and are widely distributed across a variety of terrestrial ecosystems, even in human-altered habitats (Kemp et al., 2019; Long, Kurta, & Clemans, 2013). Moreover, the possibility to concomitantly explore both the

diet of a species and its population structure was recently explored using the common vampire bat *Desmodus rotundus* as focal species (Bohmann et al., 2018). Thus, bats might be highlighted as model species for the investigation of the diet of vertebrates in terrestrial ecosystems. In comparison, a single aquatic species was most widely used in the studies here reviewed, the harbor seal *P. vitulina*. This species was used as a model for both specific (Kvitrud et al., 2005; Purcell et al., 2004) and broader methodological improvements to assess aquatic vertebrates' diets through barcoding approaches (Thomas, Deagle, Eveson, Harsch, & Trites, 2016). Moreover, other studies have inspected the spatial differences in diet composition (Granquist et al., 2018; Thomas, Nelson, Lance, Deagle, & Trites, 2017) and investigated the seasonal variation in this seals' dietary habits (Voelker, 2018). The red lionfish, Adelie penguins, and the gray seal *H. grypus* were used in three different studies each, mostly for methodological purposes (Harms-Tuohy et al., 2016; Jarman et al., 2004, 2002; Parsons et al., 2005; Tverin et al., 2019), but also to investigate the species' foraging habits (Côté et al., 2013; Jarman et al., 2013; Méheust, Alfonsi, Le Méné, Hassani, & Jung, 2015; Valdez-Moreno et al., 2012). As such, all these species are good candidate models for future barcoding-based dietary studies.

The increased value of feeding ecology studies for the assessment of aquatic environments is further reinforced by the number of species with conservation status of “Data Deficient” or “Not Evaluated” by the (IUCN, 2019), which is three times higher among the studies surveyed on aquatic vertebrates when compared to the terrestrial taxa (Figure 2c; Table S1). Firstly, poorly understood taxonomic groups, such as sharks, rays, and cephalopods, still present high percentages of data-deficient taxa (IUCN, 2019). Secondly, aquatic environments hold thousands of vertebrate species (Balian et al., 2008; Costello et al., 2010), with a large fraction estimated to remain undescribed (e.g., Mora, Tittensor, Adl, Simpson, & Worm, 2011) and tens of new species being described every year (WoRMS Editorial Board, 2019).

Finally, we found a considerable geographical bias in the studies here compiled toward the Palearctic and Nearctic regions (Figure 2b), and this trend is most striking among the terrestrial ecosystems. More than an half of the studies on terrestrial ecosystems were focused in these two regions, with few species' diets assessed in the biodiversity-rich tropical realms. Similarly, for aquatic species, there is almost no geographical overlap between the studied areas and the global distribution of marine and freshwater biodiversity hotspots, which are mostly located in the tropics and southern hemisphere (Collen et al., 2008; Ramírez, Afán, Davis, & Chiaradia, 2017). Trophic webs in biodiversity hotspots may be complex due to the high number of species interactions, which is likely to lead to high number of trophic levels (Vander Zanden, Shuter, Lester, & Rasmussen, 1999) or increased diet diversity (e.g., Xiong et al., 2017). Thus, this geographical trend is not congruent with the current knowledge caveat but likely reflects geographical patterns of funding availability and technological resources, needed to implement a metabarcoding strategy.

4 | IMPORTANT NEW RESEARCH AVENUES FROM DIET STUDIES USING DNA METABARCODING

4.1 | Detecting dietary shifts in the Anthropocene: A global changing scenario

How species will cope with the effects of anthropogenic-driven alterations to ecosystems and rapidly changing climate remains a pivotal question for ecologists. Due to physiological limits, habitat, or trophic specialization, the plasticity of a population to buffer the effects of habitat degradation and/or climatic alterations may include behavioral responses, genetic adaptation, or a geographical shift (Moritz & Agudo, 2013). Due to these global changes, spatiotemporal discrepancies may arise between predators and prey, enhancing the impacts of the original perturbation (Hazen, Nowacek, Laurent, Halpin, & Moretti, 2011). In this regard, it is expected that some species will be forced to adjust their dietary requirements in response to environmental change or perturbation (e.g., Wong & Candolin, 2015). The studies compiled herein show that multiple terrestrial (Bohmann et al., 2011; Clare et al., 2014; Czenze et al., 2018) and aquatic species (Albaina et al., 2016; McInnes, Alderman, et al., 2017; Oyafuso, Toonen, & Franklin, 2016) have adaptive dietary preferences, presenting natural spatiotemporal changes in their diets' composition related to prey species' availability. However, generally, these studies are mostly descriptive, and only about one third (47 out 150) addressed impacts of human-mediated changes in diets (37% of the studies on terrestrial organisms and 22% of the studies on aquatic taxa, see Table S1). For terrestrial species, most of these studies focused on the impacts of urbanized areas (Bohmann et al., 2018; Coghlan et al., 2013; Cravens et al., 2018; Forin-Wiart et al., 2018; Groom et al., 2017; Khanam, Howitt, Mushtaq, & Russell, 2016; Lim, Ramli, Bhassu, & Wilson, 2018; Quéméré et al., 2013; Smith, Thomas, Levi, Wang, & Wilmers, 2018; Sullins et al., 2018) and landscape changes due to agriculture (e.g., Branco et al., 2019; Clare et al., 2014, 2011; Sugimoto et al., 2018) in trophic interactions. Importantly, the results obtained so far provide substantial knowledge toward effective conservation planning and management, by identifying the impacts of introduced species, both revealing competition with native fauna (Brown et al., 2014; Egeter, Bishop, & Robertson, 2015; Robeson II et al., 2018; Zarzoso-Lacoste et al., 2016) and uncovering the importance of introduced plant species to the diet of endangered species (Ando et al., 2013). There have also been some studies focusing on the diets of reintroduced or recovering populations (Kowalczyk et al., 2011; Pansu et al., 2019; Pinho et al., 2018) and on the effects of hunting in niche partitioning between both herbivores (Pansu et al., 2019) and carnivores (Smith et al., 2018). Additionally, the effects of pollution have been addressed twice, with light pollution having species-specific effects on bats' feeding habits (Cravens et al., 2018) and trace metal pollution modulating food preferences of the wood mouse *Apodemus sylvaticus* (Ozaki et al., 2018). So far, and to the best of our knowledge and based on our compilation, only one study focused on the effects of

climate change on bats trophic niche, uncovering a profound impact on trophic webs (de Oliveira, 2018).

On the other hand, among the 13 studies on interactions between humans and aquatic taxa, the majority focused on the pressure top predators, mostly seals, can exert on marine fisheries and/or on endangered fish (Granquist et al., 2018; Méheust et al., 2015; Purcell et al., 2004; Schwarz et al., 2018; Thomas et al., 2016; Tverin et al., 2019), or on the impacts of two invasive species, the lionfish on Caribbean coral reef ecosystems (Côté et al., 2013; Harms-Tuohy et al., 2016; Valdez-Moreno et al., 2012) and the catfish in Chesapeake Bay, USA (Aguilar et al., 2017). One study measured the consumption of fishery discards by birds, revealing that, although breeding success was higher in populations with access to discarded fish, mortality could also be enhanced by the increased interacting with fishing vessels (McInnes, Jarman, et al., 2017). Finally, another study focused on investigating the diets of recovering harbor seal populations, postcessation of sealing, showing no significant differences with populations at the center of distribution (Hardy et al., 2017).

Notably, with only one exception (Cravens et al., 2018), all the aforementioned studies targeted generalist species. It is intuitive that generalist foragers display more flexible behaviors, allowing them to adapt more easily to changes in resource by switching foraging strategies (Gray, Burwell, & Baker, 2017; O'Donoghue, Boutin, Krebs, Murray, & Hofer, 1998), contrarily to specialist predators that should be highly susceptible to the changes introduced in the Anthropocene. It is clear that studies designed to directly address the impacts of human-related activities or climate change on trophic niches are still lacking and are urgent, especially among aquatic specialists.

4.2 | dDNA and dietary sampling as an environmental DNA approach to study aquatic ecosystems

The emerging analysis of environmental DNA (eDNA—a molecular-based approach to identify remnant DNA in the environment) is another significant advance of metabarcoding approaches (reviewed by Cristescu & Hebert, 2018). In practice, eDNA involves the extraction of imprinted exogenous, usually highly fragmented, DNA from the environmental samples, without a prior isolation of target organisms for sequencing that is then used for biodiversity assessments (Taberlet, Coissac, Hajibabaei, & Rieseberg, 2012). This technique has considerable importance, for example, in the assessment of the diversity of macro-organismal communities (Thomsen & Willerslev, 2015) and soil monitoring (e.g., for agroecosystem evaluation, assess overall taxonomic richness and relative biomass of indicator species) (Anderson-Carpenter et al., 2011). Moreover, the use of eDNA analysis together with longitudinal sampling could result in the most powerful tool to assess and monitor both biodiversity changes in remote ecosystems and measure ecosystems resilience to global changes (Berry et al., 2019; Bohan et al., 2017; Evans et al., 2016).

Water, soil, and even air samples have been discussed to be suitable for eDNA studies (Brennan et al., 2019; George et al., 2019;

Taberlet, Coissac, Hajibabaei, et al., 2012). Similarly, gut contents and scats have been highlighted as useful in describing regional biodiversity and used in eDNA-like surveys (Boyer et al., 2015; Siegenthaler et al., 2019; Taberlet, Coissac, Pompanon, Brochmann, & Willerslev, 2012). Boyer et al. (2015) first suggested this approach, considering that feces from generalist predators could be regarded as "biodiversity capsules," used to infer on species occurrence, distribution, and abundance. In their study, these authors further suggested the potential value of such approach for detecting small and "cryptic" species in remote environments, and in their closing arguments, they suggest that the efficiency of such an approach should be empirically compared to traditional biodiversity surveys. The guidelines established by Boyer et al. (2015) differed according to the proposed study goals. For instance, the authors recommended the use of universal primers for species inventory and estimates of local species abundances, whereas more specific primers should be preferred for dietary and food web assessments (Boyer et al., 2015). Although this approach has still limited empirical application and has been mostly viewed as a promising tool (Berry et al., 2017), a similar methodology has been applied to determine whether fecal samples from the generalist Bryde's whale *Balaenoptera edeni brydai* could be used as proxies for zooplankton diversity across seasons (Carroll et al., 2019). However, despite being generalists, Bryde's whales show preferences for certain prey, irrespectively of their availability in the environment, which could result in biased estimates of local prey availability (Carroll et al., 2019). We consider that, as the knowledge on species dietary habits progresses, more informed choices on the biological models will allow successful spatiotemporal biodiversity assessments.

The extension of this approach to stomach contents yielded promising results, as recently shown by the use of stomach contents from shrimps *Crangon crangon* to assess local fish communities, assisted by sediment samples and traditional net sampling (Siegenthaler et al., 2019). Comparatively to fecal, DNA from stomach contents is of better quality due to its collection at an earlier stage of digestion. Additionally, although the use of this "biodiversity capsules" method is ideal to study terrestrial ecosystems, it is only applicable to a small fraction of aquatic organisms, such as sea lions (Berry et al., 2017), as access to scats from other organisms is mostly restricted to opportunistic sampling (Carroll et al., 2019). In aquatic systems, especially the deep sea, where sampling can be more challenging, the use of gut and stomach contents could be highly valuable as a biodiversity information source. The results from Valdez-Moreno et al. (2012), where a "simple" dietary analysis of the invasive lionfish resulted in seven new records of fish species for the Mexican Caribbean coast, confirm this expectation. Henceforth, we propose the use of the term dDNA, dietary DNA, to refer to eDNA approaches that use DNA extracted from gut, stomach, or fecal contents with the purposes of describing local biodiversity, complemented or not by other means of sampling strategies. In this definition, we include ancient DNA extracted from preserved stomachs and coprolites. In fact, ancient eDNA recovery using from permafrost sediment samples revealed the dominance of forb (herbaceous flowering plant) species

questioning feeding habits inferred for extinct herbivores, which based on fossil pollen was expected to be mostly based on graminoid herbaceous plants (Willerslev et al., 2014). Indeed, molecular analyses of coprolites and preserved stomach contents from these herbivores confirmed a predominance of forbs. In this sense, the use of traditional eDNA opened new hypotheses on dietary habits of wild species, and the joint eDNA/dDNA approach led to a more comprehensive understanding of past terrestrial ecosystems, even without a thorough knowledge on overall species diversity in the study area. To the best of our knowledge, the first usage of a dDNA approach using stomach contents from a vertebrate was only recently applied with notable results. de Luna Sales et al. (2019) used the stomach contents from the red snapper *Lutjanus purpureus* to successfully characterize the community of cephalopodes in Amazonian reefs, describing a new cephalopod genus.

This dDNA approach is also promising in disentangling food webs and trophic interactions. In this regard, diet items identified as potentially derived from secondary predation have been often viewed as problematic due to their inflation effects on the diversity of diets of single species (e.g., Galan et al., 2018; Jakubavičiūtė et al., 2017; Jarman et al., 2013). While many of the studies here reviewed do not take into account the effects of secondary predation, others have discarded these items from their analyses. This latter approach has been possible due to previous good knowledge of the predator and prey diets (from conventional field observations), and/or through the use of abundance thresholds below which putative prey are eliminated (DNA of secondary prey should appear at a much lower quantity compared with primary prey), or through the identification of a consistent association with a specific prey item, the primary consumer (Hardy et al., 2017; Jakubavičiūtė et al., 2017; McInnes, Jarman, et al., 2017). However, accounting for and even targeting secondary prey items, for instance by complementing the sampling of top predator(s) with that of potential prey(s), brings a broader view on species interactions and consequently a better understanding of food webs (Bowser et al., 2013; Galan et al., 2018). In this regard, dDNA approaches simultaneously targeting multiple species (both top predators and prey) (e.g., Bowser et al., 2013; Carroll et al., 2019), combined with environmental samples (e.g., samples from water column and/or soil) to determine prey availability (e.g., Willerslev et al., 2014), can be tremendous robust in providing comprehensive species inventories and provide insights regarding their interactions. We thus predict that in a near future, studies using dDNA will assist on regional and global biodiversity surveys, contributing to the identification of potentially undescribed species (e.g., Corley et al., 2019; de Luna Sales et al., 2019), as well as foster new hypotheses on the hierarchical organization of the trophic webs.

5 | LIMITATIONS AND PITFALLS OF BARCODING APPROACHES TO DIETARY STUDIES

Although metabarcoding applied to dietary studies is now well established, there are still some important limitations to the method,

the most important being the lack of a standardized method for quantitative assessment of prey items, as the use of the number of reads belonging to a certain prey as a proxy for its abundance or biomass is not straightforward, nor are the simple counts of sequence reads to estimate the relative levels of prey diversity available (reviewed by Deagle et al., 2019). This is due to many factors, which include biological issues, such as the number of target genes present in each prey cell, body size, and prey digestibility, and also technical factors such as primer annealing bias and PCR random effects (Corse et al., 2019; Darby, Todd, & Herman, 2013; Deagle, Thomas, Shaffer, Trites, & Jarman, 2013; Mumma et al., 2016). The inclusion of mock communities of known composition, sequenced alongside the samples of interest, might allow establishing a direct correlation between the number of reads and the biomass/number of individuals or estimating a correction factor for both biological and technical biases (Thomas et al., 2016), but different outcomes have been obtained from different experiments and the feasibility of such trials varies (reviewed by Deagle et al., 2019). For a successful quantification of prey items from sequence reads, it is necessary to (a) design a good sampling scheme, covering both prey and predator putative spatiotemporal diversities while ensuring the distinction between rare and frequent prey taxa; (b) delimit and describe bioinformatic thresholds, from quality control of raw reads to counting of sequence reads, increasing both accuracy of taxonomic assignments and allowing reproducibility; (c) choose the most appropriate metrics (i.e., using an oversimplification, estimates based on relative abundances for generalist predators or per cent of occurrence for less generalist feeders); and d) test new methodologies (from species sampling and laboratory procedures to description of better-suited mathematical models and tools for their application; Deagle et al., 2019). For aquatic organisms, the search for standardized methodologies accounting for differential digestibility of prey items is particularly important and challenging, since mainly stomach and intestinal contents can be assessed (but see Jarman et al., 2002; Jarman & Wilson, 2004), with prey items at different stages of digestibility significantly influencing the amount of DNA recovered for each prey. Experimental studies have shown how prey detectability can vary between species (Egeter et al., 2015; Nordøy, Sørmo, & Blix, 1993), but also that it is possible to estimate correction factors to account for differential digestibility in studies using DNA strategies (Egeter et al., 2015). The results obtained by using these methodologies are promising since they are less affected by differential digestibility than morphologic methodologies (Deagle et al., 2009; Egeter et al., 2015), so this is still a developing line of research, and ongoing improvements are expected (Deagle et al., 2019). Importantly, although molecular barcoding allows the identification of a species regardless of the life stage, which is often difficult when using morphological traits (Packer et al., 2009; Pereira et al., 2019), this may preclude valuable insights regarding the foraging strategies of a predator. For example, in the case of insectivores, the preference for eggs, larvae, or adult stages may provide valuable information regarding foraging strategies as early life stages of insects are often less mobile than adults (e.g., Pereira et al., 2019). Another caveat of using

metabarcoding in diet studies, especially in the cases where “universal” instead of group-specific primers are used, relates to the high prevalence of host DNA in gut or stomach contents and feces, which can be preferentially amplified over prey, hampering taxonomic inference (Jarman et al., 2004; Leray, Agudelo, Mills, & Meyer, 2013; Leray, Yang, et al., 2013). There are, however, a few technical adjustments that can be performed to remove or inhibit amplification of host DNA. In this regard, the use of blocking primers is the one more frequently adopted (e.g., Jakubavičiūtė et al., 2017; Leray et al., 2015; Sousa et al., 2016). This method consists of adding to the PCR reaction, one annealing inhibiting primer overlapping with the 3' end of one of the universal primers used for amplification (Vestheim & Jarman, 2008). This blocking primer needs to be custom-designed to perfectly hybridize with the DNA of the predator, thereby preventing its amplification even when predator DNA is 1000-fold in excess of the prey template (Vestheim & Jarman, 2008), and to be used in high concentrations (higher than the universal primers) so it can bind to all available predator DNA.

Lastly, metabarcoding ultimately relies on the quality of data deposited in public repositories such as GenBank and BOLD. It has been argued that independently of the marker used, reference databases are still incomplete (Bartley et al., 2015), with increased uncertainty for the aquatic species (Radulović, Archambault, & Dufresne, 2010) and terrestrial realms within the southern hemisphere, where only an estimated fraction of all known metazoan species has been barcoded (BOLD, last access 15.04.2019). The quality control and ongoing curation of such databases is crucial, and has been, and should continue to be, scrutinized and extensively debated (Santos & Branco, 2012; Whitlock, 2011). However, misidentifications of species are extensive (Harris, 2003). Apart from different taxonomic concepts and erroneous identifications of species which cause mislabeling in such databases, another common problem in metabarcoding of prey items from gut or feces is the co-amplification of bacterial and fungal DNA. These data can be easily eliminated from downstream analysis through comparison with DNA databases; however, the DNA of microorganisms may also be wrongly cataloged both in GenBank and in BOLD, particularly in marine ecosystems, where there are documented cases of common bacteria are identified as metazoan marine species (Siddall, Fontanella, Watson, Kvist, & Erséus, 2009). Having this in mind, it is advisable to add an extra step in data curation, such as checking multiple best matches for every query sequence. A dDNA approach, as above mentioned, cataloging and describing new sequences of unknown taxa, and linking these to a taxonomic classification, will also improve the amount and quality of information on reference databases (de Luna Sales et al., 2019), especially in diverse but still understudied biodiversity hotspot regions of the world.

6 | CONCLUDING REMARKS

DNA-based techniques are powerful tools to address diet-related hypotheses, as they consistently outperform morphological

identification approaches. Specifically, metabarcoding has allowed an increasing number of dietary studies to be undertaken, since it requires little or no taxonomic expertise. It is true that the technology and associated bioinformatics behind this approach are still far from obtainable for many ecologists. Nonetheless, many commercial companies can provide the full service from sample preparation to data analysis, although at considerable costs.

Our review shows that the recent work using DNA barcoding approaches to study aquatic species has a high potential to uncover novel information and provide substantial advancements to the knowledge of aquatic ecosystems. We do not intend to devalue the importance of the application of diet barcoding for terrestrial species. Rather, this study highlights the power of such approaches in the aquatic environment, where direct observation of feeding events and species interactions is often much more difficult relative to terrestrial environments (Shehzad, Riaz, et al., 2012).

Moreover, metabarcoding is also a powerful tool to monitor ecosystems and the effects of ongoing climatic changes, particularly in association with eDNA approaches. We advocate that dDNA studies should be used to enable the concomitant disclosure of species dietary habits, inventory of regional biodiversity and its spatiotemporal dynamics, and trophic interactions.

So far, the impacts of human actions on the dietary dynamics of aquatic vertebrates have been seldom assessed, calling for an even more expansive increase in studies on diet, food webs, and trophic niches using barcoding approaches in the next few years. From a conservation point of view, marine hotspots such as the Southwest Atlantic, Tropical Pacific, and western Indian Ocean, which are presently under immense pressure due to climate change and intensive fisheries (Ramírez et al., 2017), remain largely underrepresented in dietary studies. We therefore emphasize the urgent need of studies on aquatic taxa in such regions which could elucidate processes of ecological adaptation and help understand the cascading impacts occurring throughout aquatic food webs.

ACKNOWLEDGMENTS

The research was supported by the European Regional Development Fund (ERDF) through COMPETE program and by National Funds through FCT—Foundation for Science and Technology (project PTDC/MAR-BIO/0902/2014-POCI-01-0145-FEDER-016550). SMS was funded by ERDF through COMPETE program and by FCT (project PTDC/BIA-MIC/27995/2017-POCI-01-0145-FEDER-027995). RX is supported by FCT under the Programa Operacional Potencial Humano—Quadro de Referência Estratégico Nacional funds from the European Social Fund and Portuguese Ministério da Educação e Ciência (IF-FCT contract IF/00359/2015).

CONFLICT OF INTEREST

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

All data used in this review are available in Table S1.

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SUPPORTING INFORMATION

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How to cite this article: deSousa LL, Silva SM, Xavier R. DNA metabarcoding in diet studies: Unveiling ecological aspects in aquatic and terrestrial ecosystems. *Environmental DNA*. 2019;1:199–214. <https://doi.org/10.1002/edn3.27>