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Metabarcoding reveals waterbird diet in a French Ramsar wetland: implications for ecosystem management

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Abstract – Environmental and/or climate changes, occurring at a global or local scale, can significantly impact the diets, health, and population dynamics of waterbirds. This study aimed to develop an effective tool, using DNA metabarcoding of fecal samples, for monitoring waterbird diets during the breeding season in a Ramsar freshwater wetland in Northern France. We collected bird feces across eight marshes with varying anthropic usage. The majority of samples (69%) were from five waterbird species: Eurasian coot (*Fulica atra*), Eurasian moorhen (*Gallinula chloropus*), mallard (*Anas platyrhynchos*), mute swan (*Cygnus olor*), and grey heron (*Ardea cinerea*). DNA was extracted from 116 samples, with plant and invertebrate primers used to undertake multi-marker metabarcoding. Despite a negative impact of uric acid on DNA amplification, we observed significant dietary variations among bird species and sampling sites. Wetland bird diets primarily consisted of four arthropod families, dominated by Chironomidae and Asellidae. The number of plant families detected was higher, consisting of 33 families, with Poaceae highly prevalent within wetland bird diets. This study shows that using DNA metabarcoding to explore interactions between waterbirds and trophic resources is a promising approach to assist wetland management and assess the effect of environmental changes.

Keywords: Molecular ecology / high-throughput sequencing / food webs / bird communities

1 Introduction

Wetlands are among the most productive ecosystems in the world and provide critical services, such as carbon storage, water purification, fish production and biodiversity conservation (Moreno-Mateos *et al.*, 2012). However, these ecosystems have faced an alarming reduction over the past two centuries. According to the 2019 report from the IPBES, 87% of these environments have disappeared since the 18th century (source: https://www.ipbes.net/global-assessment). In France, according to the French Office for Biodiversity (OFB), 50% of terrestrial aquatic ecosystems disappeared between 1960 and 1990, and 41% of emblematic wetland areas were degraded between 2010 and 2020 (source: http://www.zones-humides.org).

Meanwhile, the Living Planet Index, which synthesizes trends in global vertebrate populations, indicates an exacerbation in the decline of freshwater species compared to terrestrial and marine ones between 1970 and 2018. The drop represents 84% for freshwater species, 40% for terrestrial species, and 35% for marine species (WWF, 2022). In France, among nesting waterbirds, four Anatidae species are classified as Vulnerable on the Red List of Threatened Species: the Eurasian teal (*Anas crecca*), the garganey (*Spatula querquedula*), the greylag goose (*Anser anser*), and the common pochard (*Aythya ferina*). Two are listed as Critically Endangered, the red-breasted merganser (*Mergus serrator*) and the common eider (*Somateria mollissima*). Two species are Extinct in the metropolitan area, the marbled teal (*Marmaronetta angustirostris*) and the white-headed duck (*Oxyura leucocephala*) (https://inpn.mnhn.fr/docs/LR_FCE/UICN-LR-Oiseaux-diffusion.pdf). Out of eight Rail species, two are classified as Vulnerable (the Western swamphen, *Porphyrio porphyrio*; and the spotted crake, *Porzana porzana*), while three are Critically Endangered (the little crake, *Zapornia parva*; the Baillon's crake, *Zapornia pusilla*; and the common crane, *Grus grus*). Additionally, among herons, out of nine species, the Eurasian bittern (*Botaurus stellaris*) is classified as Vulnerable,

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and the little bittern (*Ixobrychus minutus*) is Critically Endangered.

These figures reveal a biodiversity crisis largely caused by anthropogenic activities. Indeed, the main threats to freshwater ecosystem biodiversity, including waterbirds, are well-documented and include habitat loss, fragmentation, and degradation, alterations in water flow, pollution, overfishing, hunting pressure, infectious diseases, and invasive exotic species (Dudgeon *et al.*, 2006; Reid *et al.*, 2019).

The alterations in habitat quality can lead to significant direct and indirect repercussions for wildlife. Ornithological research has long focused on avian diets, a critical area of study that has provided essential insights into the role of dietary composition in avian evolution (Hoenig *et al.*, 2022). By studying avian prey dietary composition, we can effectively identify dietary shifts resulting from disturbances, which can have profound impacts on bird populations and communities. Studies have shown that changes related to human land use, urban expansion, water quality, and the introduction of non-native plants have an impact on the feeding habits of wetland-dependent birds, causing modifications to their trophic niches (Brochet *et al.*, 2012; Murray *et al.*, 2018; Riedl *et al.*, 2018; Trevelline *et al.*, 2018; Evans and Gawlik, 2020). In Camargue, a Mediterranean wetland in southern France, practices such as agriculture, salt extraction, industrial extension, and intensified marsh management (partly for hunting and tourism purpose) have led to shifts in the diets of mallards and teals. These shifts resulted from an overall loss of plant diversity in their natural foraging areas, as well as the emergence of new food items such as a cultivated species (rice) and exotic seeds (Brochet *et al.*, 2012). Some Anatidae species (the Eurasian wigeon (*Anas penelope*), the greylag goose (*Anser anser*), the pink-footed goose (*Anser brachyrhynchus*), and the Barnacle goose (*Branta leucopsis*)), were determined to have foraged on barley and wheat in the surrounding agricultural areas in Denmark (Svendsen *et al.*, 2023). Similarly, diet expansion to compensate for the loss of preferred prey has been reported in riparian habitats in the case of insectivorous songbirds exposed to pollution: (i) the Louisiana waterthrush (*Parkesia motacilla*) opportunistically exploited terrestrial prey taxa due to the loss of their preferred prey, the pollution-sensitive Ephemeroptera, Plecoptera, and Trichoptera due to stream acidification (Trevelline *et al.*, 2018); and (ii) meanwhile, foliage gleaners such as the Virginia's warbler (*Leiothlypis virginiae*) and the warbling vireo (*Vireo gilvus*) consumed more aquatic insects in sites invaded by the non-native woody plant *Robinia neomexicana* (Riedl *et al.*, 2018). Diet expansion was also documented in urban wetlands with the emergence of anthropogenic food. However, the consequences on the health and breeding success are poorly known. In urban habitat, easily accessible anthropogenic food such as bread and chips consumed by the American white ibis (*Eudicumus albus*) did not support higher mass, but may have increased time available for anti-parasite behaviors, such as preening (Murray *et al.*, 2018). Meanwhile, the consumption of anthropogenic food such as chicken wings or hotdogs during suboptimal hydrologic conditions may have increased breeding success in the wood stork (*Mycteria americana*) (Evans and Gawlik, 2020).

While exploring food webs is a key prerequisite for understanding how species can adapt their diets in changing environments (*e.g.* Villsen *et al.*, 2022a) and for species conservation and management (*e.g.* Zarzoso-Lacoste *et al.*, 2019), the study of dietary compositions presents inherent challenges related to field data collection. This process is often expensive, time-consuming, and demands a large number of samples. Common visual techniques, including gut, stomach, or fecal content analyses, as well as stable isotope analyses of bulk or specific compounds, have been frequently employed (Nielsen *et al.*, 2018). Over the past two decades, metabarcoding has rapidly emerged as a powerful and cost-effective tool for unraveling food webs, including those involving birds across various trophic guilds and habitats (Pompanon *et al.*, 2012; Jarman *et al.*, 2013; Jedlicka *et al.*, 2013; Kress *et al.*, 2015; Taberlet *et al.*, 2018; Dunn *et al.*, 2018; Laviad-Shitrit *et al.*, 2019; Huang *et al.*, 2021). This method, which identifies multiple species through high-throughput sequencing of a specific DNA marker, is commonly employed in fecal sampling due to its non-invasiveness. Moreover, it has been proposed that a metabarcoding analysis of fecal samples may also be a relevant method for exploring wildlife richness and evolutionary diversity for conservation biology purposes. Boyer *et al.* (2015) suggested that feces from generalist predators may be considered as "biodiversity capsules" containing a representative sample of prey species to assess biodiversity of the predator's foraging area. More generally, Sousa *et al.* (2019) have introduced the term of "dietary DNA" to describe environmental approaches aiming to assess both dietary habits and describe local biodiversity. Thus, fecal samples analyzed by molecular methods are expected to reveal a wealth of ecological information, including dietary composition and the quality of the foraging area of the sampled community. This information is crucial not only for understanding the functioning of wetland ecosystems under growing human pressure, but also for evaluating restoration success, for providing essential information for habitat management and conservation efforts (Ontiveros *et al.*, 2005; Poulin *et al.*, 2010; Edwards *et al.*, 2013; Loch *et al.*, 2020; Muro-Torres *et al.*, 2020; Cabodevilla *et al.*, 2021; Li *et al.*, 2023).

Yet obtaining reliable molecular data from avian feces is still complicated by its specific chemical composition, as digestive excreta are mixed with urinary products such as uric acid that can degrade DNA or interfere with DNA extraction (Eriksson *et al.*, 2017; Vargas-Pellicer *et al.*, 2019). The result is that DNA yields from avian feces are typically low, making amplification difficult, and rendering bioinformatics pipelines more sensitive to contamination. A recent study has explored the dietary habits of herbivorous anatids using two analysis methods in fecal samples: microscopic analysis and metabarcoding of environmental DNA (Svendsen *et al.*, 2023). Out of 56 plant species detected, 12 were identified by both methods, 8 solely by microscopy, and 36 exclusively by metabarcoding. However, eight of the latter were unexpected, considering their rarity throughout the entire country (Denmark). Therefore, each method was found to carry its own set of advantages and limitations. Microscopic analysis faces challenges in taxonomy and potential biases stemming from variations among researchers. Conversely, DNA-based methods are expected to offer a higher taxonomic resolution and enable more frequent and stable detection of consumed taxa (Jakubavičiute *et al.*, 2017; Zarzoso-Lacoste *et al.*, 2016).

Especially, DNA metabarcoding may detect prey that might be missed during hard-part dietary studies, such as soft-bodied species (Berry *et al.*, 2015; Egeter *et al.*, 2015). Notwithstanding potential challenges in amplification with very low DNA quantities, DNA metabarcoding is also anticipated to reveal inadvertently ingested food items and false positives more frequently. Generally, as a relatively recent approach, metabarcoding faces challenges related to protocol standardization, data analysis, as well as the potential for environmental contamination and technical artefacts (Alberdi *et al.*, 2019; Zinger *et al.*, 2019; Hoenig *et al.*, 2022).

In this context, the primary objective of our study was to investigate trophic interactions among waterbirds in a vast wetland situated in Northern France (see: https://www.somme.fr/plan-gestion-ramsar/). This site, with its close vicinity to the heavily avian-frequented Bay of Somme, and its diverse habitats comprising a mosaic of ponds and marshes, holds great potential for hosting waterbirds. However, the site faces various anthropogenic pressures, mainly associated with hunting, agriculture, and urbanization, and is known for hosting only small bird populations. During breeding season, these populations primarily include common species such as the Eurasian coot (*Fulica atra*), which is predominant, the mallard (*Anas platyrhynchos*), the mute swan (*Cygnus olor*), and the Eurasian moorhen (*Gallinula chloropus*), and may also shelter protected species. Our study focused on the breeding season, a critical period for bird food resources essential for the success of their reproduction. Notably, the selection of food by waterbirds is known to undergo significant changes between seasons (Murkin and Batt, 1987). In particular, the Eurasian coot, highly territorial during breeding season, shifts from a primarily vegetarian diet, composed mostly of submerged plants, to an omnivorous diet that includes invertebrates. Additionally, soft-bodied invertebrates are used to feed chicks (Brinkhof, 1997). Coots exhibit considerable flexibility in their foraging behavior: (i) upending in shallow water or diving in deeper water to forage on macrophytes, algae, detritus, and benthic invertebrates; (ii) cropping emergent or bankside vegetation from water or land, gleaning insects, seeds, and fruits from the water surface or vegetation; and/or (iii) taking handouts or leftovers from human visitors (Perrow *et al.*, 1997). To analyze dietary composition, we undertook multi-marker DNA metabarcoding, using plant and invertebrate primers on fecal samples opportunistically collected within wetlands. We (i) evaluated the impact of uric acid in the analysis of bird diets, (ii) we implemented a robust experimental protocol and bioinformatics pipeline to minimize potential biases as effectively as possible (Corse *et al.*, 2019), and (iii) we analyzed variations in diets based on bird species and ponds. Given that the biodiversity of urban ponds generally appeared to be lower than that in rural ponds, we hypothesized that sites may differ in terms of resource availability and diversity depending on their localization in urban or rural environment (Oertli and Parris, 2019). This work serves as a pilot study, establishing the groundwork for a protocol as adaptable as possible for future investigations into the dietary habits of waterbirds.

2 Materials and methods

2.1 Study area

The study area encompasses wetlands located in the lower reaches of the Somme River and its main tributary, the Avre River, located in Northern France in the Atlantic Biogeographic Region (Channel/North Sea, Fig. 1). Since 2017, this site, covering a total area of 13,100 hectares, has been designated as the 48th Ramsar site in France (see: https://www.somme.fr/plan-gestion-ramsar/). The "Ramsar Convention on Wetlands" was established in February 1971 in the city of Ramsar, Iran (Gell *et al.*, 2023). A Ramsar Site refers to wetlands specifically designated by the Contracting Parties for inclusion in the List of Wetlands of International Importance. These wetlands are identified based on their fulfillment of one or more of the Ramsar Criteria, emphasizing factors such as representativeness, uniqueness, and biodiversity (Stroud and Davidson, 2021). Unlike the Picardy Littoral (Bay of Somme), which meets criterion 5 (>20 000 waterbirds) of the Ramsar Convention, the inland Somme supports small populations of waterbirds (Schmaltz *et al.*, 2020). Among these waterbirds, certain species are classified as Vulnerable on the national Red List, such as the common teal (*Anas crecca*) or the Eurasian bittern (*Botaurus stellaris*). Others are Endangered, including the little bittern (*Ixobrychus minutus*) and the Savi's warbler (*Locustella luscinioides*), while some are Near Threatened, like the Western marsh harrier (*Circus aeruginosus*) (see: https://www.somme.fr/plan-gestion-ramsar/). The peatlands in the Somme region represent one of the largest alkaline peatlands in Western Europe. Manual peat extraction and grazing have led to the formation of extensive ponds, reed beds, and damp meadows, which are currently becoming more fragmented and susceptible to reforestation. These wetlands serve as reservoirs of biodiversity amidst intense human activities such as hunting, fishing, hiking, and canoeing. According to the Corine Land Cover cartography, the site primarily consists of water bodies and marshes (44%). However, the Somme River catchment area hosts over 65% of the human population of the "Département de la Somme" within 5 km of the Ramsar site. Along the margins, the Ramsar site faces significant urban pressure, and the area is also characterized by intensive agriculture. Within the Ramsar site, we have monitored seven marshes that vary in their level of human impact (Tab. 1) and in the richness and density of their waterbird populations. Among these seven marshes were: two urban parks (Marais des Trois Vaches and Parc de la Bouvaque), one large rural pond (Marais de Cléry-sur-Somme), one vast stretch of smaller marshes (Marais de Long), and three smaller and more isolated marshes (Marais de Hailles, Marais de Morcourt, Marais de Tirancourt). The "Marais d'Isle", a National Nature Reserve adjacent to an urban park located on the outskirts of the Ramsar site, was the eighth site sampled. According to the collaborative wildlife

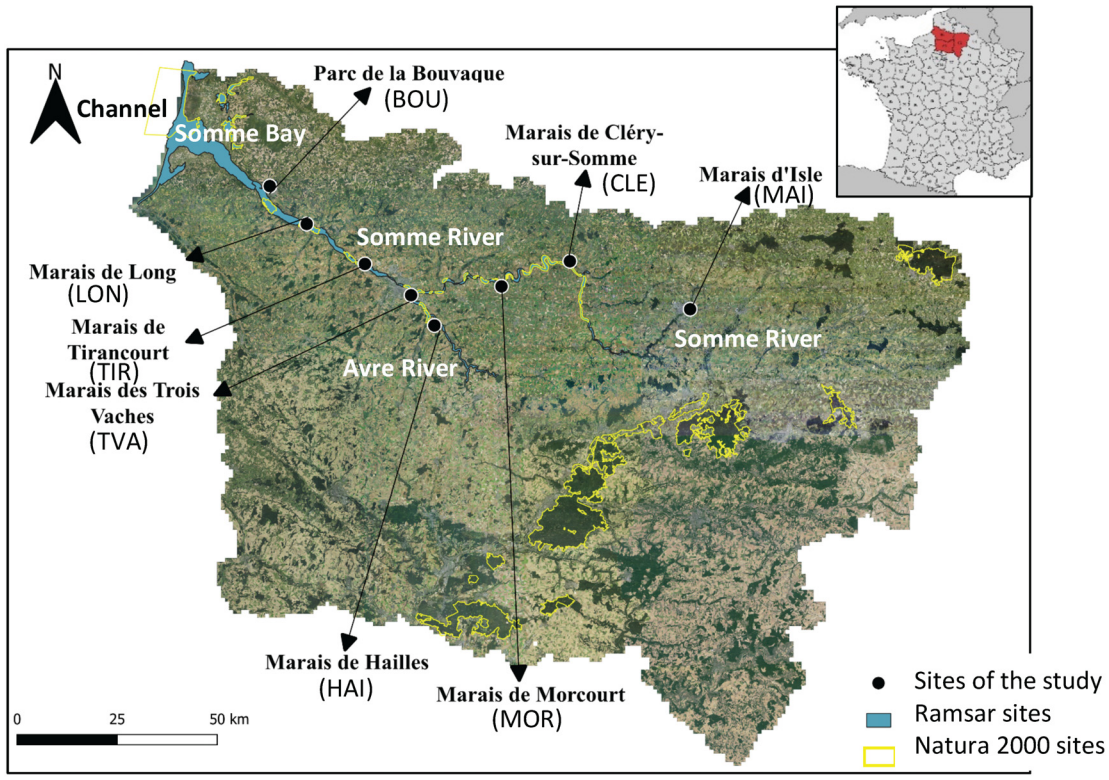

Fig. 1. Sampling sites. The eight marshes included in this study are located in Northern France within the former French Administrative Region of Picardy. The French "Département de la Somme" comprises two Ramsar sites: "*Baie de Somme*", a natural estuary, and "*Marais et tourbières des vallées de la Somme et de l'Avre*" which encompasses the lower stretches of the Somme River and its main tributary, the Avre River, as well as adjacent marshes and peatlands. Sampling sites are situated in the latter, except the National Nature Reserve "*Marais d'Isles*", located amid the Saint-Quentin urban area. The inset in the top right corner depicts the position of Picardy on the map of France and its division into three "Départements". The Département of Somme is located Northwest of Picardy.

Table 1. Information on sampling sites. The eight sampled marshes exhibit variations in their geographic location (rural or urban), anthropic usage and pound surface areas, providing insights into the diversity of habitats.

Site name	Abbreviation	Town	Geographic location	Geographical setting	River	Anthropic usage	Total pond surface (ha)
Parc de la Bouvaque	BOU	Abbevile	50°6′59″N, 1°50′34″E	Peri-urban	Scardon	Urban park, fishing	7.7
Marais de Cléry-sur-Somme	CLE	Cléry-sur-Somme	49°57′14″N, 2°53′44″E	Rural	Somme	Waterfowl hunting, fishing	46.2
Marais de Hailles	HAI	Hailles	49°48′31″N, 2°25′23″E	Rural	Avre	Waterfowl hunting, fishing	7.1
Marais de Long	LON	Long	50°1′36″N, 1°58′33″E	Rural	Somme	Waterfowl hunting, fishing	63.0
Marais d'Isle	MAI	Saint-Quentin	49°50′36″N, 3°18′22″E	Urban	Somme	Urban park, National Nature Reserve	14.6
Marais de Morcourt	MOR	Morcourt	49°53′47″N, 2°39′53″E	Rural	Somme	Waterfowl hunting, fishing	12.4
Marais de Tirancourt	TIR	Tirancourt	49°56′23″N, 2°10′52″E	Rural	Somme	Waterfowl hunting	9.2
Marais des Trois Vaches	TVA	Amiens	49°52′38″N, 2°20′22″E	Urban	Avre	Urban park, fishing	12.0

observation database *clicnat*, managed by the NGO "Picardie Nature" (Amiens, France) and fed by volunteer naturalists and partner organizations (https://clicnat.fr), the main breeding waterbird species in the areas containing sampling sites include Anatidae such as the common teal (*Anas crecca*), the common pochard (*Aythya ferina*), the mallard (*Anas platyrhynchos*), the Northern shoveler (*Anas clypeata*), and the tufted duck (*Aythya fuligula*). Additionally, Anatinae are represented by the greylag goose and the mute swan, while Ardeidae species consist of the great egret (*Ardea alba*), the grey heron (*Ardea cinerea*), the little bittern (*Ixobrychus minutus*), and the little egret (*Egretta garzetta*). Charadriidae is represented by the Northern lapwing (*Vanellus vanellus*), and Laridae includes the black-headed gull (*Larus ridibundus*), the herring gull (*Larus argentatus*), and the lesser black-backed gull (*Larus fuscus)*. Phalacrocoracidae is represented by the

European Great cormorant (*Phalacrocorax carbo*), and Podicipedidae includes the great crested grebe (*Podiceps cristatus*). Rallidae species consist of the common moorhen (*Gallinula chloropus*), the Eurasian coot, and the water rail (*Rallus aquaticus*). Finally, Sternidae is represented by the common tern (*Sterna hirundo*).

2.2 Field sampling and data collection

To avoid disturbance, we sampled each marsh only once during the breeding season from April 19 to April 30, 2021. In some cases, we employed a kayak to access survey points that were not reachable on foot. It is important to note that field conditions during the collection of fecal samples can significantly affect the results of molecular analyses of bird diets (Oehm *et al.*, 2011; Menke *et al.*, 2015; McInnes *et al.*, 2017; Ando *et al.*, 2018). Factors such as exposure to sunlight or rain a few days or the deposition on wet soil or dirt substrate over a few hours, have been shown to reduce the proportion of food DNA in bird feces (Oehm *et al.*, 2011; McInnes *et al.*, 2017; Ando *et al.*, 2018). To mitigate these effects, several precautions have been taken. We collected samples between 8 a.m. to 12 p.m. when the mean temperature ranged from 3 to 11°C, and there was no rainfall during the collection period or the night before. We selected the freshest bird scats based on their dry or moist appearance and intact shape, using non-talc gloves and sterile cotton swabs (see: Tab. S1 for sample distribution). These samples were then placed in a plastic bag, immediately transferred to a field cooler, and stored at −20 °C in the evening ($n = 146$, Tab. S2). Following the field campaign, the samples were further preserved at −80 °C until DNA extraction.

2.3 DNA extraction

DNA extraction was conducted in a dedicated laboratory room under a laminar flow hood, using the DNeasy® *mericon*® food kit by QIAGEN. This kit has been previously recommended for extracting DNA from fecal samples to minimize the co-extraction of contaminants and enhance PCR success (Zarzoso-Lacoste *et al.*, 2013). The protocol designed for small DNA fragments and small-scale samples (200 mg) was performed following the supplier's recommendations. Whenever possible, areas with white uric acid traces were avoided during DNA extraction. Mechanical lysis was carried out by freezing the samples in a nitrogen bath and subsequently using 3mm zirconium beads (FisherScientific) with a Mixer Mill MM 400 (Retsch) at 2 × 45s at 30Hz. At the end of the protocol, two column washes were conducted using 500 µl of AW2 before DNA elution. To prevent cross-contamination, each extraction series included the fecal samples from a specific marsh. We incorporated two negative controls into each extraction series. Negative controls for extraction (TnegExt) consisted of 500 µl of DNA-free water that underwent the entire extraction protocol. Negative controls for DNA aerosols (TnegAer) comprised of a vial containing 50 µl of DNA-free water that remained open under the laminar flow hood throughout the extraction protocol (see: Corse *et al.*, 2017). We prepared three separate mock communities (Tpos; Tab. S3) by extracting DNA from tissues using the same extraction kit. The bird mock community consisted of DNA extracted from ostrich (*Struthio camelus*), chicken (*Gallus gallus*) and turkey (*Meleagris gallopavo)* meat. The plant mock community consisted mainly of DNA from indoor plants (*Chlorophytum comosum, Kalanchoe blossfeldiana*, *Crassula ovata*, *Plectranthus scutellarioides*, *Sansevieria stuckyi*, *Schlumbergera truncate*, and *Sedum palmeri)* except for *Olea europaea*, which was cultivated outdoors. The invertebrate mock community primarily consisted of marine (*Anemonia viridis, Eunicella singularis,* Eunicidae, Paguridae, *Pisa* sp., and *Synalpheus gambarelloides*), terrestrial Mediterranean species (*Bactrocera oleae*, *Derbentina xeropicta*, and *Scolopendra forficata)*, and *Armadillidium vulgare*, a widespread terrestrial woodlouse in France.

DNA concentrations and A260/A280 absorbance ratios were assessed using a NanoPhotometer NP80 (IMPLEN, Munich, Germany). Whenever possible, DNA concentrations were standardized to a maximum of 20 ng/µl for fecal samples or 15 ng/µl for mock community samples.

2.4 PCR reactions

PCR mixes and plates were prepared in a dedicated room, in triplicates, with a total volume of 25 µl using the QIAGEN® Multiplex PCR kit. Primer pairs and annealing temperatures are shown in Table 2. To facilitate the tracing of amplicons back to the respective samples, 12–14 nucleotide-long sequence tags were added to the 5' end of each primer, creating a potential pool of 96 forward and reverse tag combinations (Corse *et al.*, 2017).

At this stage, we introduced two additional negative controls. Negative controls for PCR (TnegPCR) consisted of 2 µl of DNA-free water subjected to PCR reaction. Negative controls (Ttag) for assessing mistagging levels ('Tag-jump') due to sequence recombination from different samples (Ttag) consisted of empty wells in the 96-well PCR plate, leaving particular tag combinations unused (Schnell *et al.*, 2015).

PCR reactions were performed in two GeneAmp® PCR System 9700 and a GeneAmp® PCR System 2700 (Thermo Fisher Scientific, MA, USA). The PCR programs were as follow: initial denaturation (15 min at 95 °C), bird and invertebrate runs: 35 cycles (30 s at 94 °C; 40 s at the annealing temperature, and 60 s at 72 °C); for plant runs: 5 cycles (30 s at 94 °C, 40 s at 50 °C, and 60 s at 72 °C), followed by 30 cycles (30 s at 94 °C, 40 s at 53 °C, and 60 s at 72 °C); and final extension (10 min at 72 °C).

Amplicons were assessed using gel electrophoresis (1.25% agarose), then pooled by replicate series and purified using the Wizard® SV Gel and PCR Clean-Up Systems by Promega.

2.5 Illumina sequencing libraries and high-throughput sequencing

We employed a two-step, tailed PCR approach to construct the paired-end ready-to-pool amplicon libraries. The locus-specific primers contained sequence tails that allow a second PCR to add Nextera® XT indexed adapters. The Nextera XT library preparation, qualification, quantification, and sequencing were conducted on the sequencing platform iGenSeq (Institut du Cerveau, Paris). After purification, libraries were

Table 2. DNA primer pairs used for PCR. Modified primer sequences (ZF2 and HC) are indicated, with variations in degenerated bases marked in bold. Primer pairs were designed for amplifying specific regions, including mt COI (mitochondrial cytochrome *c* oxidase I gene), 12S mt RNA (mitochondrial 12S RNA gene), and cp *tnr*L intron (P6 loop of the *tnr*L intron chloroplast DNA), Annealing temperature (°C) is also provided. n.a. : not applicable.

Primer name	Target taxa	Target gene	Primer version	Primer type	Primer sequence (5'-3')	Annealing temperature	Reference
MiBird-U-F	Birds	mt 12S rRNA	Original	Forward	GGGTTGGTAAATCTTGTGCCAGC	50 °C	Ushio *et al.* (2018)
MiBird-U-R	Birds	mt 12S rRNA	Original	Reverse	CATAGTGGGGTATCTAATCCCAGTTTG	50 °C	Ushio *et al.* (2018)
ZBJ-ArtF1C	Arthropods	mt COI	Original	Forward	AGATATTGGAACWTTATATTTTATTTTTGG	n.a.	Zeale *et al.* (2011)
HCO1777	Invertebrates	mt COI	Original	Reverse	ACTTATATTGTTTATACGAGGGAA	n.a.	Brown *et al.* (2012); Verkuil *et al.* (2022)
ZF2	Invertebrates	mt COI	Modified from ZBJ-ArtF1C	Forward	GATATTGGWACHTTWTAYTTTHTHTTYGG	48 °C	this study
HC	Invertebrates	mt COI	Modified from HCO1777	Reverse	ACTTATATTRTTTATACGAGGGAA	48 °C	this study
trnL_g	Plants	cp *tnr*L intron	Original	Forward	GGGCAATCCTGAGCCAA	53 °C	Taberlet *et al.* (2007)
trnL_h	Plants	cp *tnr*L intron	Original	Reverse	CCATTGAGTCTCTGCACCTATC	53 °C	Taberlet *et al.* (2007)

pooled at equimolar concentrations. Sequencing was performed with a partial run on a NovaSeq 6000 Illumina System (Illumina, CA, USA) with an SP-500 cycle cartridge (2*800 Millions of 250 base read) for the 12 pools. The sequencing run yielded a total of 1845 million sequences, of which 746 million sequences originating from fecal and control samples were integrated in this study. Per-base read quality plots are available in Figs. S1–S3Figs. S1 S3.

2.6 Sequence filtering and taxonomic assignation

The data filtration and taxonomic assignment of taxa from the NovaSeq sequencing were conducted using the bioinformatics pipeline known as VTAM (Validation and Taxonomic Assignation of Metabarcoding data; González *et al.*, 2023). This pipeline was developed to explicitly integrate technical controls and replicates as part of the metabarcoding workflow to parametrize the denoising high-throughput datasets and the validation of sequence data. VTAM's purpose was to minimize artifacts that could artificially inflate sample diversity, ensuring the reliability of the analyses, a concern raised by several authors (Ando *et al.*, 2018; Alberdi *et al.*, 2019; Corse *et al.*, 2017; Zinger *et al.*, 2019) and proved to be effective in providing accurate and robust diet data for conducting very fine ecological analyses (Villsen *et al.*, 2022a, 2022b). VTAM was designed to address all types of sample contamination known, including tag-jump. As recommended, to improve data quality, we removed taxa with low read counts, thereby reducing the presence of environmental and experimental contaminants, mis-tagged sequences, and sequencing and PCR errors. In conjunction with negative controls, we used mock community samples to determine optimal parameters for filtering sequences, tailored to each dataset. The specific optimized filter parameters employed for data curation are detailed in Table S4. The numbers of reads validated after each filtering step are presented in Table S5. The initial number of read pairs per replicate varied between 34.0 and 39.4 million for the bird dataset, 23.0 and 53.2 million for the invertebrate data set, and 43.4 and 55.2 million for the plant dataset. Among all the filters, except for replicate 1 in the plant dataset, it was the LFN filters (low-frequency noise) that significantly reduced the number of reads, implying that low read counts were the primary reason for the removal of filtered occurrences. The remaining taxa still represented between 10.3 and 11.7 million sequences in the bird dataset, 6.4 and 19.0 million in the invertebrate dataset, and 13.0 and 25.2 million sequences in the plant dataset. The final output of VTAM was an amplicon sequence variant (ASV) table.

For taxonomic assignation, we created a custom database using 12S mt rRNA and tnrL sequences. This database construction was facilitated by python package nucleotide or NCBI sequence downloader (NSDPY; Hebert and Meglécz, 2022). Additionally, we obtained a non-redundant COI database (COInr) from https://doi.org/10.5281/zenodo.6555985 (Meglécz, 2023). Taxonomic assignments were based on the top hits with a sequence identity of at least 97%.

2.7 Method evaluation procedures

2.7.1 DNA extraction

We successfully extracted DNA from 116 samples out of a total number of 146 samples (Tab. S2). A total of 30 fecal samples could not be extracted: 25 samples were deemed challenging to handle after thawing or were too small relative to the 200 mg minimum material required for extraction, and an additional five fecal samples were lost due to a thermomixer failure during incubation with lysis buffer. To assess DNA sample purity, the A260/A280 ratio, commonly used to evaluate protein contamination (with an expected value between 1.8 and 2.0), and the A_{260}/A_{230} ratio, used to assess the presence of undesired organic compounds (with an expected value between 2.0 and 2.2), were analyzed (Wilfinger *et al.*, 1997). Furthermore, uric acid has been previously shown to exhibit two distinct peaks in UV spectra, with a major peak around 288 nm and a minor peak around 230 nm (Fischer, 1995). Consequently, the presence of uric acid in DNA samples after purification may not only lead to an overestimation of DNA concentration when using spectrophotometric quantification

but can also adversely affect sample purity, as indicated by the A_{260}/A_{230} ratio.

2.7.2 DNA amplification

Before conducting the PCR reactions for sequence analysis, we initially assessed each sample for amplification by performing PCR reaction using MiBird-U primers to confirm the presence of amplicons on agarose gels.

2.7.3 Sequence filtering and taxonomic identification

Following the curation of MiBird-U sequences, 26 distinct taxa were retained, of which 92% were identified as belonging to the expected Taxon, with the remaining 8% identified as bacteria. The final ASVs table containing reads counts, amplicon lengths and sequences, taxonomic identification, and percentage of identity are displayed in Table S6. Most bird taxa (80%) were identified at the species level.

After quality filtering of the ZF2HC sequences with the optimized VTAM parameters, some contaminant taxa remained in the negative controls. If these were also present in the fecal samples, they were removed, resulting in the elimination of five taxa and 65 occurrences across 34 samples. Among the remaining ZF2HC sequences, 103 distinct taxa passed through the filters, with the majority (84%) being identified as Arthropoda, 8.5% as birds, 3.8% as Rotifera (Tab. S7). Furthermore, 100% of the taxa were identified at the family level, and 58% were identified at the species level. A few taxa that could not be identified using the COI database were assigned using the NCBI nucleotide database. At least one invertebrate Taxon was identified in 50 samples. However, to address potential biases previously mentioned, since birds are likely to unintentionally ingest Rotifera and *Piona immunita*, a water mite typically associated with an invertebrate host, these organisms were excluded as potential prey (Svendsen *et al.*, 2023).

In the plant dataset, a total of 113 taxa were attributed to the fecal samples. No significant similarities were found with BLAST in 19% of taxa. The remaining taxa were all identified as Streptophyta, with 64% identified at the family rank, 45% at the genus level, and 8% at the species level (Tab. S8).

2.8 Statistical analysis

All analysis were undertaken in R 4.2.3 software (R Core Team, 2022). Quantitative data were presented as means ± standard errors.

For all the comparative analyses related to DNA quality and quantity, the normality of the datasets was assessed using the Shapiro–Wilk test. Since the data did not pass the normality test, non-parametric analyses were performed using the Wilcoxon rank sum test.

For dietary analysis, to mitigate PCR biases, we chose to not use relative read abundances that may not reflect food proportions (see: Ando *et al.*, 2018; Lamb *et al.*, 2019). Instead, we chose a more conservative indicator: the frequency of occurrence (FOO) per bird species, calculated as a FOO % = (x/n) * 100, where 'x' represented the number of samples in which the Taxon was detected, and 'n' was the total number of fecal samples of the considered bird species (Dunn *et al.*, 2018; Davies *et al.*, 2022; Schumm *et al.*, 2023). It is worth noting however that this metric may overemphasize the significance of rare food items, since main and rare food are equally weighted (Ando *et al.*, 2018). To illustrate the frequency of predator-prey interactions, bipartite food webs were constructed using the *plotweb* function from the BIPARTITE package (Dorman *et al.*, 2008).

For diet similarity between bird species and/or sites, we conducted Non-metric Multidimensional Scaling (NMDS) analysis using the *metaMDS* function from the R package VEGAN (Dixon, 2003). We used Gower dissimilarities estimated using the square root of the occurrence number to avoid the over-representation of the most abundant taxa when constructing the distance matrix and perform the NMDS. The stress parameter was used to evaluate the quality of the representation in space, where a stress value below 0.1 indicated a good representation, and a value above 0.2 was considered inaccurate (Clarke, 1993). To assess the dispersion of observations around their centroid in the reduced space generated by the NMDS analysis, we calculated the average distance to the centroid. These distances indicate variations in diet composition for species, and variations in resource diversity for sites. The coordinates of the centroids were obtained by averaging the coordinates of the points per species (or site) using the *kmeans* function in R, with a single center (centers = 1). Subsequently, for each species (or site), the average distances between the observation points and their respective centroids were calculated using the Euclidean formula, which involves summing the squares of the differences between each component of the point and the centroid, and then taking the square root of this sum. We tested the significance of dietary differences (the response variable) considering the interaction between three predictors: species, site, and geographic location (urban *versus* rural), using a PERmutational Multivariate ANalysis Of VAriance using distance matrices (PERMANOVA). This test was performed using the function *adonis2* in the R package VEGAN. To test for significant differences in diet between species or sites, we performed pairwise comparisons using a permutation MANOVA with the *pairwise.perm.manova* function, from the RVAideMemoire R package (Hervé, 2020). The ratio of consumed plant richness to consumed invertebrate richness was calculated using the complete set of samples for each species.

3 Results

3.1 Methodological insights

A total of 146 fecal bird samples were collected, with 18 ± 4 samples per site. Notably, no fecal material was found in the "Marais de Long". This site, managed by the "Conservatoire d'Espaces Naturels des Hauts-de-France" (CEN-HDF), is known to have low waterbird activity. Of the collected samples, 116 could be extracted for DNA (Tab. S2). Post-extraction, the DNA concentration ranged from 0 to 615 ng/µl, with a mean value of 33.5 ± 6.9 (n = 116). Notably, the DNA concentration fell below the NanoPhotometer detection limit in 9 samples. To investigate factors affecting DNA yield, we categorized fecal samples based on (i) their freshness determined by their shape and dry or humid appearance

(ii) the percentage of surface area covered with uric acid, and (iii) the primary substrate from which they were collected (Tab. S1; Fig. 2a, b, and c). The data suggested that the presence of uric acid significantly reduced DNA concentrations in the extracts, whereas freshness and the substrate of collection have no significant effects. In the fecal samples, purity was notably poor, with an A_{260}/A_{280} ratio ranging from 0.242 to 2.182 (mean value of 0.796 ± 0.048; $n = 105$) and an A_{260}/A_{230} ratio ranging from 0.128 to 2.242 (mean value of 0.551 ± 0.048; $n = 100$). To investigate whether uric acid contributed to DNA sample contamination, DNA purity was analyzed in samples grouped by the percentage of their surface area covered with uric acid (Fig. 2d). The results indicated that the A_{260}/A_{230} ratio significantly decreased in samples with an increasing surface area covered with uric acid. Additionally, the reduction in variance of this ratio with increasing percentages of uric acid provided further evidence that this compound is likely the primary factor causing the low quality of the samples. These findings suggested that after extraction, impurities may hinder the PCR reactions. Therefore, we assessed each sample for amplification by performing PCR reaction using MiBird-U primers to verify the presence of amplicons on agarose gels. Amplicons were detected in only 64% of the extracted samples (Tab. S2). Samples were categorized as successfully amplified or unsuccessfully amplified by MiBird-U primers. We then compared the absorbance ratios in these groups (Fig. 2e). The A260/A280 ratio showed no significant difference between both groups of samples (amplification: 0.86 ± 0.06; no amplification: 0.64 ± 0.06). However, the 260/A230 ratio was significantly lower in the group with no amplification (amplification: 0.65 ± 0.06, no amplification: 0.32 ± 0.003; $P < 0.001$), suggesting that uric acid was likely the primary factor inhibiting the PCR reaction (it is noteworthy that sample freshness was similar in both groups, data not shown). Subsequently, we conducted further analysis on the 74 samples that exhibited successful amplification with the MiBird-U primers.

3.2 Sample composition

After data curation, we successfully identified MiBird-U amplicons at either the species (55 samples) or family level (10 samples) from 65 out of 74 fecal samples (Fig. 3). Fig. S4 illustrates the correspondence between some of these samples photographed in the field and their identification by metabarcoding. The 65 fecal samples identified as originating from birds came from nine different bird families, with a predominant representation of waterbirds (69%) with, notably the Rallidae family (constituting 37% of the total, as shown in Fig. 3).

At least one COI amplicon was sequenced in 69 samples and one *trn*L amplicon in 68 samples out of 74. After data curation, among the 65 fecal samples identified as bird species, at least one ingested prey was found in 31 samples and at least one plant in 52. The number of invertebrate prey items detected per sample ranged from 0 to 11, while the number of ingested plants ranged from 0 to 7. Notably, the number of food items detected in the samples did not correlate with the A_{260}/A_{230} ratio (Fig. S5). The composition of prey items varied among the samples, with a total of 26 different valid invertebrate taxa from 16 families being identified (Tab. 3). Species identification was successfully achieved for 65% of the taxa. At the family level, Chironomidae was present in 63% of the samples, followed by Asellidae in 30%, Caenidae in 26%, Ephydridae in 21%, Noctuidae in 12%, and Baetidae in 9% (Fig. 4). The remaining 11 families were found in only 2% of the samples. Notably, Chironomidae represented 46% of the total number of ASVs (comprising *Cricotopus* sp. at 23 %, and *Paracladius quadrinosus* at 18%), while *Asellus aquaticus* accounted for 17%, *Caenis robusta* and *Scalella paludum* for 7%, and the remaining species each represented equal to or less than 3%.

A total of 55 valid taxa from 33 plant families was detected in the bird feces (Tab. 4). Only 15% of the taxa were identified at the species level. The six most frequently encountered plant families in the samples were Poaceae (present in 59% of the samples), Salicaceae (36%), Betulaceae (21%,) Asteraceae (19%), Cyperaceae (17%), and Rosaceae (17%) (Fig. 4). At the genus level, *Salix* sp. (26%) and *Carex* sp. (17%) were prevalent, while aquatic species *Ceratophyllum demersum* (9%) and *Phragmites australis* (9%) were the most frequently detected (Tab. 4)

Finally, the ratio between plant and invertebrate richness in dietary composition of bird species varied from 0.6 (mute swan) to 4 (mallard), with the majority of ratios being greater than one (Tab. 5). Data suggest that plant items were generally more diverse than invertebrate items, and that the dietary composition varied between species.

3.3 Diet similarities among waterbird species and sampling sites

To analyze the diet similarities between bird species and/or sites, we conducted an NMDS analysis (Fig. 5). The representation in the reduced space showed a stress parameter of 0.141, indicating an acceptable representation. The multivariate statistical analysis of the community revealed a distinct pattern in the distribution of invertebrate preys and plants at the family level across various bird species (Fig. 5b) and sampling sites (Fig. 5c). A permutation test indicated that the variation was explained by differences in bird species and sites, accounting for 27% ($p = 0.001$) and 18% ($p = 0.002$) of the total variation, respectively, while the interaction between species and site was not significant ($p = 0.246$). Additionally, we revealed that the localization of the sites in rural or urban environments did not significantly explain the differences in dietary patterns ($p = 0.238$).

The pairwise comparison test for significant differences in diet between species or sites did not show significant differences, likely because of the small number of samples. However, some tendencies were observed; the ring-necked pheasant exhibited the most divergent diet ($p = 0.063$, compared to mallard, woodpigeon (*Columba palumbus*), Eurasian Coot, Eurasian moorhen, and Turdidae), followed by mute swan ($p = 0.063$, compared to woodpigeon, Eurasian moorhen, and Turdidae).

The mute swan included in its diet *Callitriche stagnalis*, an aquatic plant with floating leaves, *Micronecta scholtzi*, an aquatic insect, and *Parapoynx stratiotata*, whose caterpillar feeds on aquatic plants (Fig. 4 and 5; Tab. 3 and 4). Meanwhile,

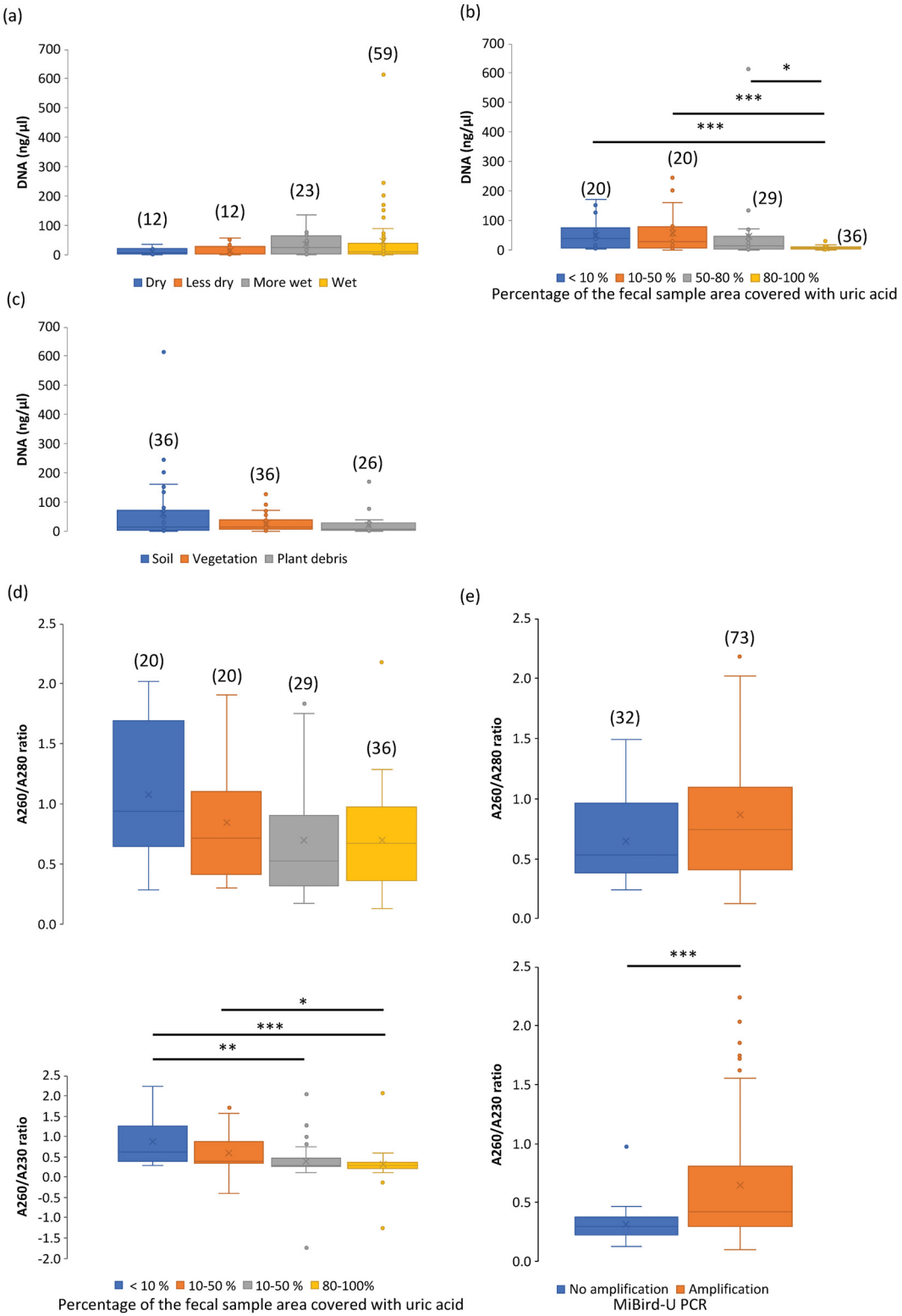

Fig. 2. Comprehensive analysis of factors influencing DNA extraction and PCR results. Panels (a), (b) and (c) illustrate the impact of (a) sample freshness, (b) the presence of uric acid on the entire fecal surface, and (c) the dominant sampling substrate on DNA concentration, measured immediately after fecal sample extraction. The samples were categorized based on their "swollen" and "shiny appearance for fresh samples and "contracted" and "dull" for dry samples. Panel (d) depicts the relationship between DNA purity, assessed through absorbance ratios at 260 nm and 280 nm (upper panel), and 260 nm and 230 nm (lower panel), and the presence of uric acid on the fecal sample surface. Panel (e) shows the effect of DNA purity, assessed through absorbance ratios at 260 nm and 280 nm (upper panel), and 260 nm and 230 nm (lower panel) on MiBird-U PCR result. PCR products were analyzed by agarose gel electrophoresis. The numbers in brackets indicate the sample count. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, analyzed using the Wilcoxon rank-sum test.

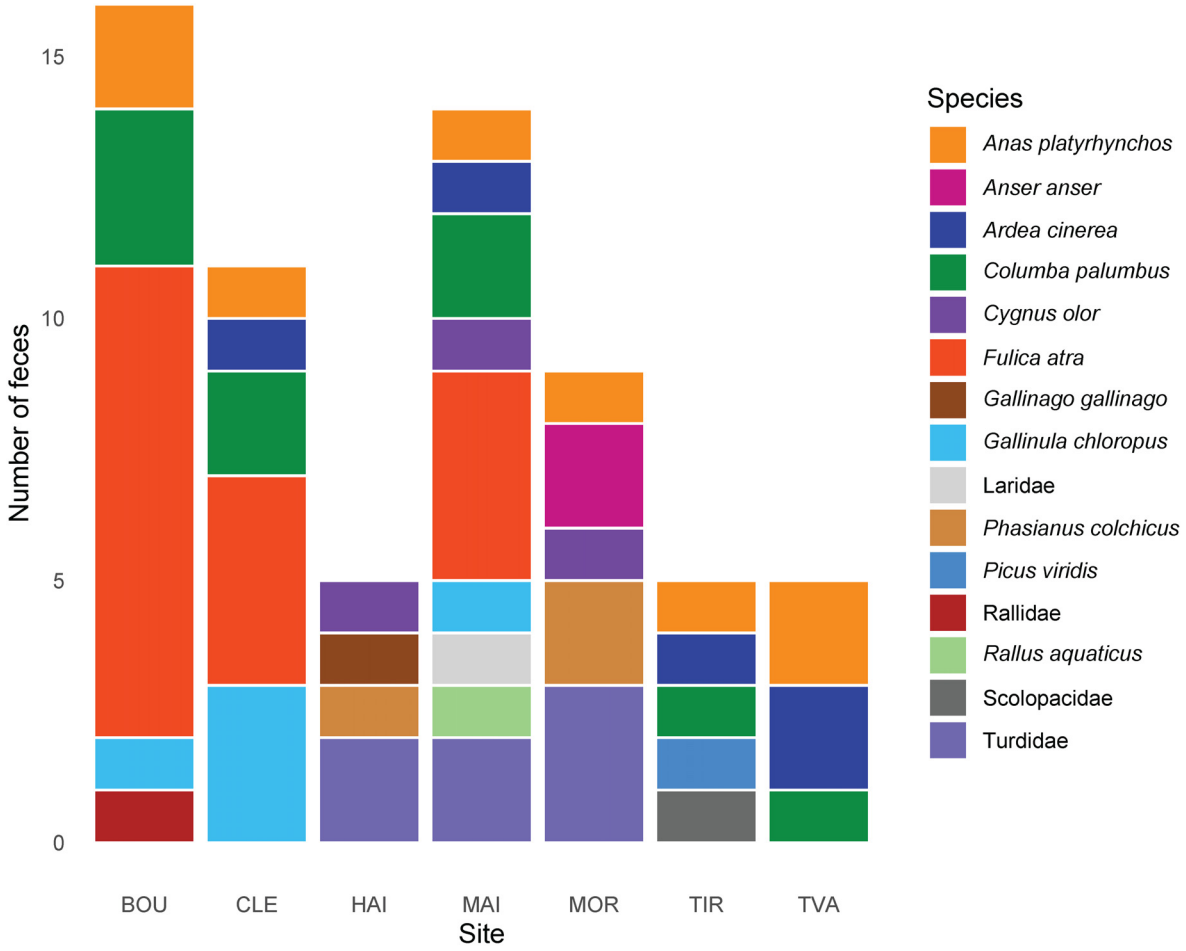

Fig. 3. Metabarcoding identification of bird species from fecal samples collected at each sampling site.

the ring-necked pheasant included terrestrial plants, such as *Galium* sp. and *Apiaceae*, as well as *Tipula helvola* (Diptera) and *Deroceras leave*, a marsh slug. Dispersion was evaluated by calculating the distances to the centroids (Tab. 5); data suggest that the Eurasian moorhen had the least diversified regime, dominated by Poaceae and Asellidae (Figs. 4 and 5). Interestingly, the Eurasian coot included a significant portion of Poaceae in its diet, which was absent in the dietary composition of the mallard. Meanwhile, the mallard introduced families of terrestrial plants absent in the diet of the Eurasian coot, such as Rosaceae, Cupressaceae, Fabaceae, or Ranunculaceae. Both species had also ingested a variety of aquatic insects (Figs. 4 and 5).

The pairwise comparison test showed dissimilarity tendencies between sites: Marais d'Isle ($p = 0.052$, compared to Bouvaque, Cléry, and Morcourt) and between Bouvaque and Cléry ($p = 0.052$). Cléry and Morcourt were the sites where the more dispersed dietary compositions were found. Specifically, Cléry included some aquatic Ephemeroptera from the Caenidae and Baetidae families, as well as aquatic plants such as *Potamogeton* and *Ceratophyllum demersum*, while Morcourt was more dominated by terrestrial plant items such as Poaceae, Asteraceae, Rubiaceae, Fagaceae, and Rosaceae (Fig. 5). Bouvaque was the site where the less dispersed diet composition was found, with a dominance of Asteraceae, Fagaceae, Poaceae, Salicaceae, and Chironomidae (Tab. 5; Fig. 5).

4 Discussion

In recent decades, fecal DNA metabarcoding has been successfully used for high taxonomic resolution and non-invasive monitoring of diet in a number of wild species (Pompanon *et al.*, 2012; Taberlet *et al.*, 2018). However, due to PCR bias, metabarcoding is not a strictly quantitative method, preventing the precise quantification of prey ingested by an individual and present some other limitations that may be exacerbated when studying bird species (Ando *et al.*, 2018; Lamb *et al.*, 2019; Liu *et al.*, 2023). Nevertheless, the fecal DNA metabarcoding method offers significant advantages, including high sensitivity, good taxonomic resolution, time- and cost-effectiveness, and the ability to systematically and non-invasively monitor wild communities and their habitats. This method can provide valuable data for conservation and management decisions while minimizing disturbances to wildlife and ecosystems.

Table 3. Presence of invertebrate taxa in the diet of ten bird species and one genus. Results are presented as the Frequency Of Occurrence (FOO%) with "*n*" indicating the number of samples.

Presence [FOO%]											Arthropoda & Mollusca		
Anas Platyrhynchos	*Anser anser*	*Ardea cinerea*	*Columba palumbus*	*Cygnus olor*	*Fulica atra*	*Gallinula chloropus*	*Phasianus colchicus*	*Picus viridis*	*Rallus aquaticus*	Turdidae	Order	Family	Taxon
n = 6	*n* = 2	*n* = 5	*n* = 7	*n* = 3	*n* = 16	*n* = 5	*n* = 3	*n* = 1	*n* = 1	*n* = 6			
							33				Stylommatophora	Agriolimacidae	*Deroceras laeve*
					6						Amphipoda	Crangonyctidae	*Crangonyx* sp.
33		20	43		25	40			100	83	Isopoda	Asellidae	*Asellus aquaticus*
			14			20				17			*Asellus* sp.
					13	20					Ephemeroptera	Baetidae	*Cloeon dipterum*
				33	13								*Cloeon* sp.
17					19							Caenidae	*Caenis robusta*
				67									*Caenis horaria*
		20			13	20					Diptera	Chironomidae	Chironomidae
					6								*Paratendipes albimanus*
					6								*Cryptochironomus* sp.
17	250		29							33			*Paracladius quadrinodosus*
17					6								*Cricotopus* sp.
17													*Parachironomus arcuatus*
				33									*Procladius* sp.
										17		Ephydridae	*Scatella paludum*
					6							Lymnaeidae	*Ampullaceana balthica*
					6							Mycetophilidae	Mycetophilidae sp.
										17		Polleniidae	*Pollenia griseotomentosa*
							33					Tipulidae	*Tipula helvola*
					6						Hemiptera	Corixidae	*Sigara striata*
				67								Micronectidae	*Micronecta scholtzi*
		20									Coleoptera	Dytiscidae	*Dytiscus marginalis*
									100		Megaloptera	Sialidae	*Sialis lutaria*
				33							Lepidoptera	Crambidae	*Parapoynx stratiotata*
					13							Noctuidae	Noctuidae sp.
		20					33						*Noctua pronuba*

In this context, the aim of the present study was to evaluate the feasibility of using bird fecal samples simply collected from the ground for DNA metabarcoding to investigate the diet composition and habitat quality of waterbirds in a French Ramsar wetland characterized by small waterbird populations. During the breeding season, when breeding success may be dependent on emerging insects and vegetation, we sampled eight marshes with various waterbird-related activities and anthropic uses (Tab. 1). We anticipated that these human activities might alter food sources by reducing natural food diversity and introducing anthropogenic food.

Collecting fecal samples from scarce and small populations of wild waterbirds in the marshes can be challenging, and harvesting fecal matters immediately after defecation may be very difficult. Feces can be deposited on water or in hard-to-reach places, making their collection a logistical challenge. Furthermore, trapping options should be avoided during the breeding season to minimize disturbance to the birds. When using feces deposited on the ground, both biotic and abiotic factors can introduce biases into the results. For example, samples found in moist soil have been shown to contain a higher proportion of food contaminants, and the time elapsed after defecation is often unknown (Ando *et al.*, 2018). Considering all the known parameters that could introduce bias into our sampling process, we were able to collect an average of 21 bird samples per marsh under favorable conditions in seven of the marshes (Tab. S2).

Several types of avian feces are distinguished based on their content, consistency, and shape (Fig. S4). In birds, as in other species with a cloaca, feces are typically accompanied by urine, and the presence of uric acid can make DNA extraction from avian feces particularly challenging (Eriksson *et al.*, 2017; Davies *et al.*, 2022). Using commercial kits commonly employed for the bird DNA extraction, Erickson *et al*. (2017) demonstrated the difficulty of extracting DNA from feces from the mallard, an omnivorous species (Tab. S9), resulting in either null or very low DNA yields. While one of the kits they tested showed improved results, it was primarily designed for DNA extraction from pathogens, rendering it unsuitable for the analysis of degraded DNA often encountered in diet studies. Therefore, selecting a DNA extraction method capable of analyzing diet in a bird community remains a challenging task. For these reasons, we opted to use a DNA extraction kit that had previously yielded favorable results in the study of the diet

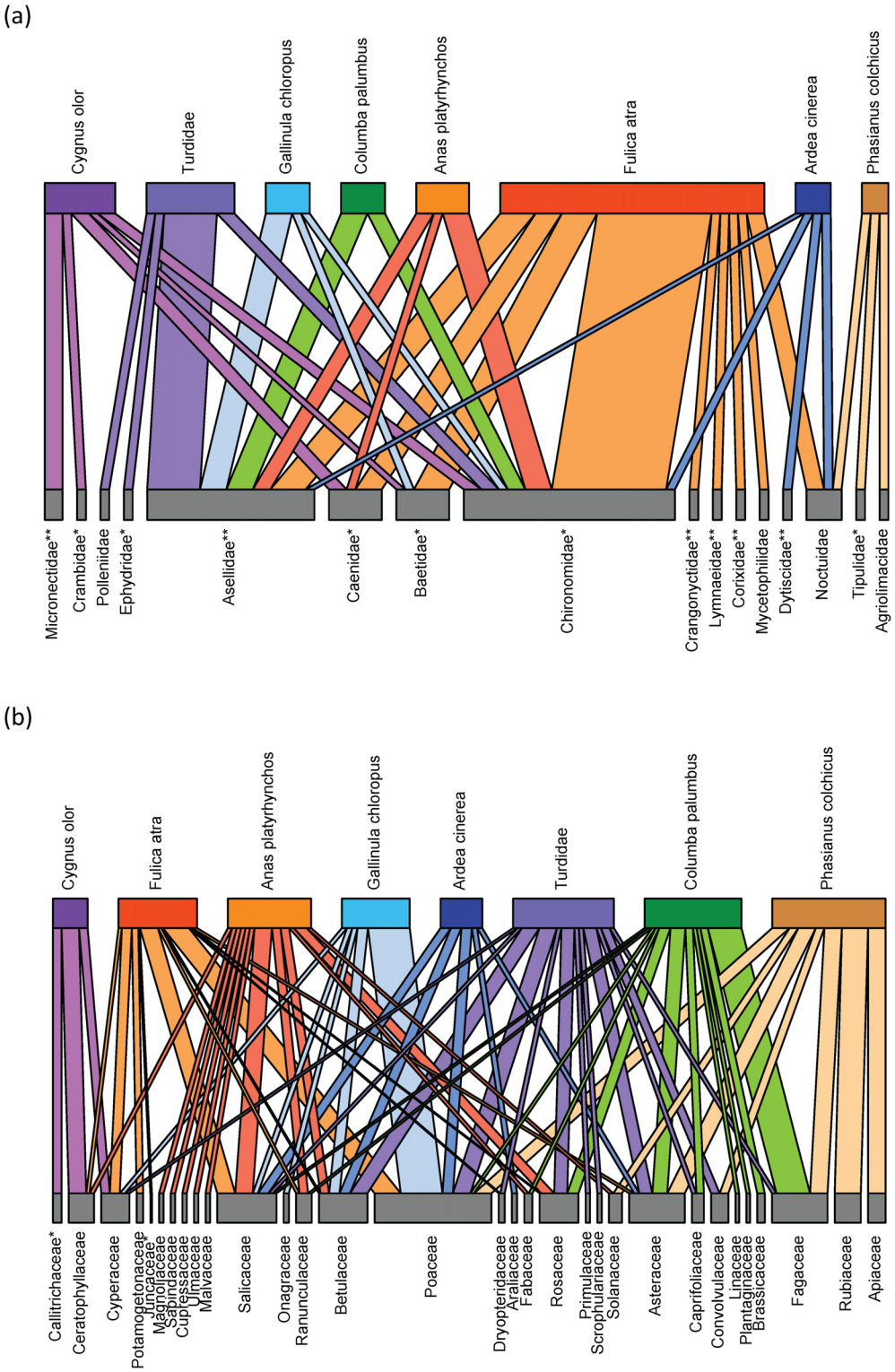

Fig. 4. Bipartite food webs illustrating the frequency of predator-prey interactions. The upper bars represent the bird species, and the lower bars represent the invertebrate families (a) and the plant families (b). The lines connecting the bars depict interactions between species, with the line width proportional to the number of interactions.

Table 4. Presence of plant taxa of in the diet of ten bird species and one genus. Results are presented as the Frequency Of Occurrence (FOO%) with "*n*" indicating the number of samples.

Presence [FOO%]											Streptophyta		
Anas Platyrhynchos	*Anser anser*	*Ardea cinerea*	*Columba palumbus*	*Cygnus olor*	*Fulica atra*	*Gallinula chloropus*	*Phasianus colchicus*	*Picus viridis*	*Rallus aquaticus*	Turdidae	Order	Family	Taxon
n = 6	*n* = 2	*n* = 5	*n* = 7	*n* = 3	*n* = 16	*n* = 5	*n* = 3	*n* = 1	*n* = 1	*n* = 6			
							67				Apiales	Apiaceae	Apiaceae
		20										Araliaceae	Araliaceae
		20	57		19		67		100	50	Asterales	Asteraceae	Asteraceae
17						20			100		Fagales	Betulaceae	*Betula* sp.
17		20			6	20				33			*Alnus* sp.
		20								33			Betulaceae
			14								Brassicales	Brassicaceae	*Cardamine* sp.
			14										Brassicaceae
				33							Callitrichales	Callitrichaceae	*Callitriche stagnalis*
										17	Dipsacales	Caprifoliaceae	*Lonicera* sp.
			29										*Sambucus* sp.
17				67	13						Ceratophyllales	Ceratophyllaceae	*Ceratophyllum demersum*
							33			33	Solanales	Convolvulaceae	Convolvulaceae
17											Cupressales	Cupressaceae	*Hesperocyparis* sp.
	100			33	38	20				17	Poales	Cyperaceae	*Carex* sp.
					6				100	17	Polypodiales	Dryopteridaceae	Dryopteridaceae
17			14								Fabales	Fabaceae	*Robinia* sp.
	50		43				67			17	Fagales	Fagaceae	*Quercus* sp.
			86										*Fagus* sp.
									100		Saxifragales	Grossulariaceae	*Ribes nigrum*
					6						Poales	Juncaceae	*Juncus compressus*
			14								Malpighiales	Linaceae	*Linum* sp.
									100		Myrtales	Lythraceae	Lythraceae
17											Magnoliales	Magnoliaceae	*Magnolia* sp.
17											Malvales	Malvaceae	*Tilia* sp.
						20					Myrtales	Onagraceae	*Epilobium* sp.
			14								Lamiales	Plantaginaceae	*Veronica* sp.
	50						33				Poales	Poaceae	*Secale* sp.
		20			25								*Festuca* sp.
	100	20	14		56	120	33			33			Poaceae
	50				6				100	33			*Phragmites australis*
	50												*Holcus* sp.
					6	40							*Dactylis* sp.
					6								*Poa* sp.
					13						Alismatales	Potamogetonaceae	*Zannichellia* sp.
					13								*Potamogeton* sp.
									100	17	Ericales	Primulaceae	*Lysimachia* sp.
33											Ranunculales	Ranunculaceae	*Clematis* sp.
			14										*Ficaria verna*
					13								*Ranunculus* sp.
17											Rosales	Rosaceae	*Rosa* sp.
33													*Filipendula ulmaria*
			29										*Potentilla* sp.
	50				6					33			Rosaceae
			14										*Prunus* sp.
										17			*Geum* sp.
							100				Gentianales	Rubiaceae	*Galium* sp.
50			14		56					17	Malpighiales	Salicaceae	*Salix* sp.
17		40			13								*Populus* sp.
						20							Salicaceae
17											Sapindales	Sapindaceae	*Sapindaceae*
										17	Lamiales	Scrophulariaceae	*Scrophulariaceae*
17											Solanales	Solanaceae	*Solanum* sp.
							33						*Solanum dulcamara*
17											Rosales	Ulmaceae	*Ulmaceae*

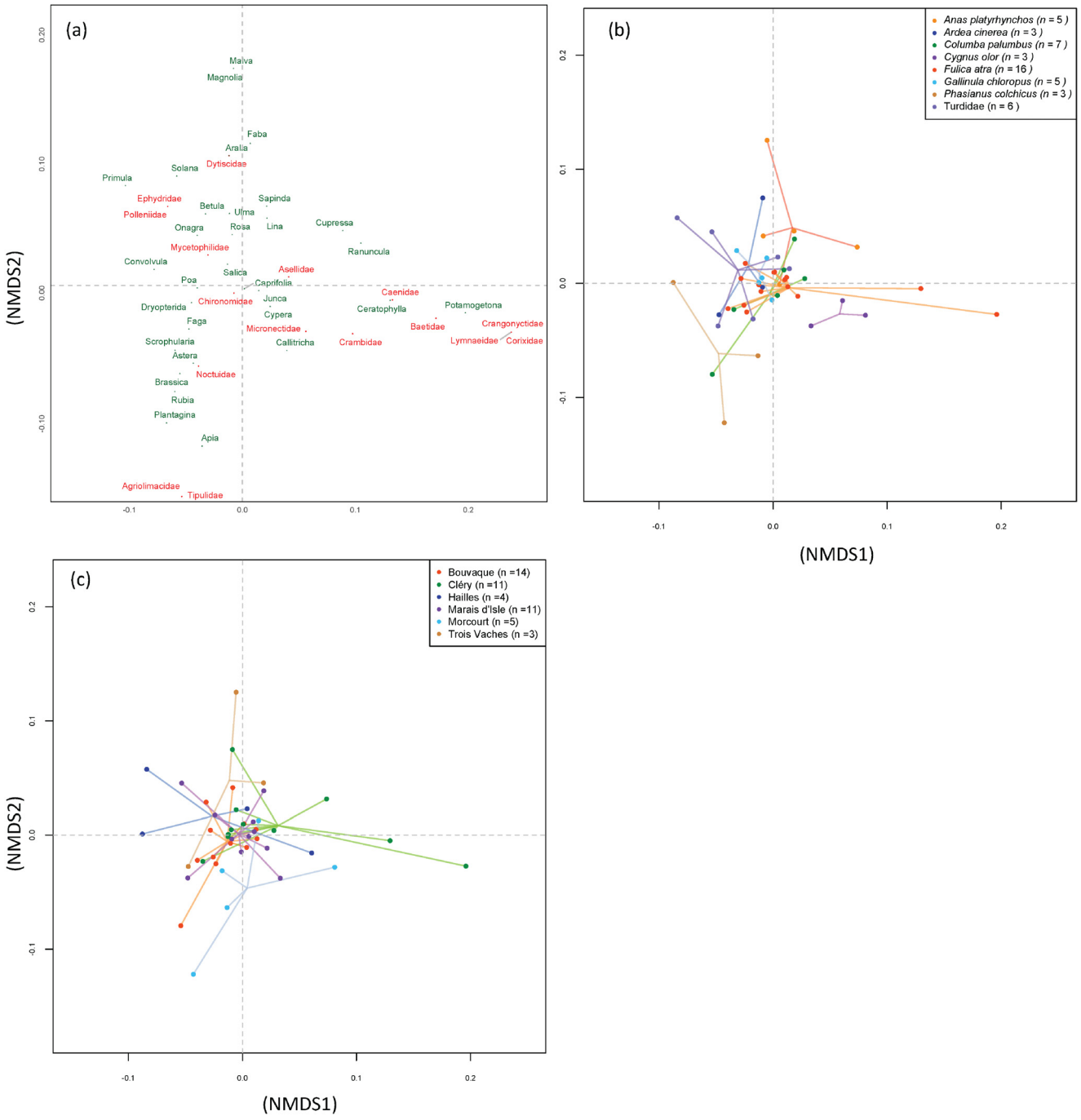

Fig. 5. Similarity analysis of the diet composition among bird species and sampling sites through Non-metric Multidimensional Scaling (NMDS). Only species with at least three samples that provided valid data are represented. (a) Distribution plot in a two-dimensional space of the plant families (family name's ending "ceae" was removed from the graph to prevent label overlap) appearing in green, and invertebrate families appearing in red. Distribution plot of the samples by (b) species and (c) sites. The quality of the representation in space, or stress value, was 0.141. Numbers in brackets indicate the sample size.

of two omnivorous species (*Rattus exulans* and *Rattus rattus*). This kit was specifically designed for extracting total DNA from complex and heavily processed samples in which DNA is highly degraded (Zarzoso-Lacoste *et al.*, 2013).

Studies have shown uric acid's distinct peaks in UV spectra (Fischer, 1995), potentially influencing DNA concentration and sample purity post-purification, as reflected by the A_{260}/A_{230} ratio. Our findings demonstrate a clear correlation between the visible presence of uric acid on the surface of feces and reduced DNA yield and purity (Fig. 2). Moreover, the reduced purity negatively impacted DNA amplification using avian primers, only 64% of the extracted samples were successfully amplified using primers targeting the 12S gene of birds (Tab. S2). Surprisingly, in samples successfully amplified

Table 5. Dispersion of observations around their centroid in the reduced space generated by the NMDS analysis and the ratio of plant richness consumed to invertebrate richness consumed for each species. Average distances to centroids were calculated only for species with at least 5 analyzed samples. n.d.: not determined. BOU: Bouvaque, CLE: Cléry-sur-Somme, HAI: Hailles, MAI: Marais d'Isle, MOR: Morcourt, TVA: Trois Vaches.

Bird species	*n*	Average distance from centroids	Diet taxonomic richness		Plants/Invertebrates ratio	Main diet
			Plants	Invertebrates		
Anas platyrhynchos	5	0.059 ± 0.013	12	3	4.0	Omnivorous
Ardea cinerea	3	n.d.	5	4	1.3	Piscivorous
Columba palumbus	7	0.045 ± 0.011	11	2	5.5	Herbivorous
Cygnus olor	3	n.d.	3	5	0.6	Herbivorous
Fulica atra	16	0.051 ± 0.012	11	9	1.2	Omnivorous
Gallinula chloropus	5	0.023 ± 0.005	5	3	1.7	Omnivorous
Phasianus colchicus	3	n.d.	7	3	2.3	Omnivorous
Turdidae	6	0.066 ± 0.008	12	4	3.0	Omnivorous
Sites						
BOU	14	0.035 ± 0.004				
CLE	11	0.069 ± 0.013				
HAI	4	n.d.				
MAI	11	0.049 ± 0.012				
MOR	5	0.060 ± 0.013				
TVA	3	n.d.				

by avian primers, the number of food items detected by PCR remained unaffected by sample purity (Fig. S5).

Using these primers, we were able to identify the bird species at the origin of 88% of the sequenced fecal samples. Notably, we observed a strong correspondence between visual identification of fecal samples based on their appearance and their identification through metabarcoding (Fig. S4). As expected, at least for waterbirds, the number and origin of fecal samples identified by marsh did not serve as a reliable proxy for richness and abundance. For instance, no feces from the mute swan were identified in the marsh of "Cléry-sur-Somme", despite the presence of a large flock in the water (Fig. 3).

Due to its high sensitivity, metabarcoding results may be affected by sample contamination as well as technical inherent errors. On one hand, fecal samples are susceptible of environmental DNA contamination, particularly when collected from wet soil (Ando *et al.*, 2018). To mitigate this risk, we implemented several precautionary measures during our sampling process. These measures included collecting fresh fecal samples, avoiding samples from deposits in waterlogged soil, and taking photographs for later verification to confirm if the vegetation present in the sampling substrate matched the species identified through metabarcoding (Fig. S4). Additionally, during bioinformatics data analysis, we filtered out occurrences with low counts to reduce the likelihood of contamination affecting our results (Ando *et al.*, 2018). On the other hand, to minimize technical contaminations, we employed the VTAM analysis pipeline, specifically designed to address cross-sample contamination and tag-jumps through rigorous filtration procedures (González *et al.*, 2023). We also assessed the potential contamination of samples with plant DNA by examining the presence of plant taxa in samples from carnivorous birds. The grey heron is primarily piscivorous but also opportunistic, notably known to consume mollusks, crustaceans, and aquatic insects (Tab. S9). However, it is not known to eat plants. In our study, we found DNA from five different plant families (Betulaceae, Salicaceae, Araliaceae, Poaceae, and Asteraceae) in heron feces. These contaminations could be linked to secondary predation, pollens, or plant litter, although the latter was not readily apparent based on field observations. Conversely, evaluating contamination of feces with arthropod DNA presented a challenging issue, given that none of the species in our dataset were strictly herbivorous. Birds may incidentally ingest insects when foraging on plants. Contamination of fecal matter on the ground may also result from detritivorous insects or egg deposition; however, these behaviors are expected to be rare, for example, in the case of Chironomidae and Asellidae, which are primarily aquatic (Oertli and Frossard, 2013).

Among the 55 plant taxa detected in our fecal samples, 8 have been identified to the species level (*Callitriche stagnalis*, *Ceratophyllum demersum*, *Ribes nigrum*, *Juncus compressus*, *Phragmites australis*, *Ficaria verna*, *Filipendula ulmaria*, and *Solanum dulcamara*), and all have been reported in the management plans of the CEN-HDF (personal communication) for one or more of the sampled sites included in this study. Among the 33 taxa identified at the genus level, only 9% have not been reported in the management plans; these are *Hesperocyparis* sp., *Magnolia* sp., and *Secale* sp. The first two genera are likely cypress and magnolia, commonly found in gardens and parks in France. The genus *Secale* was detected in the feces of graylag goose at the Morcourt site and of ring-necked pheasant at the Hailles site. This genus includes wild species and the cultivated rye (*Secale cereale*), closely related to barley and wheat. As Morcourt and Hailles are hunting sites, these seeds were likely left by hunters, possibly to feed live

decoys for waterfowl hunting that remains on the sites even outside the hunting season.

Our study identified 27 invertebrate taxa in the diet of the sampled birds, of which 18 were identified to the species level. Most of the species and genera found in our fecal samples are recorded in the participatory database *clicnat* (https://clicnat.fr), confirming their presence in the region. However, only four species had already been mentioned in the management plans of the CEN-HDF: *Deroceras laeve*, a marsh slug, *Ampullaceana balthica*, a freshwater pulmonated snail, and two Lepidoptera: *Parapoynx stratiotata*, whose larvae are aquatic, and *Noctua pronuba* (personal communication). It is worth noting that aquatic invertebrate families such as Asellidae, Baetidae, Caenidae, or Chironomidae, despite their interest as bioindicators, are not among the species listed by site managers due to time constraints. Finally, among the five taxa not mentioned in local and regional inventories are: *Micronecta scholtzi*, the lesser water boatman, *Pollenia griseotomentosa*, a fly, and *Crangonyx*, a genus of freshwater crustaceans. However, these species are present in France and are listed in the National Inventory of Natural Heritage (https://inpn.mnhn.fr/accueil/index). Additionally, according to the same database, *Scatella paludum* is a fly that has not been recorded in northern France but is present in neighboring countries: Belgium and southeastern Great Britain. *Paracladius quadrinodosus* is a midge whose presence has been primarily reported in Scandinavia, but also in southern Germany. In conclusion, the comparison with databases confirms our findings, while our study provides a significant contribution by complementing local and regional inventories of invertebrate taxa.

Despite the small number of samples obtained per species, our study reveals emerging trends suggesting dietary variation among bird species, supported by a PERMANOVA. Some of the plant and invertebrate species that we have detected coincide with other dietary composition studies, while some other plant species, to our knowledge, were not previously reported as part of waterbird diet. The mute swan is primarily herbivorous, but may occasionally consume animals such as frogs, toads, tadpoles, mollusks, insects, and their larvae, especially during the annual molt and cool springs when plant growth is inhibited (Mathiasson, 1973; Tab. S9). Among the plant species detected in the present study, *Ceratophyllum demersum* and *Callitriche* sp. coincide with other studies (Billerman *et al.*, 2022). The specific plant richness observed in the diet of the mute swan was low compared to that of other species such as the Eurasian coot or the mallard (Tab. 5). Therefore, the swans may maintain a relatively limited diversity of a plant-based diet and with the occasional consumption of aquatic insects or their larvae, occurring either deliberately or incidentally while ingesting plants or grit. In general, swans show little preference among plants (*e.g.* Bailey *et al.*, 2008), but there are instances where they may exclusively consume one or two plant species, which may lead to the elimination of those plants from the ecosystem. For example, it has been demonstrated in Sweden that, within one month and a half, 45 individuals of molting swans, showing a clear preference for *Zostera marina* and *Ulva lactuca*, eliminated this sea lettuce from a one-hectare bed (Mathiasson, 1973).

The Eurasian coot is an omnivorous species during breeding season: its diet is dominated by invertebrates and filamentous algae, while macrophytes make up a low proportion of the diet (Perrow *et al.*, 1997). This is explained by these soft-bodied invertebrates being fed to chicks that need protein-rich food for growing (Brinkhof, 1997). Among the plant species detected in the present study, *Carex* sp., *Phragmite australis*, *Zannichellia* sp., *Potamogeton* sp., and *Ranunculus* sp., were already found in the diet of the Eurasian coot (Billerman *et al.*, 2022). Additionally, we observed consumption of Ceratophyllaceae and Cupressaceae. Furthermore, Eurasian coot showed a distinct attraction towards invertebrate taxa such as Chironomidae, Asellidae, Caenidae, and Baetidae, as well as families such as Noctuidae, Lymnaeidae, Corixidae, Mycetophilidae and Crangonyctidae (Fig. 4 and 5). While many of these families have not been well-documented in the Eurasian coots' diet, our findings confirm their significant reliance on Chironomidae, a vital food source for their chicks (Hansson *et al.*, 2014). However, Hansson *et al.* (2014) have shown that common waterbirds are unlikely to adjust their reproduction to align with the anticipated shift in insect emergence timing due to climate change. This discrepancy could lead to a mismatch between food availability and waterbirds requirements, ultimately impacting their reproductive success and population dynamics.

The diet variability among samples of the Eurasian moorhen was less pronounced compared to other waterbird families (Fig. 5; Tab. 5), resulting in a lower mean distance to the centroid when compared with other species. Therefore, the feeding behavior of the moorhen showed tendencies toward being less omnivorous and/or adaptable. The diet of the Eurasian moorhen was dominated by Poaceae and Asellidae (Fig. 4). Their consumption of *Dactylis* sp. aligned with their feeding behavior, which includes not only feeding while swimming or walking on floating vegetation, but also feeding on land, where they graze and glean over open grass. They also glean insects, seeds, and fruits from the ground and from plants. *Carex* sp. and Diptera have already been shown to compose the diet of the Eurasian moorhen (Billerman *et al.*, 2022).

The mallard is an omnivorous, opportunistic, and generalist feeder. The breeding season is known to reveal an increase in invertebrates in their diet; including insects such as midge larvae (Chironomidae) and other Diptera, dragonflies (Odonata), and caddisfly (Trichoptera) larvae, aquatic invertebrates such as snails and freshwater shrimp, and terrestrial earthworms (Dessborn *et al.*, 2011; Billerman *et al.*, 2022). Laying females in North Dakota were shown to consume more invertebrates than non-laying females and males during the same period (Dessborn *et al.*, 2011). The diets of ducklings and adults overlap widely. Both adults and ducklings eat a variety of animals and vegetable matter; however, benthic invertebrates and aquatic plant parts (other than seeds) are more common in adult diet, whereas emerging invertebrates such as Chironomidae are more commonly found in ducklings, likely reflecting differences in feeding method. Our results have shown a greater diversity of plants than insects, with a ratio of 4 in the diet of the mallard, including a greater diversity of terrestrial plants than in coots, suggesting that ducks have fed outside of water. Nevertheless, there was a significant overlap between the diet of the mallard, the Eurasian coot, and the Eurasian moorhen, while the mute swan exhibited minimal dietary overlap with other waterbird species (Fig. 4).

Among the waterbird feces we found, there were some samples from ring-necked pheasant, woodpigeon, and birds of the Turdidae family. The ring-necked pheasant takes food primarily from the ground; our results suggest that the ring-necked pheasant did not depend on aquatic species, and its diet did not overlap with that of waterbirds (Fig. 4). Among the plant species detected in the present study, *Solanum dulcamara,* Acorns (Quercus spp.), and *Secale* sp. are known to be part of the pheasant diet (Billerman *et al.*, 2022). The woodpigeon also takes most of his food from the ground, but feeds on trees as well. Most of its diet is known to be made up of plant matter; various invertebrates are also occasionally eaten, including earthworms, gall wasps, beetles, pupae of Lepidoptera, spiders, slugs, and snails (Billerman *et al.*, 2022). The diet is dominated by the fruit and seeds of trees in spring, which is in agreement with the elevated plant/invertebrate ratio that we have found for this species (Tab. 5). This varied diet allows common wood-pigeons to feed on seasonally abundant food sources with high calorific content, which was shown to be ignored by most other seed-eating birds in Spain (Billerman *et al.*, 2022). Acorns of Quercus spp. were the most consumed item in winter, cereals dominated the summer diet, and tree fruits predominated in spring and autumn. *Quercus* spp. and *Fagus* sp. were among the main food items that we identified in the woodpigeon's diet, along with other terrestrial plants such as Asteraceae, *Sambucus* sp., and *Potentilla* sp.. Finally, Turdidae's diet was found to be quite diverse as well (Tab. 5), and it contained, together with terrestrial plants, some macrophytes such as *Carex* sp. and *Phragmite australis*. This is compatible with the highly flexible and adaptative diet that has been described for the Eurasian blackbird (*Turdus merula*) (Billerman *et al.*, 2022). What was more surprising, was to find Asellidae, a family of benthic isopod, in the diet of Turdidae and woodpigeon. However, detritivorous Asellidae are frequently encountered in ponds rich in decomposing organic matter (tree leaves, vegetation...) and have been detected in tire track pools and puddles (Armitage *et al.*, 2012). Therefore, ingestion by these birds could be voluntary or incidental while drinking water.

Our results also suggest differences in dietary patterns among sampled sites, regardless of the bird species sampled (Fig. 5). However, unlike other sites, the analyzable feces collected at Morcourt and Hailles were predominantly from terrestrial birds: ring-necked pheasant and Turdidae. This resulted in Hailles in dietary compositions that mainly consisted of terrestrial plants, such as Convolvulaceae, Rosaceae, *Quercus* sp., *Lysimachia* sp., *Galium* sp., and *Solanum dulcamara*. In contrast, Morcourt was more dominated by Asteraceae, Rubiaceae, Fagaceae, and Rosaceae. The presence of Poaceae was also detected in both sites. However, this family can include both terrestrial and aquatic plants, such as *Phragmites australis*. In Cléry, La Bouvaque, and Marais d'Isle, where the collected feces primarily came from waterbirds, the dietary composition included numerous aquatic species (Fig. 5). Cléry was the site that exhibited the greatest variety of invertebrate and plant resources, as suggested by a greater average distance to the centroid (Tab. 5). Moreover, this site included some aquatic Ephemeroptera from the Caenidae and Baetidae families. According to the AFNOR NF T90-350, 2004, standard, referenced in the European Water Framework Directive, these Ephemeroptera are classified as taxa in group 2 due to their pollution-sensitive nature, while Chironomidae and Asellidae, which are less sensitive, belong to group 1. This classification is used to calculate the standardized global biological index, suggesting that Cléry has a higher environmental quality. Marais d'Isle, in addition to terrestrial plant species showed a large majority of Carex sp., many of which thrive in wetland areas. There were also aquatic plants such as *Phragmites australis*, *Salix* sp., *Callitriche stagnalis*, and *Juncus compressus*, as well as aquatic invertebrates: *Asellus aquaticus*, *Micronecta scholtzi*, and Chironomidae (including *Paracladius quadrinosus*). Finally, the least dispersed dietary compositions were found in Bouvaque, with a dominance of Asteraceae, Fagaceae, Poaceae, Salicaceae, and Chironomidae.

Eutrophication and urbanization can lead to the simplification of macrophyte and aquatic invertebrate communities (Rejmankova, 2011; Oertli and Parris, 2019). We had anticipated that the localization of sites in rural or urban environments could affect the diet of waterbirds. However, this variable did not explain the variation in dietary composition in our samples. It is noteworthy that Cléry, which appears to be the most favorable in terms of dietary variety, is not only located in a rural environment but also represents the largest pond, which may also explain its greater richness in invertebrate species (Oertli and Parris, 2019).

In conclusion, our study delved into the dietary patterns of waterbirds in diverse wetland environments, shedding light on the intricate relationships between avian species, their prey, and the surrounding ecosystems. The identification of various arthropod families, such as Chironomidae, Asellidae, Baetidae, and Caenidae, underscores the importance of aquatic invertebrates in supporting wetland ecosystems. This aligns with existing knowledge that aquatic invertebrates serve as crucial prey for a multitude of wetland predators (Baxter *et al.*, 2005; Oertli and Frossard, 2013; Davies *et al.*, 2022). Our results revealed a greater diversity of plants in bird diets compared to insects, including various terrestrial aquatic plants such as *Ceratophyllum demersum*, *Phragmites australis*, *Potamogeton* sp., and *Callitriche stagnalis*. This observation resonates with the prevailing vegetation in the studied region's ponds, affirming the ecological relevance of our findings (Oertli and Frossard, 2013).

5 Perspectives

While our study has provided valuable insights, we acknowledge the need for further protocol optimizations. Enhancements, such as obtaining a larger sample size per marsh through multiple sampling campaigns or exploring alternative PCR primer pairs like ITS2 primers, could improve the resolution of plant taxa identification at the species level (Dunn *et al.*, 2018; Prewer *et al.*, 2023; Schumm *et al.*, 2023). Despite these considerations, our results suggest that the combination of collecting fecal samples from the ground and employing metabarcoding holds promise as a practical approach for regular monitoring of waterbird community diet and habitat quality. This method, facilitating long-term ecosystem monitoring, could offer valuable insights for ecosystem management and aid in predicting the impacts of environmental changes associated with anthropogenic activities. As we move forward, such integrative approaches will

prove essential for the conservation and sustainable management of wetland habitats.

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Supplementary material

Supplemental Information S1. Distribution of feces samples by three variables: "Area covered with uric acid", "Freshness" and "Sampling substrate" (Tab. S1); Number of samples collected at each site and remaining after each step of the experimental procedure (Tab. S2); Community composition of mock samples used as positive controls (Tab. S3); The numerical parameters used for metabarcoding data curation (Tab. S4); NovaSeq metabarcoding DNA sequence read counts recovered from all fecal and control samples after each HTS data filtering step using the VTAM pipeline (Tab. S5). Food habits of the birds identified in this study (Tab. S9); Per-base sequence quality plots for the bird dataset (Fig. S1); Per-base sequence quality plots for (Fig. S2) the invertebrate dataset, and (Fig. S3) the plant dataset; Comparison between fecal samples photographed in the field and the results of their identification through metabarcoding (Fig. S4); DNA purity (A260/A230) measured in samples grouped based on the number of food items identified by metabarcoding (Fig. S5).

Supplemental Information S2. Taxa read counts retrieved after the last filtering step using the VTAM pipeline and their taxonomic assignment. (Tab. S6) bird dataset, (Tab. S7) invertebrate dataset and (Tab. S8) plant dataset.

The Supplementary Material is available at https://www.kmae-journal.org/10.1051/kmae/2024005/olm.

References

Alberdi A, Aizpurua O, Bohmann K, Gopalakrishnan S, Lynggaard C, Nielsen M, Gilbert MTP. 2019. Promises and pitfalls of using high-throughput sequencing for diet analysis. *Mol Ecol Resour* 19: 327–348.

Ando H, Fujii C, Kawanabe M, Ao Y, Inoue T, Takenaka A. 2018. Evaluation of plant contamination in metabarcoding diet analysis of a herbivore. *Sci Rep* 8: 15563.

Armitage PD, Hawczak A, Blackburn JH. 2012. Tyre track pools and puddles – Anthropogenic contributors to aquatic biodiversity. *Limnologica* 42: 254–263.

Bailey M, Petrie SA, Badzinski SS. 2008. Diet of mute swans in lower Great Lakes coastal marshes. *J Wildl Manage* 72: 726–732.

Baxter CV, Fausch KD, Carl Saunders W. 2005. Tangled webs: Reciprocal flows of invertebrate prey link streams and riparian zones. *Freshw Biol* 50: 201–220.

Berry O, Bulman C, Bunce M, Coghlan M, Murray DC, Ward RD. 2015. Comparison of morphological and DNA metabarcoding analyses of diets in exploited marine fishes. *Mar Ecol Prog Ser* 540: 167–181.

Billerman SM, Keeney BK, Rodelwald PG, Schulenberg TS. 2022. *Birds of the World.* Ithaca, *NY, USA: Cornell Laboratory of Ornithology.* https://birdsoftheworld.org/bow/home

Boyer S, Cruickshank RH, Wratten SD. 2015. Faeces of generalist predators as 'biodiversity capsules': A new tool for biodiversity assessment in remote and inaccessible habitats. *Food Webs* 3: 1–6.

Brinkhof M. 1997. Seasonal variation in food supply and breeding success in European Coots *Fulica atra*. *Ardea* 85: 51–65.

Brochet A-L, Mouronval J-B, Aubry P, Gauthier-Clerc M, Green AJ, Fritz H, Guillemain M. 2012. Diet and feeding habitats of Camargue dabbling ducks: What has changed since the 1960s? *Waterbirds* 35: 555–576.

Brown DS, Jarman SN, Symondson WOC. 2012. Pyrosequencing of prey DNA in reptile faeces: analysis of earthworm consumption by slow worms. *Mol Ecol Resour* 12: 259–266.

Cabodevilla X, Mougeot F, Bota G, Mañosa S, Cuscó F, Martínez-García J, Arroyo B, Madeira MJ. 2021. Metabarcoding insights into the diet and trophic diversity of six declining farmland birds. *Sci Rep* 11: 21131.

Clarke KR. 1993. Non-parametric multivariate analyses of changes in community structure. *Austr J Ecol* 18: 117–143.

Corse E, Meglécz E, Archambaud G, Ardisson M, Martin J-F., Tougard C, Chappaz R, Dubut V. 2017. A from-benchtop-to-desktop workflow for validating HTS data and for taxonomic identification in diet metabarcoding studies. *Mol Ecol Resour* 17: e146–e159.

Corse E, Tougard C, Archambaud-Suard G, Agnèse J-F., Messu Mandeng FD, Bilong Bilong CF, Duneau D, Zinger L, Chappaz R, Xu CCY, Meglécz E, Dubut V. 2019. One-locus-several-primers: a strategy to improve the taxonomic and haplotypic coverage in diet metabarcoding studies. *Ecol Evol* 9: 4603–4620.

Davies SR, Vaughan IP, Thomas RJ, Drake LE, Marchbank A, Symondson WOC. 2022. Seasonal and ontological variation in diet and age-related differences in prey choice, by an insectivorous songbird. *Ecol Evol* 12: e9180.

Dessborn L, Brochet AL, Elmberg J, Legagneux P, Gauthier-Clerc M, Guillemain M. 2011. Geographical and temporal patterns in the diet of pintail *Anas acuta*, wigeon *Anas penelope*, mallard *Anas platyrhynchos* and teal *Anas crecca* in the Western Palearctic. *Eur J Wildl Res* 57: 1119–1129.

Dixon, P. (2003). VEGAN, a package of R functions for community ecology. *J Veg Sci* 14: 927–930.

Dorman C, Gruber B, Fründ J. 2008. Introducing the bipartite package: analysing ecological networks. *R News* 8: 8–11

Dudgeon D, Arthington AH, Gessner MO, Kawabata Z-I., Knowler DJ, Lévêque C, Naiman RJ, Prieur-Richard A-H., Soto D, Stiassny MLJ, Sullivan CA. 2006. Freshwater biodiversity: importance, threats, status and conservation challenges. *Biol Rev* 81: 163–182.

Dunn JC, Stockdale JE, Moorhouse-Gann RJ, McCubbin A, Hipperson H, Morris AJ, Grice PV, Symondson WOC. 2018. The decline of the Turtle Dove: Dietary associations with body condition and competition with other columbids analysed using high-throughput sequencing. *Mol Ecol* 27: 3386–3407.

Edwards DP, Woodcock P, Newton RJ, Edwards FA, Andrews DJR, Docherty TDS, Mitchell SL, Ota T, Benedick S, Bottrell SH, Hamer KC. 2013. Trophic flexibility and the persistence of understory birds in intensively logged rainforest: trophic responses of birds to logging. *Conserv Biol* 27: 1079–1086.

Egeter B, Bishop PJ, Robertson BC. 2015. Detecting frogs as prey in the diets of introduced mammals: a comparison between morphological and DNA-based diet analyses. *Mol Ecol Resour* 15: 306–316.

Eriksson P, Mourkas E, González-Acuna D, Olsen B, Ellström P. 2017. Evaluation and optimization of microbial DNA extraction from fecal samples of wild Antarctic bird species. *Infect Ecol Epidemiol* 7: 1386536.

Evans BA, Gawlik DE. 2020. Urban food subsidies reduce natural food limitations and reproductive costs for a wetland bird. *Sci Rep* 10: 14021.

Fischer J. 1995. Specific detection of nucleotides, creatine phosphate, and their derivatives from tissue samples in a simple, isocratic, recycling, low-volume system. *LC-GC International* 8: 254–264.

Gell PA, Finlayson CM, Davidson NC. 2023. An introduction to the Ramsar Convention on Wetlands. In Gell PA, Davidson NC Finlayson CM, eds. *Ramsar Wetlands: Values*, *Assessment, Management*, Elsevier, Amsterdam, 1–36.

González A, Dubut V, Corse E, Mekdad R, Dechatre T, Castet U, Hebert R, Meglécz E. 2023. VTAM: a robust pipeline for validating metabarcoding data using controls. *Comput Struct Biotechnol J* 21: 1151–1156.

Hansson L-A., Ekvall MK, Ekvall MT, Ahlgren J, Holm WS, Dessborn L, Brönmark C. 2014. Experimental evidence for a mismatch between insect emergence and waterfowl hatching under increased spring temperatures. *Ecosphere* 5: 1–9.

Hebert R, Meglécz E. 2022. NSDPY: A python package to download DNA sequences from NCBI. *SoftwareX* 18: 101038.

Hervé M. 2020. Aide-mémoire de statistique appliquée à la biologie – Construire son étude et analyser les résultats à l'aide du logiciel *R. R Package version 0.9- 83-7*. https://cran.r-project.org/web/packages/RVAideMemoire/

Hoenig BD, Snider AM, Forsman AM, Hobson KA, Latta SC, Miller ET, Polito MJ, Powell LL, Rogers SL, Sherry TW, Toews DPL, Welch AJ, Taylor SS, Porter BA. 2022. Current methods and future directions in avian diet analysis. *Ornithology* 139: ukab 077.

Huang P-Y., Poon ESK, Wong ATC, So IWY, Sung Y-H., Sin SYW. 2021. DNA metabarcoding reveals the dietary composition in the endangered black-faced spoonbill. *Sci Rep* 11: 18773.

Jakubavičiute E, Bergström U, Eklöf JS, Haenel Q, Bourlat SJ. 2017. DNA metabarcoding reveals diverse diet of the three-spined stickleback in a coastal ecosystem. *PLoS ONE* 12: e0186929.

Jarman SN, McInnes JC, Faux C, Polanowski AM, Marthick J, Deagle BE, Southwell C, Emmerson L. 2013. Adélie penguin population diet monitoring by analysis of food DNA in scats. *PLoS ONE* 8: e82227.

Jedlicka JA, Sharma AM, Almeida RPP. 2013. Molecular tools reveal diets of insectivorous birds from predator fecal matter. *Conserv Genet Resour* 5: 879–885.

Kress WJ, García-Robledo C, Uriarte M, Erickson DL. 2015. DNA barcodes for ecology, evolution, and conservation. *Trends Ecol Evol* 30: 25–35.

Lamb PD, Hunter E, Pinnegar JK, Creer S, Davies RG, Taylor MI. 2019. How quantitative is metabarcoding: a meta-analytical approach. *Mol Ecol* 28: 420–430.

Laviad-Shitrit S, Izhaki I, Lalzar M, Halpern M. 2019. Comparative analysis of intestine microbiota of four wild waterbird species. *Front Microbiol* 10: 1911.

Li C, Chen J, Liao X, Ramus AP, Angelini C, Liu L, Silliman BR, Bertness MD, He Q. 2023. Shorebirds-driven trophic cascade helps restore coastal wetland multifunctionality. *Nat Commun* 14: 8076.

Liu M, Burridge CP, Clarke LJ, Baker SC, Jordan GJ. 2023. Does phylogeny explain bias in quantitative DNA metabarcoding? *MBMG* 7: e101266.

Loch JMH, Walters LJ, Cook GS. 2020. Recovering trophic structure through habitat restoration: a review. *Food Webs* 25:e 00162.

Mathiasson S. 1973. A moulting population of non-breeding Mute Swans with special reference to flight-feather moult, feeding ecology and habitat selection. *Wildfolw* 24: 43–53.

McInnes JC, Alderman R, Deagle BE, Lea M, Raymond B, Jarman SN. 2017. Optimised scat collection protocols for dietary DNA metabarcoding in vertebrates. *Methods Ecol Evol* 8: 192–202.

Meglécz E. 2023. COInr and mkCOInr: Building and customizing a nonredundant barcoding reference database from BOLD and NCBI using a semi-automated pipeline. *Mol Ecol Resour* 23: 933–945.

Menke S, Meier M, Sommer S. 2015. Shifts in the gut microbiome observed in wildlife faecal samples exposed to natural weather conditions: lessons from time-series analyses using next-generation sequencing for application in field studies. *Methods Ecol Evol* 6: 1080–1087.

Moreno-Mateos D, Power ME, Comín FA, Yockteng R. 2012. Structural and functional loss in restored wetland ecosystems. *PLoS Biol* 10:e1001247.

Murkin HR, Batt BDJ. 1987. The interactions of vertebrates and invertebrates in peatlands and marshes. *Mem Entomol Soc Can* 119: 15–30.

Muro-Torres VM, Amezcua F, Soto-Jiménez M, Balart EF, Serviere-Zaragoza E, Green L, Rajnohova J. 2020. Primary sources and food web structure of a tropical wetland with high density of mangrove forest. *Water* 12: 3105.

Murray MH, Kidd AD, Curry SE, Hepinstall-Cymerman J, Yabsley MJ, Adams HC, Ellison T, Welch CN, Hernandez SM. 2018. From wetland specialist to hand-fed generalist: Shifts in diet and condition with provisioning for a recently urbanized wading bird. *Phil Trans R Soc B* 373: 20170100.

Nielsen JM, Clare EL, Hayden B, Brett MT, Kratina P. 2018. Diet tracing in ecology: Method comparison and selection. *Methods Ecol Evol* 9: 278–291.

Oehm J, Juen A, Nagiller K, Neuhauser S, Traugott M. 2011. Molecular scatology: how to improve prey DNA detection success in avian faeces? *Mol Ecol Resour* 11: 620–628.

Oertli B, Frossard P-A. 2013. *Mares et étangs: Ecologie, gestion, aménagement et valorisation*, EPFL Press, Lausanne, 512p.

Oertli B, Parris KM. 2019. Review: Toward management of urban ponds for freshwater biodiversity. *Ecosphere* 10: e 02810.

Ontiveros D, Pleguezuelos JM, Caro J. 2005. Prey density, prey detectability and food habits: The case of Bonelli's eagle and the conservation measures. *Biol Conserv* 123: 19–25.

Perrow MR, Schutten JH, Howes JR, Holzer T, Madgwick FJ, Jowitt AJD. 1997. Interactions between coot (*Fulica atra*) and submerged macrophytes: the role of birds in the restoration process. *Hydrobiologia* 342: 241–255.

Pompanon F, Deagle BE, Symondson WOC, Brown DS, Jarman SN, Taberlet P. 2012. Who is eating what: Diet assessment using next generation sequencing. *Mol Ecol* 21: 1931–1950.

Poulin B, Lefebvre G, Paz L. 2010. Red flag for green spray: adverse trophic effects of *Bti* on breeding birds. *J Appl Ecol* 47: 884–889.

Prewer E, Vilaça ST, Bird S, Kutz S, Leclerc L, Kyle CJ. 2023. Metabarcoding of fecal pellets in wild muskox populations reveals negative relationships between microbiome and diet alpha diversity. *Ecol Evol* 13: e10192.

R Core Team. 2022. *R: A language and environment for statistical computing. Vienna: R Foundation for Statistical Computing.* https://www. R-project.org

Reid AJ, Carlson AK, Creed IF, Eliason EJ, Gell PA, Johnson PTJ, Kidd KA, MacCormack TJ, Olden JD, Ormerod SJ, Smol JP, Taylor WW, Tockner K, Vermaire JC, Dudgeon D, Cooke SJ. 2019. Emerging threats and persistent conservation challenges for freshwater biodiversity. *Biol Rev* 94: 849–873.

Rejmankova E. 2011. The role of macrophytes in wetland ecosystems. *J Ecol Environ* 34: 333–345.

Riedl HL, Stinson L, Pejchar L, Clements WH. 2018. An introduced plant affects aquatic-derived carbon in the diets of riparian birds. *PLoS ONE* 13: e0207389.

Schmaltz L, Quaintenne G, Gaudard C, Dalloyau S. 2020. *Comptage des Oiseaux d'eau à la mi-janvier en France. Résultats 2020 du comptage Wetlands International*, LPO France, Rochefort, 26 p.

Schnell IB, Bohmann K, Gilbert MTP. 2015. Tag jumps illuminated – Reducing sequence-to-sample misidentifications in metabarcoding studies. *Mol Ecol Resour* 15: 1289–1303.

Schumm YR, Masello JF, Vreugdenhil-Rowlands J, Fischer D, Hillerich K, Quillfeldt P. 2023. Diet composition of wild columbiform birds: Next-generation sequencing of plant and metazoan DNA in faecal samples. *Sci Nat* 110: 38.

Sousa LL, Silva SM, Xavier R. 2019. DNA metabarcoding in diet studies: unveiling ecological aspects in aquatic and terrestrial ecosystems. *Environmental DNA* 1: 199–214.

Stroud DA, Davidson NC. 2021. Fifty years of criteria development for selecting wetlands of international importance. *Mar Freshw Res* 73: 1134–1148.

Svendsen A-SL, Nielsen LB, Schmidt JB, Bruhn D, Andersen LH, Pertoldi C. 2023. eDNA metabarcoding- and microscopic analysis for diet determination in waterfowl, a comparative study in Vejlerne, Denmark. *Biology* 12: 1272.

Taberlet P, Coissac E, Pompanon F, Gielly L, Miquel C, Valentini A, Vermat T, Corthier G, Brochmann C, Willerslev E. 2007. Power and limitations of the chloroplast *trn* L (UAA) intron for plant DNA barcoding. *Nucleic Acids Res* 35: e14.

Taberlet P, Bonin A, Zinger L, Coissac E. 2018. *Environmental DNA: For biodiversity research and monitoring*. Oxford, Oxford University Press, 272p.

Trevelline BK, Nuttle T, Porter BA, Brouwer NL, Hoenig BD, Steffensmeier ZD, Latta SC. 2018. Stream acidification and reduced aquatic prey availability are associated with dietary shifts in an obligate riparian Neotropical migratory songbird. *PeerJ* 6: e5141.

Ushio M, Murata K, Sado T, Nishiumi I, Takeshita M, Iwasaki W, Miya M. 2018. Demonstration of the potential of environmental DNA as a tool for the detection of avian species. *Sci Rep* 8: 4493.

Vargas-Pellicer P, Watrobska C, Knowles S, Schroeder J, Banks-Leite C. 2019. How should we store avian faecal samples for microbiota analyses? Comparing efficacy and cost-effectiveness. *J Microbiol Methods* 165: 105689.

Verkuil YI, Nicolaus M, Ubels R, Dietz MW, Samplonius JM, Galema A, Kiekebos K, de Knijff P, Both C. 2022. DNA metabarcoding quantifies the relative biomass of arthropod taxa in songbird diets: Validation with camera-recorded diets. *Ecol Evol* 12: e8881.

Villsen K, Corse E, Meglécz E, Archambaud-Suard G, Vignes H, Ereskovsky AV, Chappaz R, Dubut V. 2022a. DNA metabarcoding suggests adaptive seasonal variation of individual trophic traits in a critically endangered fish. *Mol Ecol* 31: 5889–5908.

Villsen K, Corse E, Archambaud-Suard G, Héran K, Meglécz E, Ereskovsky AV, Chappaz R, Dubut, V. 2022b. Diet metabarcoding reveals extensive dietary overlap between two benthic stream fishes (*Zingel asper* and *Cottus gobio*) and provides insights into their coexistence. *Diversity* 14: 412.

Wilfinger WW, Mackey K, Chomczynski P. 1997. Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. *BioTechniques* 22: 474–481.

WWF. 2022. Living Planet Report 2022-Building a nature-positive society. Almond, R. E. A., Grooten, M., Juffe Bignoli, D. Petersen, T. (Eds). WWF, Gland, Switzerland, 115p.

Zarzoso-Lacoste D, Corse E, Vidal E. 2013. Improving PCR detection of prey in molecular diet studies: Importance of group-specific primer set selection and extraction protocol performances. *Mol Ecol Resour* 13: 117–127.

Zarzoso-Lacoste D, Bonnaud E, Corse E, Gilles A, Meglécz E, Costedoat C, Gouni A, Vidal E. 2016. Improving morphological diet studies with molecular ecology: an application for invasive mammal predation on island birds. *Biol Conser* 193: 134–142.

Zarzoso-Lacoste D, Bonnaud E, Corse E, Dubut V, Lorvelec O, De Meringo H, Santelli C, Meunier J-Y., Ghestemme T, Gouni A, Vidal E. 2019. Stuck amongst introduced species: Trophic ecology reveals complex relationships between the critically endangered Niau kingfisher and introduced predators, competitors and prey. *NeoBiota* 53: 61–82.

Zeale MR, Butlin RK, Barker GL, Lees DC, Jones G. 2011. Taxon-specific PCR for DNA barcoding arthropod prey in bat faeces. *Mol Ecol Resour* 11: 236–244.

Zinger L, Bonin A, Alsos IG, Bálint M, Bik H, Boyer F, Chariton AA, Creer S, Coissac E, Deagle BE, De Barba M, Dickie IA, Dumbrell AJ, Ficetola GF, Fierer N, Fumagalli L, Gilbert MTP, Jarman S, Jumpponen A, Kauserud H, Orlando L, Pansu J, Pawlowski J, Tedersoo L, Thomsen PF, Willerslev E, Taberlet P. 2019. DNA metabarcoding—Need for robust experimental designs to draw sound ecological conclusions. *Mol Ecol* 28: 1857–1862.

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