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Amazonian mammal monitoring using aquatic environmental DNA

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Abstract

Environmental DNA (eDNA) metabarcoding has emerged as one of the most efficient methods to assess aquatic species presence. While the method can in theory be used to investigate nonaquatic fauna, its development for inventorying semi-aquatic and terrestrial fauna is still at an early stage. Here we investigated the potential of aquatic eDNA metabarcoding for inventorying mammals in Neotropical environments, be they aquatic, semi-aquatic or terrestrial. We collected aquatic eDNA in 96 sites distributed along three Guianese watersheds and compared our inventories to expected species distributions and field observations derived from line transects located throughout French Guiana. Species occurrences and emblematic mammalian fauna richness patterns were consistent with the expected distribution of fauna and our results revealed that aquatic eDNA metabarcoding brings additional data to line transect samples for diurnal nonaquatic (terrestrial and arboreal) species. Aquatic eDNA also provided data on species not detectable in line transect surveys such as semi-aquatic, aquatic and nocturnal terrestrial and arboreal species. Although the application of eDNA to inventory mammals still needs some developments to optimize sampling efficiency, it can now be used as a complement to traditional surveys.

KEYWORDS
Amazonian mammals, aquatic eDNA, metabarcoding, monitoring

1 | INTRODUCTION

Mammal biodiversity is currently impacted by various factors, mainly habitat modification and loss and/or illegal and unregulated hunting (Bowyer et al., 2019). Monitoring the state of biodiversity has thus become vital to assess trends and set priorities for conservation programmes (Visconti et al., 2016). Among the methods used to inventory fauna, environmental DNA (eDNA) has been recently developed and is increasingly being used (Taberlet et al., 2018). eDNA consists of collecting DNA fragments from environmental samples (e.g., soil, water, faeces or air) to detect organisms (Taberlet et al., 2018). eDNA metabarcoding surveys in aquatic environments are under active development because water acts as a collector for DNA, allowing an integrative assessment of biodiversity from a locality (Valentini et al., 2016). However, although most previous studies have focused on assessing aquatic species or communities (Bylemans et al., 2018; Cilleros et al., 2019; Civade et al., 2016; Fujii et al., 2019; Lopes et al., 2017; Tréguier et al., 2014), the approach can in theory be used to investigate nonaquatic species (Rodgers & Mock, 2015). In fact, water also receives DNA from nonaquatic organisms while they are bathing (Ushio et al., 2017), drinking (Rodgers

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& Mock, 2015) swimming or when mammals defecate in the water (Harper et al., 2019), but also potentially through soil drainage by rain.

Several studies have aimed to detect mammals or other non-aquatic vertebrates with aquatic eDNA. Early research focused on small water bodies that are expected to be intensively visited by terrestrial animals. Rodgers and Mock (2015) successfully retrieved captive coyote (*Canis latrans*) DNA in drinking water samples. Ushio et al. (2017) tested a metabarcoding approach on eDNA collected from zoo drinking water and on small natural ponds. They detected 10 out of the 13 species present in the zoo enclosure, while they retrieved 15%–89% of forest mammalian sequences in the pond samples. Similarly, Kocher et al. (2017) surveyed vertebrate species in uranium mine containment ponds and retrieved 18 terrestrial species including hard to observe taxa such as the tiger salamander (*Ambystoma tigrinum*). Egeter et al. (2018) detected four out of the 10 expected species in drinking water bodies in the Sahara desert. Waterholes left by African megafauna were also used as eDNA collectors, allowing the detection of 16 species (Seeber et al., 2019). Given the demonstrated high potential of eDNA to detect terrestrial fauna in small water bodies that are expected to be more saturated in eDNA than larger water bodies (Harper et al., 2019), studies then focused on collecting eDNA from larger water bodies. Harper et al. (2019) evaluated eDNA metabarcoding of pond samples as a tool for monitoring semi-aquatic, ground-dwelling and arboreal mammals. They led a comparative study on how animal behaviour affects the release of eDNA in artificial vs. natural environments. While mammal life habits and behaviour did not influence eDNA detection in artificial ponds, it played a major role in natural systems. Attempts to detect mammals in natural aquatic systems remain scarce and,to date, only a few studies have explored the reliability of eDNA metabarcoding to detect nonaquatic species in rivers and streams. Among those studies, Sales, Kaizer, et al. (2020) retrieved 14 mammal families in the Amazon river and nine mammal families in the Brazilian Atlantic forest from aquatic eDNA. While their study highlights the potential of aquatic eDNA metabarcoding to detect nonaquatic species, the reliability of the method remained to be tested by investigating the spatial concordance between the species occurrences and their expected distribution. Indeed, one of the greatest challenges is that nonaquatic species are not in permanent contact with the water, potentially resulting in smaller amounts of DNA released in the water (Harper et al., 2019; Sales, McKenzie, et al., 2020). Consequently, false negatives (i.e., missing detections when species are present) may be more frequent than for aquatic fauna, particularly in large water bodies (Harper et al., 2019; Seeber et al., 2019).

We here investigate the potential of aquatic eDNA metabarcoding to detect aquatic, semi-aquatic, terrestrial and arboreal mammal fauna along three rivers of the Amazonian biome. For this, we used a comparative analysis between aquatic eDNA metabarcoding and standardized visual faunistic inventories classically used for mammal monitoring. We then discuss the spatial concordance between the observations of several emblematic Amazonian mammals retrieved with aquatic eDNA metabarcoding and their expected distribution.

2 | MATERIALS AND METHODS

2.1 | Study rivers

We collected aquatic eDNA in three large French Guianese rivers (Figure 1): the Maroni river (612 km in length), the watershed of which extends over Suriname and French Guiana; the Oyapock river (404 km in length), the watershed of which extends over Brazil and French Guiana; and the Sinnamary river (262 km in length) situated within the territory of French Guiana. The three river basins are characterized by an equatorial climate with annual rainfall ranging from 3,600 mm (northeast) to 2,000 mm (south and west). These three rivers face different levels of anthropogenic pressures unevenly distributed along the watercourses as most people are concentrated in the coastal area. The Maroni river is the most inhabited with ~83,000 habitants (INSEE, 2020) unevenly distributed from Saint-Laurent-du-Maroni to Pidima village, which constitutes the most upstream human settlement on the Maroni river (Figure 1). The Maroni river is the most affected by human activities, mainly legal and illegal gold mining, which represented 8,058 ha of deforestation (0.37% of the catchment area in 2014) spanning from Saint-Laurent-du-Maroni to upstream of Maripasoula (Gallay et al., 2018). Only the most upstream part of the Maroni river (upstream from Pidima, Figure 1) has not been impacted by human activities. The Oyapock river is more preserved with only three villages and ~6,000 habitants (INSEE, 2020). Gold mining is much less developed than on the Maroni drainage, and represented 1,547 ha of deforestation in 2014 (0.06% of the catchment area), mainly concentrated near the village of Camopi (Gallay et al., 2018). The Sinnamary river is not exploited for gold but the building of a large hydroelectric dam (Petit saut dam) in 1994–1995 has severely modified the landscape: 365 km^2 of primary rain forest were flooded, leaving hundreds of islands of various sizes covering a total area of 105 km^2 (Vié, 1999). Several human settlements are located downstream from the dam while the upstream part of the river remains free from human settlements, with only occasional recreational fishing. Hunting activities also occur along the watercourses, subsistence hunters being frequent in remote isolated areas. In small rural villages or gold mining camps, hunting for meat represents a non-negligible disturbance to large vertebrate fauna (Richard-Hansen & Hansen, 2004). The impact of hunting on wildlife populations is nevertheless concentrated in small areas around human settlements and access paths (Richard-Hansen et al., 2019). The Sinnamary river being the least populated, it shows the weakest hunting pressure. Moreover, its upstream course lies within the core area of the Guianese National Park (Parc Amazonien de Guyane) where access is restricted and hunting is prohibited. Hunting is also prohibited in the Petit-Saut dam area. In contrast, hunting pressure is important along the course of the Maroni river, and only the upstream areas remain little influenced by hunting because of their distance to human settlements. Along the Oyapock river, hunting pressure is lower than

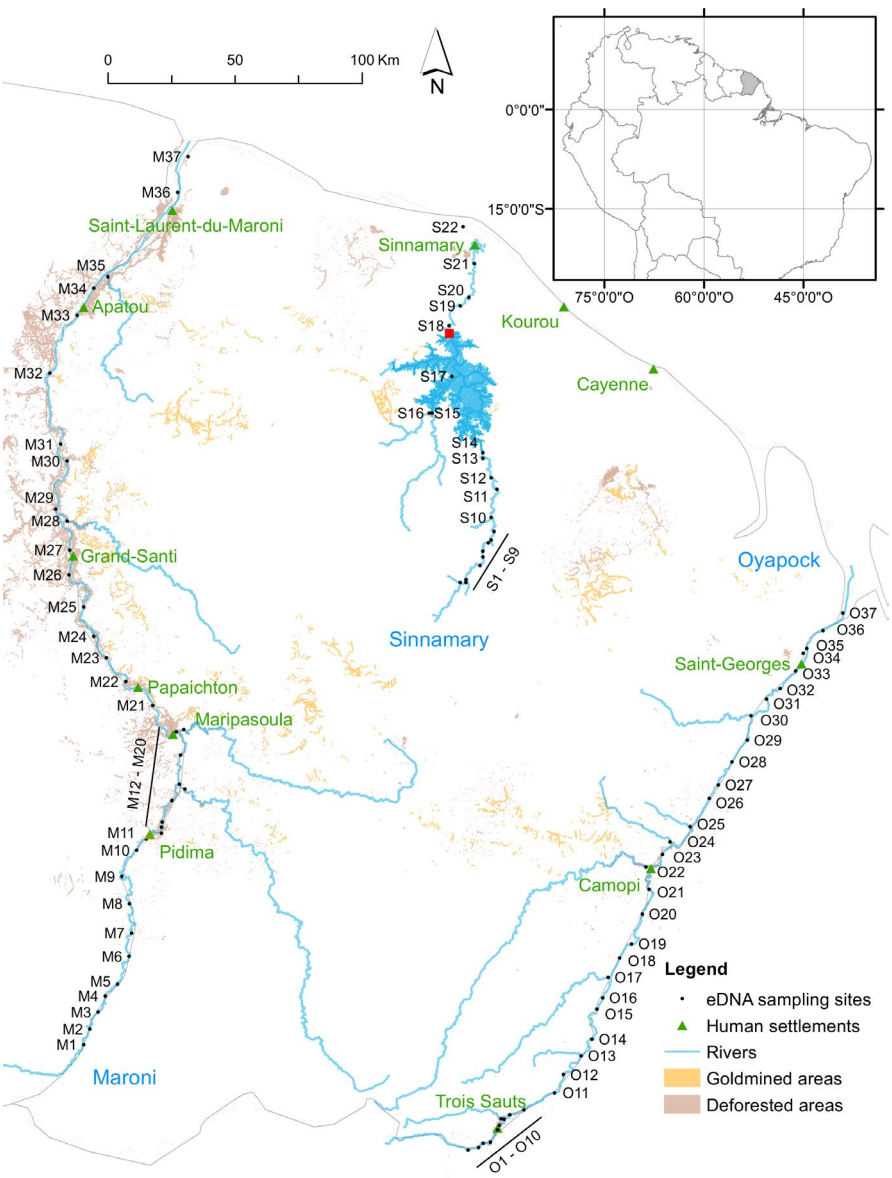

FIGURE 1 eDNA sampling sites. M1 to M37 indicate the sites sampled on the Maroni river, S1 to S22 the sites sampled on the Sinnamary river and O1 to O37 those sampled on the Oyapock river. Information about gold-mined surfaces was compiled by the WWF using Landsat satellite images of deforestation due to gold-mining in 2015 (WWF, 2016). This data set represents the most recent information available on gold-mining over the Guianese territory. Forest loss surfaces were extracted using the Global Forest Change data set (Hansen et al., 2013). This data set identifies the areas deforested from 2001 to 2017 using global Landsat satellite image at 30 m spatial scale. The red rectangle on the Sinnamary river represents the dam location. Inset map indicates the location of French Guiana in South America

on the Maroni river due to a lower human population density, but is expended all along the watercourse, because human settlements, although concentrated in three main villages, are dispersed all along the watercourse, including the most upstream areas (Figure 1).

2.2 | Water collection and sampling

The eDNA sampling was conducted in November (dry season) 2017 for the Maroni river, November 2018 for the Oyapock river and November 2019 for the Sinnamary river. In total, 96 sites were sampled using VigiDNA 0.45-µm filters (SPYGEN). These encapsulated filters possess a 500-cm^2 membrane surface made of polyethersulfone and can process up to 50 L of water (Coutant et al., 2020). Following Cantera et al. (2019), two samples were taken per site, with 34 L of water filtered per sample in 30 min. A peristaltic pump (Vampire sampler, Burlke) and a single-use tube were used to pump the water through the encapsulated filtering cartridges. The input part of the tube was held a few centimetres below the water surface and sampling was achieved in rapids where eDNA is continuously homogenized in the water column. To avoid DNA contamination, the operators remained downstream from the filtration either on the boat or on emerging rocks. After the filtration, the capsules were emptied and filled with 80 ml of CL1 conservation buffer (SPYGEN) and stored in sterile individual plastic bags in the dark. The samples were kept at room temperature until DNA extraction, performed within 1 month.

2.3 | eDNA laboratory and bioinformatics

Each filtration cartridge was agitated for 15 min on an S50 shaker (cat Ingenieurbüro) at 800 rpm, emptied into a 50-ml tube and then centrifuged for 15 min at 15,000 *g*. The supernatant was then

discarded with a sterile pipette leaving 15 ml of liquid at the bottom of the tubes. After the addition of 33 ml of ethanol and 1.5 ml of 3 M sodium acetate, the 50-ml tubes were stored at 20°C over at least one night. The tubes were subsequently centrifuged at 15,000 *g* for 15 min at 6°C and the supernatants were removed. Then, 720 µl of ATL buffer from the DNeasy blood & Tissue Extraction Kit (Qiagen) was added to the tubes. The tubes were vortexed and the supernatants were transferred to 2-ml tubes with 20 µl of Proteinase K. The tubes were incubated at 56°C for 2 hr. After this step, DNA extraction was via a NucleoSpin Soil (Macherey-Nagel) beginning at step 6 and following the manufacturer's instructions.

After the extraction step, the samples were tested for inhibition following the protocol of Biggs et al. (2015). Briefly, qPCR was performed in duplicate for each sample. If at least one of the replicates showed a different Ct (cycle threshold) than expected (at least 2 Cts), the sample was considered inhibited and diluted five-fold before the amplification. DNA amplification was performed in a final volume of 25 µl including 1 *U* of AmpliTaq Gold DNA Polymerase (Applied Biosystems), 10 mM Tris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of 12S-V5 vertebrate marker (12S-V5 R 5′-TTAGATACCCCACTATGC-3′ and 12S-V5 F 5′-TAGAACAGGCTCCTCTAG −3′, Riaz et al., 2011) and 3 µl of DNA template. Then, 4 mM of human blocking primer for 12S-V5 (5′-CTA TGCTTAGCCCTAAACCTCAACAGTTAAATCAACAAAACTGCT -C3 −3′ (De Barba et al., 2014) and 0.2 mg/ml of bovine serum albumin (BSA; Roche Diagnostic) were also added to the mixture.

The choice of the primer set used to identify the targeted fauna is critical as it may impact the composition of the inventories if eDNA markers do not have the same taxonomic resolution across clades (Zinger et al., 2020). We here used the "12S-V5" marker (Riaz et al., 2011) as it presents interesting features to identify the fauna considered in this study. Even though it was originally designed as a generic vertebrate marker, we have previously shown that it is very well suited to study the local mammal fauna (Kocher, de Thoisy, Catzeflis, Huguin, et al., 2017; Kocher, de Thoisy, Catzeflis, Valière, et al., 2017). The local reference database is nearly exhaustive and the marker provides very good accuracy (99.6% of the assignations are correct) and very high specificity (90% of the assignation are done at the species level). The 12S-V5 binding sites are extremely conserved for Amazonian mammals, thus minimizing mismatches that could lead to a lack of amplification of rare species. Finally, the marker is short (96–103 bp) and well suited for the amplification of degraded DNA (Kocher, de Thoisy, Catzeflis, Huguin, et al., 2017).

We performed 12 PCR replicates per field sample. In order to assign the sequences to the appropriate sample, the forward and reverse primers were 5′-labelled with a unique eight-nucleotide tag for each PCR replicate. Both forward and reverse primers used an identical tag in order to minimize tag-switching issues (Sales et al., 2020). The PCR mixture was denatured at 95°C for 10 min, followed by 50 cycles of 30 s at 95°C, 30 s at 55°C and 1 min at 72°C, and a final elongation step at 72°C for 7 min. Running 50 cycles as in Valentini et al. (2016) and Cilleros et al. (2019) represents a compromise between the detection power of rare species and the risk of generating artefacts. The amplification step was performed in a dedicated room with negative air pressure and physical separation from the DNA extraction rooms (with positive air pressure). The purified PCR products were then pooled in equal volumes to reach a sequencing depth of 500,000 reads per sample before library preparation. Library preparation was performed at Fasteris facilities (Geneva, Switzerland) using a Metafast protocol (www.fasteris.com/metafast). Two libraries were sequenced using an Illumina HiSeq 2500 (2 × 125 bp) (Illumina) on a HiSeq Rapid Flow Cell version 2 using the HiSeq Rapid SBS Kit version 2 (Illumina), three using a MiSeq (2 × 125 bp) (Illumina) and the MiSeq Flow Cell Kit version 3 (Illumina) and three using a NextSeq (2 × 150 bp+8) (Illumina) and the NextSeq Mid kit (Illumina). The libraries ran on the NextSeq were equally distributed in four lanes. Sequencing was performed at Fasteris. Fourteen negative extraction controls and four negative PCR controls (ultrapure water, 12 replicates) were amplified per primer pair and sequenced in parallel to the samples to monitor possible contaminants.

The EMBL-EBI vertebrate database was downloaded from the European Nucleotide Archive (ENA) (http://ftp.ebi.ac.uk/pub/databases/embl/release/std/, release 134 for Maroni river sample, 138 for Oyapock and 140 for Sinnamary samples). The three releases were compared and the new mammalian species incremented in each new version did not belong to French Guiana. Our results were therefore uninfluenced by EMBL release number. We extracted from this database the relevant metabarcoding fragment using ECOPCR (Ficetola et al., 2010) and OBITOOLS (Boyer et al., 2016). Our reference database thus includes the local database of French Guianese mammals (Kocher, de Thoisy, Catzeflis, Huguin, et al., 2017), which references 576 specimens of 164 species as well as all the vertebrate species available in EMBL.

The analysis of marker-gene data has long resorted to the construction of operational taxonomic units (OTUs): clustering of reads sufficiently similar to a sequence in a reference database (i.e., closed-reference methods) or as a function of the read pairwise sequence similarities (i.e., *de novo* methods) (Callahan et al., 2017). Recently, amplicon sequence variant (ASV) methods, which discriminate sequencing errors from biological sequences without relying on the dissimilarity threshold defining the OTUs, has gained considerable attention (Callahan et al., 2017). In our case, we used a nearly exhaustive local reference database lacking only extremely rare species not found in specimen collections (Kocher, de Thoisy, Catzeflis, Huguin, et al., 2017). In addition, the marker used provides very good accuracy and specificity. Both OTUs and ASV methods should provide similar results in this situation.

The sequence reads were analysed using the functions of the OBITOOLS package following the protocol described in Valentini et al. (2016). Briefly, forward and reverse reads were assembled using ILLUMINAPAIREDEND. Subsequently, the NGSFILTER program was used to assign the sequences to each sample. A separate data set was created for each sample by splitting the original data set into several files using OBISPLIT. Sequences shorter than 20 bp, or occurring fewer than 10 times per sample or labelled "internal" by the OBICLEAN program,

probably corresponding to PCR errors, were discarded. The function *ecotag* was used for the taxonomic assignment of molecular operational taxonomic units (MOTUs). Taxonomic assignments from *ecotag* were also corrected to avoid over-confidence in assignments: species-level assignments were validated only for sequence identity with the reference database ≥98%. MOTUs occurring with a frequency below 0.001 per library sample were considered as tag-jumps and discarded (Sales et al., 2020). These thresholds were empirically determined to clear all reads from the extraction and PCR-negative controls included in our global data production procedure as suggested in De Barba et al. (2014). For the samples sequenced with the NextSeq, only species present in at least two lanes were retrieved.

2.4 | Line transects data

Eighty-four line transects were realized between 1998 and 2018 (Figure S1). The line transect surveys were conducted as explained in de Thoisy et al. (2008) and Richard-Hansen et al. (2015). Briefly, the line transect sampling consisted in visually recording fauna by walking slowly (0.8–1.3 km/hr) on linear forest tracks measuring 3–5 km, presenting the same forest structure (Guitet et al., 2015), but including various local habitats (i.e., hill, stream). Depending on the study, there was a single forest track (de Thoisy et al., 2008) or four tracks per site (Richard-Hansen et al., 2015). The surveys were repeated daily until a cumulated sampling distance of more than 100 km was reached in each site. Those inventories were conducted during the day (from 7 a.m. to 6 p.m.), and hence, strictly nocturnal species were not observed.

2.5 | Data analysis

Because we use the "12S-V5" vertebrate marker (Riaz et al., 2011) for amplification, we obtained broad observations for various vertebrate taxonomic groups. Data were thus first sorted to only keep mammal taxa and MOTUs assigned to the species level, thus retrieving 78 mammal species (see Tables S1 and S2). Nonmammal species (amphibians, birds, reptiles) were discarded from this study because reference databases are still largely incomplete for these taxa.

To compare eDNA results with known spatial distributions of species, we used the Faune-Guyane database (Faune-Guyane, 2020). This gathers citizen science data and observation data from scientific monitoring and represents the most detailed information on vertebrate distribution (excluding fishes) in French Guiana. We used the Faune-Guyane database to identify "emblematic mammalian fauna" used to conduct the comparative analysis with line transects and to discuss the consistency of the observations with the expected species distributions. "Emblematic mammalian fauna" included large (adult body mass >1 kg) mammals, but excluded the rarest species, those occurrences in French Guiana not being sufficient to draw a relevant distribution area. "Emblematic mammalian fauna" therefore excluded small mammals (adult body mass <1 kg) such as Chiroptera, Rodentia and Didelphimorphia which are not easily identifiable without animal capture as well as medium and large mammals (adult body mass >1 kg) considered as very rare or rare (see Table S2 for species list). This index of rarity was based on the ratio between species observation number and total number of observations, and was adjusted by experts to consider species that are difficult to observe but not necessarily rare. After the exclusion of the less documented species (47 species excluded), we focused on 31 fairly well-studied species, hereafter referred to as "emblematic mammalian fauna" to conduct the comparative analysis with line transects and to discuss the consistency of the observations with the expected distributions.

Line transects and aquatic eDNA metabarcoding survey methods are not directly comparable because they focus on different habitats/microhabitats, making site by site comparisons unrealistic. To investigate the relationship between these two methods, we used what we hereafter refer to as the *observation frequency*. Observation frequency represents the total number of sites where a species has been observed by a sampling method (line transects or aquatic eDNA metabarcoding) divided by the total number of sites. This metric differs from the species detection probability as it does not intend to define the probability of encountering at least one individual of a species present on a surveyed area (Boulinier et al., 1998), but it highlights the proportion of sites where a given species has been observed by aquatic eDNA metabarcoding or line transects across the considered region (i.e., at the scale of French Guiana). Comparison of the observation frequency obtained with both methods allows us to investigate to what extent they provide similar inventories but also indicates if the observation frequency ranking is conserved between both methods. Although eDNA metabarcoding and line transect samplings were not conducted at the same sites and at the same time, both samplings cover a substantial part of the Guianese territory and include most habitat types, levels of threats and anthropization, making relevant the broad comparison between eDNA metabarcoding and line transect sampling (see Figure 1 and Figure S1). This comparison between eDNA and line transect observation frequencies should nevertheless be considered with caution as it is only meaningful over the entire studied region (French Guiana) and cannot be used to compare local areas within this region.

The observation frequency metric was computed using species by site matrices from eDNA metabarcoding and line transect data (Tables S3 and S4). The species observation frequencies were calculated for both survey methods and compared with Mann–Whitney *U* tests after species were classified as aquatic, terrestrial, arboreal, nocturnal and/or diurnal (Emmons & Feer, 1997; Hansen et al., 2000) to determine the effect of mammal habitat and ecology on observation frequency. Simple linear regressions were then performed to test for a linear relationship between the species observation frequencies calculated for both survey methods. To estimate the spatial consistency of the species observations with their expected distributions in French Guiana, we displayed the species occurrence patterns of several emblematic mammals with fairly well-known

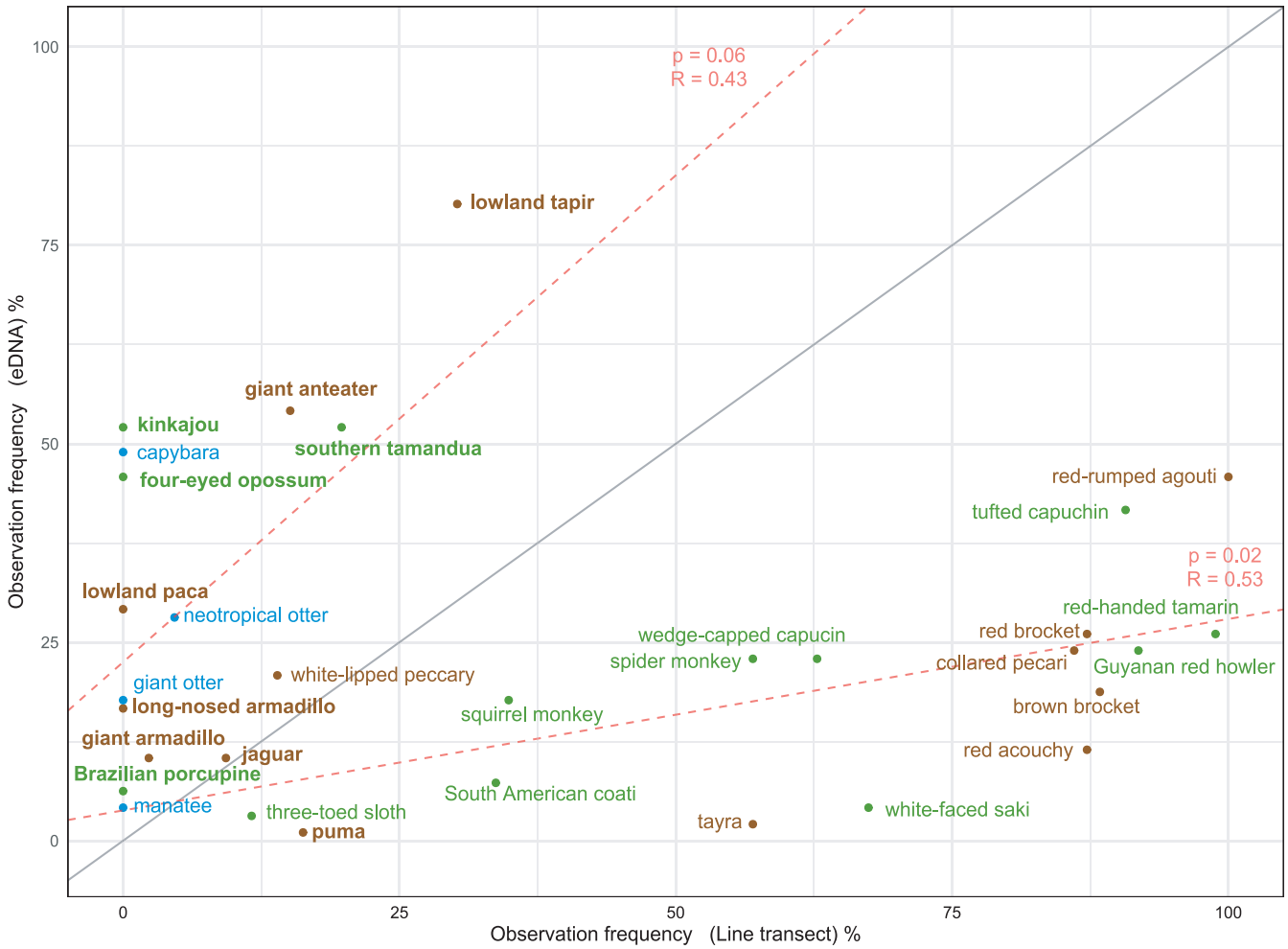

FIGURE 2 Observation frequency (%) of emblematic mammalian fauna obtained with aquatic eDNA metabarcoding and with line transects. Species in blue are aquatic or semi-aquatic, species in green are arboreal and species in brown are terrestrial. Bold species names refer to nocturnal species while regular font corresponds to diurnal species. The black solid line represents the 1:1 line. The red dotted lines above and beneath the 1:1 line refer to the linear regressions between the observation frequency obtained with eDNA and line transects for nocturnal or aquatic/semi-aquatic species and for diurnal or nonaquatic species, respectively. The *p*-value (*p*) and correlation coefficient (*R*) are indicated in red

ecologies, as well as the species richness pattern of the 31 emblematic mammal species considered in the study. All the analyses were computed in R software version 3.6.1 (May 7, 2019) (R Core Team, 2019) and the maps were edited with ArcGis software.

3 | RESULTS

A total of 152,546,060 sequences were obtained from the eDNA samples and 99,492,637 reads were kept after bioinformatics processing. Overall, we obtained 4,524,515 reads after the removal of nonmammal species, corresponding to 78 mammal species across the 96 river sites. The mammal species retrieved belonged to 72 genera, 33 families and 11 orders (Table S1).

Among the 78 species, five classified as very rare were detected using eDNA: Emilia's gracile opossum (*Gracilinanus emiliae*), Guianan white-eared opossum (*Didelphis imperfecta*), rufous mouse opossum (*Marmosa lepida*), white-faced spiny tree rat (*Echimys chrysurus*) and bush dog (*Speothos venaticus*). Moreover, among the 31 species detected and referred to as emblematic mammalian fauna, six are listed in the French-Guianese IUCN red list (UICN France et al., 2017): the giant otter (*Pteronura brasiliensis*, endangered), West Indian manatee (*Trichechus manatus*, endangered), lowland tapir (*Tapirus terrestris*, vulnerable), jaguar (*Panthera onca*, nearly threatened), puma (*Puma concolor*, nearly threatened) and white-lipped peccary (*Tayassu pecari*, nearly threatened).

3.1 | Patterns of emblematic mammalian fauna observation frequency

Eight species were only observed with aquatic eDNA metabarcoding (observation frequency in parentheses): the nocturnal kinkajou (*Potos flavus*, 52.08%), four-eyed opossum (*Philander opossum*, 45.83%), lowland paca (*Cuniculus paca*, 29.17%), long-nosed armadillo (*Dasypus kappleri*, 16.67%), Brazilian porcupine (*Coendou*

prehensilis, 6.25%), the semi-aquatic capybara (*Hydrochoerus hydrochaeris*, 48.96%), giant otter (17.71%) and the aquatic West Indian manatee (4.17%) (Figure 2).

The lowland tapir presented the highest observation frequency with aquatic eDNA metabarcoding and was observed in 80.21% of the sites, while it was observed in only 30.23% of the line transect sites. Similarly, the southern tamandua (*Tamandua tetradactyla*) and giant anteater (*Myrmecophaga tridactyla*) as well as the neotropical otter (*Lontra laugicaudis*) were observed in 52.08%, 54.17% and 28.13% of the sites with aquatic eDNA metabarcoding while they were observed in 19.77%, 15.12% and 4.65% of the sites with line transect surveys, respectively. Finally, the jaguar was observed only slightly more with eDNA metabarcoding (10.42%) than with line transects (9.30%) (Figure 2).

In contrast, the observation frequency of primates including the spider monkey (*Ateles paniscus*), wedge-capped capucin (*Cebus olivaceus*), tufted capuchin (*Sapajus apella*), red-handed tamarin (*Saguinus midas*), Guianan red howler (*Alouatta macconnelli*), white-faced saki (*Pithecia pithecia*) and squirrel monkey (*Saimiri sciureus*) did not exceed 41.67% with aquatic eDNA metabarcoding while it ranged from 34.88% to 98.84% with line transect surveys. Similarly, the observation frequency of common diurnal rodents including the red-rumped agouti (*Dasyprocta leporina*) and red acouchy (*Myoprocta acouchy*) as well as the ungulates collared pecari (*Pecari tajacu*), red brocket (*Mazama americana*), grey brocket (*Mazama nemorivaga*) and the tayra (*Eira barbara*) ranged from 56.98% to 100% with line transects while it ranged from 2.08% to 45.83% with aquatic eDNA metabarcoding (Figure 2).

Overall, aquatic/semi-aquatic and nocturnal fauna were significantly more often observed with eDNA metabarcoding than with line transects (Mann–Whitney *U* test, $U = 193$, $p < .001$, $n = 15$; Figures 2 and 3). The observation frequency of nocturnal and aquatic/semi-aquatic species ranged from 1.04% to 80.21% (median =28.13) with aquatic eDNA metabarcoding while it ranged from 0% to 30.23% (median =0) with line transects (Figure 3). Contrastingly, diurnal nonaquatic fauna was better observed with line transects than with aquatic eDNA metabarcoding (Mann–Whitney *U* test, $U = 26$, $p < .001$, $n = 16$). The observation frequency of diurnal nonaquatic species ranged from 2.08% to 45.83% (median =.87) with aquatic eDNA metabarcoding while it ranged from 11.63% to 100% (median =76.74) with line transect (Figure 3). The linear regression revealed a marginally significant linear relationship between the observation frequency of the two survey methods for aquatic/semi-aquatic and nocturnal species ($F_{(1,13)\ =4}$.20, $p = .06$, $R = .43$, slope =1.23). The observation frequency of the aquatic/semi-aquatic and nocturnal species was on average 4.7 times higher with aquatic eDNA metabarcoding than with line transects (Figures 2 and 3). By contrast, there was a significant linear relationship between the observation frequency of the diurnal nonaquatic species obtained with the two survey methods ($F_{(1,14)\ =}$ 6.73, $p = .02$, $R = .53$, slope = 0.24). The observation frequency of diurnal terrestrial and arboreal fauna was on average 3.4 times lower with aquatic eDNA metabarcoding than with line transects (Figures 2 and 3).

3.2 | Species occurrence patterns of emblematic mammalian fauna

Mammal species with a restricted distribution area were retrieved in their known habitat. The West Indian manatee was indeed observed in all three estuaries (sites M36, M37, S22 and O37). Similarly, Cetacea, although not identified to the species or genus level, were observed in estuaries using eDNA metabarcoding (sites M36, M37 and O37; Figure 4), which is consistent with their known distribution (contrary to the nearby Amazon drainage, freshwater dolphins do not occur in French Guiana).

More widespread species that inhabit the entire Guianese territory were also retrieved in a large number of the eDNA sites, or are clustered in the least anthropized areas for the species known to be sensitive to human disturbances. eDNA metabarcoding observations of the capybara, giant anteater, kinkajou and lowland tapir extended from the upstream to the downstream parts of the three rivers (excepted for the sites located at the estuaries) (Figures 4 and 5). In contrast, the giant otter, neotropical otter and spider monkey

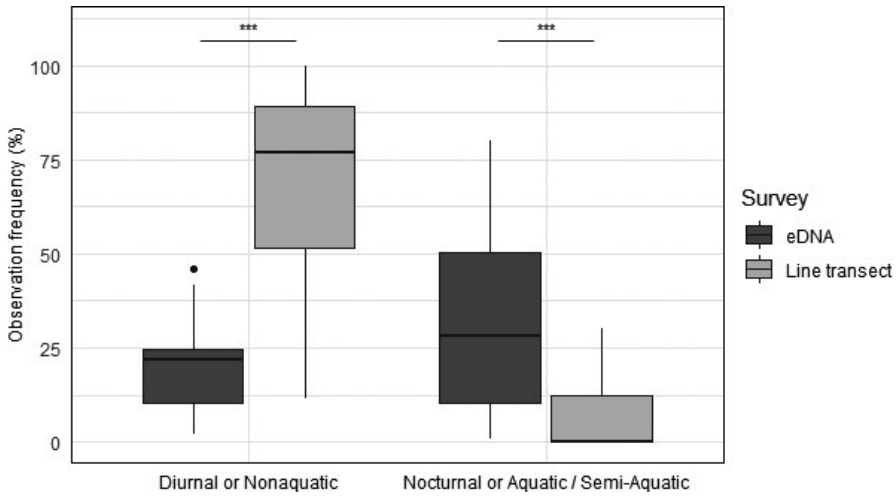

FIGURE 3 Observation frequency of emblematic mammalian fauna categorized into diurnal or nonaquatic and nocturnal or aquatic/semi-aquatic. Observation frequencies obtained with line transects and aquatic eDNA metabarcoding were compared using a Mann–Whitney *U*-test: ***$p < .001$

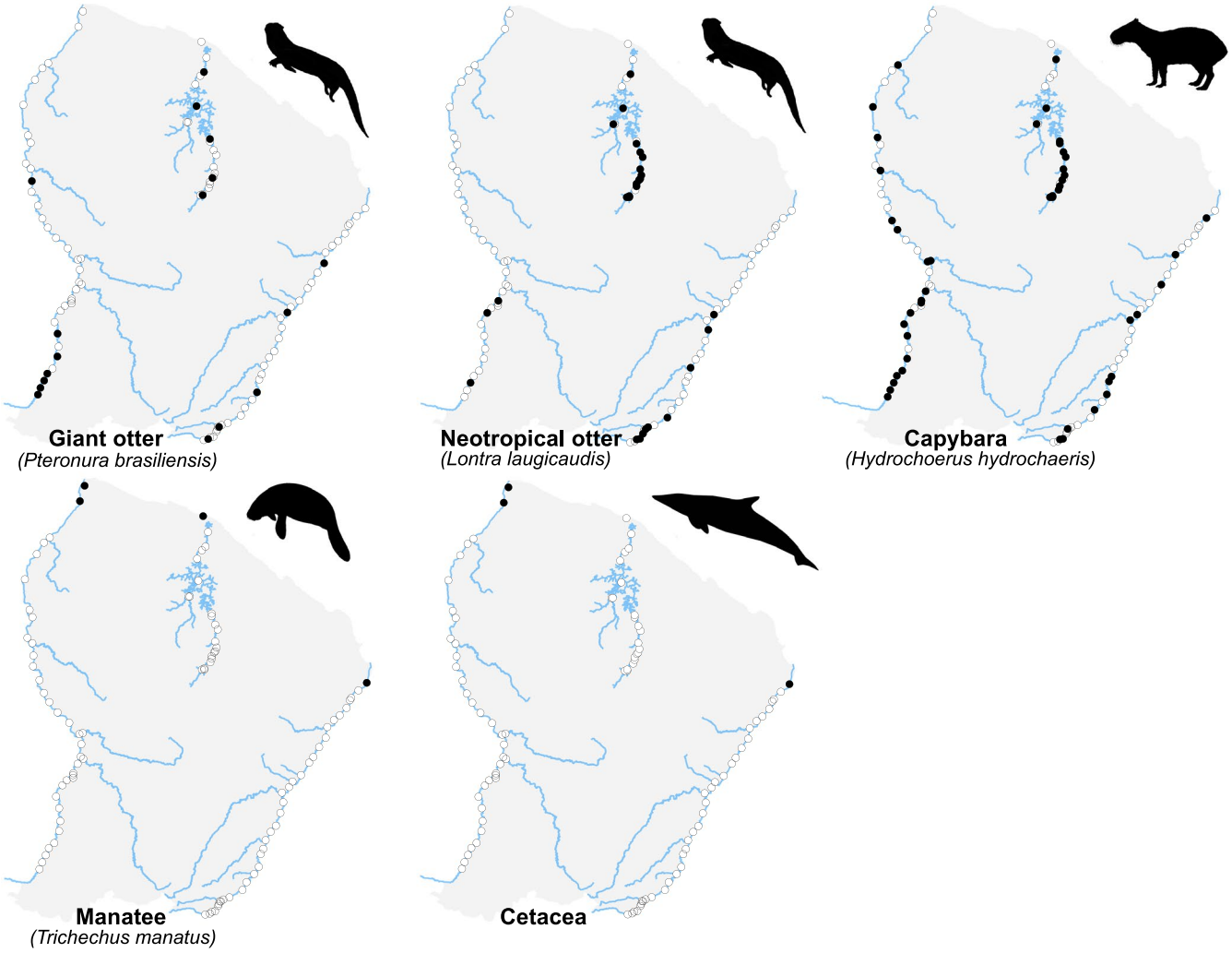

FIGURE 4 Species occurrences of several aquatic and semi-aquatic species. Presence (black dots) or absence of observations (white dots) in aquatic eDNA sampling sites are indicated on the maps

presented similar spatial patterns of distribution and were mostly observed at the upstream part of the rivers (Figures 4 and 5). Except for sites M5 and M7, the giant otter was observed in the six most upstream sites of the Maroni river. On the Oyapock river, this species was observed in five sites distributed all along the watercourse while it was retrieved in four sites located upstream of the dam, and in one site located downstream of the dam of the Sinnamary river. The neotropical otter was observed in two sites in the upstream part and in one site located near the upstream human settlements on the Maroni river. On the Oyapock river, the species was also observed in nine sites located all along the watercourse while the species was observed in 14 sites located in the upstream part of the Sinnamary river and in a single site located downstream of the 'Petit Saut' dam (Figure 4). Similarly, the spider monkey observations occurred in four sites in the upstream part of the Maroni river, in six sites distributed along the Oyapock river and in nine sites located upstream of Sinnamary dam (Figure 5). The jaguar observations were scarce, notably on the Maroni river with only one observation at the upstream part and two observations at the upstream and the downstream parts of the Oyapock river. On the Sinnamary, the species was observed in six upstream sites and in one site downstream of the dam (Figure 5).

3.3 | Richness pattern of emblematic mammalian fauna

Of the 31 emblematic mammals considered, 27, 28 and 31 mammal species were observed via eDNA metabarcoding on the Maroni, Oyapock and Sinnamary rivers, respectively. On the Maroni river, the site species richness ranged from 0 to 14 (median =5) while it ranged from 1 to 17 (median =8) and from 2 to 20 (median =14) for the Oyapock and Sinnamary river, respectively. The site species richness along the Maroni river was heterogeneous with the most upstream sites being richer than the sites located downstream of Maripasoula village, with the exception of site M35. One site located at the downstream part of the Maroni river (M33) did not provide any emblematic mammal species observations (Figure 6). Along the Oyapock river, the site species richness was distributed more homogeneously along the watercourse, with sites O9, O10, O11 and

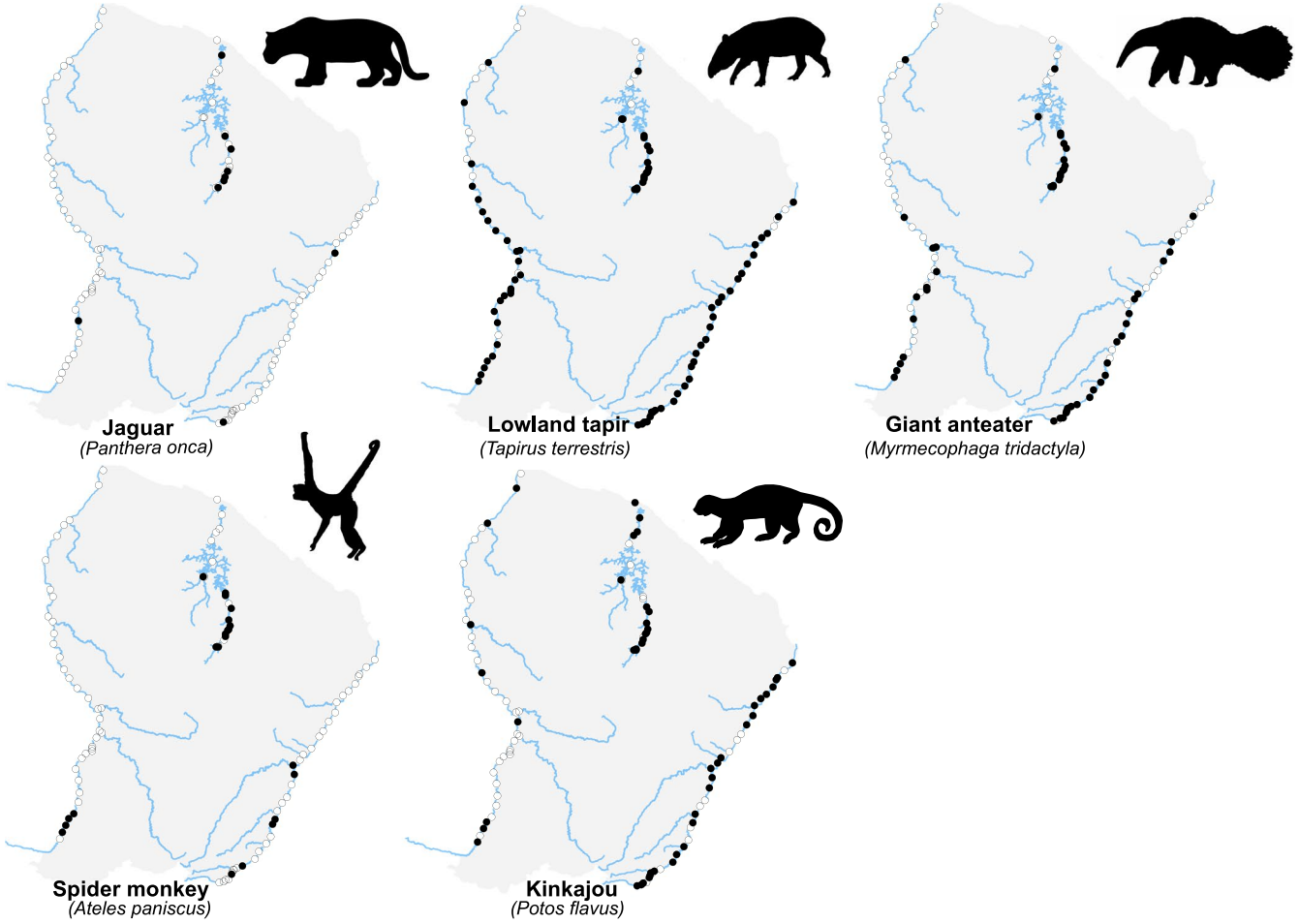

FIGURE 5 Species occurrences of several terrestrial or arboreal species. Presence (black dots) or absence of observations (white dots) in aquatic eDNA sampling sites are indicated on the maps

O30 being the richest (from 14 to 17). The upstream part of the Sinnamary river (S1–S11, S15) presented the sites with the highest species richness (14–20), which were concentrated upstream of the dam (Figure 6).

4 | DISCUSSION AND CONCLUSION

Although aquatic eDNA metabarcoding has been used widely to inventory aquatic fauna, the method is raising new interest to inventory nonaquatic species. To date, the method remains exploratory as several challenges still need to be addressed. The reliability of this survey method has already been investigated by comparing the inventoried fauna to that obtained with other methods such as camera trapping (Sales, Kaizer, et al., 2020; Sales, McKenzie, et al., 2020). Here, comparing aquatic eDNA metabarcoding inventories to line transect observations over the Guianese territory revealed consistent patterns between the expected species distributions and eDNA detections, making eDNA a promising tool to inventory both aquatic and terrestrial fauna.

4.1 | Observation frequencies between aquatic eDNA metabarcoding and line transects

Comparing eDNA metabarcoding observations to those of traditional line transects revealed that nocturnal and aquatic species were more often observed in eDNA samples than in line transects, whereas diurnal terrestrial and arboreal species were more often observed using line transects. We nevertheless detected a marginally significant relationship between the observation frequency of nocturnal and semi-aquatic species and a significant relationship between the diurnal terrestrial and arboreal species from both methods, indicating that eDNA metabarcoding retrieved a similar pattern of observation ranking compared to line transects. Although the observation frequency with aquatic eDNA metabarcoding is on average 3.4 times lower than that of line transects for diurnal terrestrial and arboreal mammals, sampling eDNA in a site is achieved in less than 1 hr. We therefore believe that aquatic eDNA metabarcoding can constitute a useful complement to line transect samples (or other sampling methods) for terrestrial and diurnal mammals given that eDNA collection by water filtration can be rapidly achieved during survey campaigns.

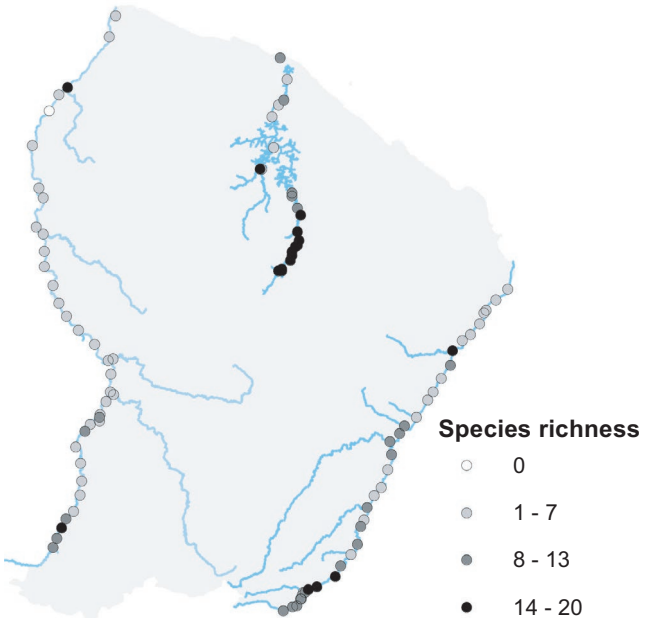

FIGURE 6 Emblematic mammalian fauna richness observed at each aquatic eDNA sampling site

A less stringent pattern was found for nocturnal and aquatic species, with giant anteater and tamandua being more frequently observed by both methods than the rare giant armadillo (Carter et al., 2016; Catzeflis & Thoisy, 2012) or the elusive jaguar which has a large individual home-range and low population densities (Petit et al., 2018). For those species, observation frequency was four-fold higher using aquatic eDNA metabarcoding than line transects. Together with the eight species observed only with eDNA metabarcoding, this testifies to the capacity of this method to detect nocturnal and aquatic species rarely or not observed in line transect inventories.

However, these relationships remain dependent on the species considered as the observation frequency of some species can be biased by different parameters. Indeed, aquatic eDNA metabarcoding may be sensitive to a particular mammal's behaviour (Harper et al., 2019). For instance, the tapir was observed in 80% of the eDNA sites regardless of the proximity to villages or land use. Yet, the tapir is a game species due to its size and the quality of its meat and is therefore under pressure in areas accessible to hunters (Richard-Hansen et al., 2019; Tobler et al., 2014). This high observation frequency was already also noted by Sales, McKenzie, et al. (2020) and may be explained by the high affinity of tapir for water, combined with its habit of defecating in water (Tobler et al., 2010). Despite such species presenting particularities hindering fine-scale observations, aquatic eDNA metabarcoding could constitute a valuable complement to traditional samples, as it allows extending the range of species and habitats to be inventoried, while saving time for biodiversity inventories.

4.2 | Species occurrence patterns

Detailing the occurrences of the West Indian manatee illustrated the capacity of aquatic eDNA metabarcoding to detect species only in their area of distribution. The West Indian manatee was observed in all the three estuaries sampled and in no other site, estuaries being the typical habitat of this species (de Thoisy et al., 2016). The Cetacea observations were also exclusively retrieved at the estuarine sites. Those observations consistent with the distribution area of the species thus constitute a proof of absence of false positives (observation of the species outside their distribution area) for those species.

The occurrences of mammals inhabiting all the territory showed that some species were observed regardless of human proximity. Among them, the capybara and the kinkajou were observed in half of the sites (50.52%). They are known as tolerant to human presence, kinkajou being a discrete nocturnal and arboreal species disregarded by hunters; and the capybara being a generalist species not appreciated by hunters because of the strong taste of its meat (hunting surveys show that they represent only 0.5% and 1.5% respectively out of 14,570 mammals hunted, Richard-Hansen et al., 2019). Other species recognized to be negatively impacted by anthropogenic activities such as the spider monkey or the neotropical and giant otter (Rheingantz et al., 2014; Richard-Hansen et al., 2019; de Thoisy et al., 2005) were preferentially observed in the upstream part of the rivers, which is free from dense human settlements or activities. For some observations, we cannot nevertheless exclude that aquatic eDNA comes from the butchering of hunted animals (animals are hunted far away, brought back and butchered in the villages), as the observations of the spider monkey near the villages of Trois-saut and Camopi are consistent with the hunting habits of Wayapi and Teko people, who utilize the spider monkey as a source of meat (Richard-Hansen et al., 2019; de Thoisy et al., 2009).

4.3 | Richness patterns

Overall, inventories of the emblematic mammalian fauna using aquatic eDNA metabarcoding revealed strong species richness variations between the three rivers, with the Sinnamary river presenting a high species richness in a large part of its course whereas the Maroni river shows rich assemblages only in restricted areas. This gradient is consistent with human presence on these rivers, the Maroni river being the most inhabited and the Sinnamary river being much less occupied by humans, with a human population density approximately 10-fold lower on the Oyapock than on the Maroni (Gallay et al., 2018).

We also outlined a trend toward highest mammal richness in the upstream part of the studied rivers, which are the least impacted by mining activities and the least densely populated by humans (Stach et al., 2009; de Thoisy et al., 2010). Maximal species richness values were indeed found in the upstream part of the Sinnamary river, which is free from any human settlements and is integrally protected as part of the core area of the Parc Amazonien de Guyane. The upstream part of the Maroni river is also free from human settlements, and traditional hunting activities by local people remain limited due to the difficulty in accessing these areas. By contrast, the upstream

part of the Oyapock river hosts around 1,700 inhabitants who rely on local fishing and hunting as sources of protein (Richard-Hansen & Hansen, 2004). However, subsistence hunting and deforestation remain scarce (only slash and burn subsistence agriculture) and this is consistent with the upstream site species richness being higher compared than the most downstream sites, despite the abundances of hunted species being shown to be locally reduced by Richard-Hansen et al. (2019).

4.4 | Challenges and applications

Although we globally retrieved consistent patterns of species distribution/richness that are comparable to line transects, aquatic eDNA metabarcoding for assessing nonaquatic species has some limitations. Among those limitations, false negatives (i.e., missing observations of present species) is a common challenge encountered in most (if not all) survey methods (Tyre et al., 2003). While with aquatic communities such as fish, the species detectability may be conditioned by species relative abundance or species morphology and physiology (Hunter et al., 2019; Lacoursière-Roussel et al., 2016), false negatives may be more frequent when assessing nonaquatic fauna as those species are less (or not directly) in contact with the water. The heterogeneous liberation of DNA in the water is then dependent on species density, species morphological and physiological characteristics, but also species behaviour and water affinity (Harper et al., 2019), probably influencing the detectability of species. Moreover, site characteristics and environmental conditions may also influence the quantity of eDNA retrieved and therefore impact the rate of false negatives and the inventories (Kocher et al., 2017; Lacoursière-Roussel, Rosabal, et al., 2016; Rees et al., 2014; Sales et al., 2020). In our study system, Cantera et al. (2019) demonstrated that for the same sampling effort, fish community inventories were significantly less exhaustive in large compared to small watercourses. We therefore cannot exclude such a sampling effect between small and large watercourses in our study as well.

Moreover, the spatial signal of eDNA (spatial extent of the downstream transport of eDNA) defining the spatial grain of the inventories may also be a determining parameter to consider when assessing the presence of species (Hauger et al., 2020). In our systems, Cantera (2020) demonstrated that the downstream detection of eDNA was short (not exceeding a few kilometres) but it might already be enough to observe vulnerable species in areas where hunting pressure is concentrated over a small spatial extent (from 2 to 5 km either side of the river; Richard-Hansen et al., 2019).

These limits influence to what extent aquatic eDNA metabarcoding should be used for biodiversity monitoring and particularly species of concern including invasive, pathogenic, threatened, endangered and other vulnerable species. In our study, incidental detections (unanticipated detection of species of concern) may be valuable to improve knowledge on species distributions, but the method's lack of regularity and exhaustivity may represent a risk if used as the sole method to assess the presence of such species or to monitor the state of biodiversity (for a review on this aspect see Darling et al., 2020).

Despite those limits, aquatic eDNA metabarcoding provides an efficient way to complement and extend traditional inventories. Although eDNA only provides presence data without information on species abundance, it allows us to detect rare, and endangered species as illustrated by the detection of six species of IUCN concern and of five species classified as very rare in the Faune Guyane database. Moreover, aquatic eDNA metabarcoding provides presence data for species not detectable in traditional surveys, be they aquatic or nocturnal. For instance, the widespread distribution of kinkajou revealed by eDNA strikingly contrasts with the rarity of visual observations, but coincides with local camera trap experiments revealing its local commonness (Coutant, 2019). Aquatic eDNA metabarcoding therefore offers a way to extend our knowledge on mammal occurrences. Despite a lower observational frequency than with the traditional line transect method for diurnal and terrestrial fauna, the sampling effort needed to collect an eDNA sample (no more than a couple of hours for a single person) makes it easily implementable together with line transects or other survey methods to complement and extend inventories. In addition, although eDNA-based methods using terrestrial substrates may be more appropriate to survey nonaquatic mammals, eDNA shed by organisms disperses less easily on the ground than in the water. Consequently, sampling strategies have significant impacts on inventories because the information collected in a single ground sample has a very restricted spatial definition (Valentin et al., 2020; Zinger et al., 2019). In contrast, aquatic eDNA-based methods use water bodies that passively aggregate eDNA shed from target species at a wider scale than the sampling point (Deiner et al., 2016; Zinger et al., 2020). However, these methods may have limited on-field applications as they require the presence of waterbodies in the surveyed areas. Nevertheless, the lack of efficient methods to collect terrestrial derived eDNA is a major limitation in eDNA-based terrestrial biodiversity monitoring. We therefore believe that mammal inventories performed with aquatic eDNA metabarcoding methods and adapted from protocols already well established to assess aquatic communities present a great potential to survey species-rich environments such as Neotropical ecosystems, and thus complement quantitative but more taxon-specific and time-consuming traditional mammal surveys using line transects.

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CONFLICTS OF INTEREST

A.V. and T.D. are research scientists in a private company specialized on the use of eDNA for biodiversity monitoring, with some patent technologies (SPYGEN).

AUTHOR CONTRIBUTIONS

O.C., S.B., J.M., C.R.H. and B.d.T conceived the ideas and designed the methodology; O.C., S.B., J.M., J.B.D. and R.V. collected the data; A.V. and T.D. conducted the laboratory work; A.V. and T.D. conducted bioinformatic analyses; O.C. analysed the data; O.C., S.B., J.M., C.R.H. and B.d.T led the writing of the manuscript. All authors contributed critically to the drafts.

DATA AVAILABILITY STATEMENT

Supplementary materials including Figure S1, Table S1, Table S2 and species by sites matrices (Tables S3 and S4) underlying the main results of the study are provided in the online version of the article. All Illumina raw sequence data are available on Figshare https://doi.org/10.6084/m9.figshare.13739086.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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