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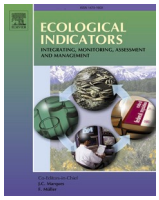

Cuvier's beaked whale (*Ziphius cavirostris*) detection through surface-sourced eDNA: A promising approach for monitoring deep-diving cetaceans

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ABSTRACT

Among cetaceans, the Cuvier's beaked whale is considered an extreme diver, thus not one of the easiest cetacean species to be studied due to its elusive behaviour and a preference for deep offshore waters. Although dedicated cetacean surveys have increased our knowledge on the distribution of Cuvier's beaked whales in the Mediterranean Sea, knowledge gaps still remain where the study effort is non-existent. In this context, the use of new molecular methodologies capable of intercepting small traces of DNA left in the environment (eDNA) by marine organisms represents a valid approach to infer species' occurrence. Moreover, the collection of water from the superficial layer is suitable for targeting marine mammals, as the constraints imposed by their nature implies periodic and frequent surfacing in order to breathe, releasing exhalants rich in their epithelial cells. Therefore, we designed and tested a taxon-specific primer set to infer Cuvier's beaked whale presence, with the aims of 1) examining the effectiveness of the eDNA technique to detect the presence of a deep-diving cetacean in open waters, using the Cuvier's beaked whale as case study; 2) providing data on the occurrence of this species within the Canyon of Caprera over a six-months study period and 3) assessing the species presence in adjacent waters in the central northern Mediterranean Sea based on the analysis of samples collected in a Citizen Science campaign. Results from this study demonstrated that superficial waters may retain biological traces of this cetacean despite the fact that it mostly inhabits deep waters. Specifically, this study provides evidence of the regular presence of Cuvier's beaked whale in the Canyon of Caprera, with a preference for bathymetry in the range of 700–1000 m. Molecular traces' distribution suggests a potential inshore movement of this species during Fall, which might be related to migration of its cephalopod prey or a shift in prey preferences, although this aspect requires further investigation. Overall, this study showed that the stronger positive signals were recorded in sampling stations located on surfaces above submarine canyon systems, demonstrating the importance of these areas as elective habitats for the Cuvier's beaked whale, thus the pivotal priority to their conservation.

1. Introduction

The identification of areas critical for species survival is essential for effective wildlife conservation, particularly when focusing on species of concern (Ambal et al., 2012; Stokes et al., 2015; Valsecchi et al., 2023). Threatened marine vertebrates are challenging to study as they are often rare or elusive, resulting in insufficient knowledge on their occurrence and distribution, which impedes management and limits effective conservation (Boldrocchi and Storai, 2021; Kiszka et al., 2007; Smith et al., 2021). These limitations seem to be somewhat mitigated by the use of new molecular methodologies capable of intercepting small traces of DNA left in the environment (eDNA) by marine organisms (*e.g.* Bohmann et al., 2014). This approach is also advantageous because it is suitable for involving citizen scientists who can easily be engaged in the sampling phase (Biggs et al., 2015), which simply consists of the collection of seawater samples (Agersnap et al., 2022), usually from the

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surface, to ease the process of sample acquisition even for non-experts using simple and readily available equipment (Valsecchi et al., 2023). Moreover, the collection of water from the superficial layer of the sea does not appear to represent a limitation when targeting marine mammals, as the constraints imposed by their nature implies periodic and frequent surfacing in order to breathe. In fact, the first research studies that used eDNA for the study of marine mammals involved the collection of seawater samples from the "footprint" left behind when the animals broke through the water surface (Alter et al., 2022; Baker et al., 2018; Székely et al., 2021). However, while marine mammals represent a perfect target for superficial eDNA sampling, recent studies have also shown that the molecular signals degrade quickly after the animal submerges: bowhead whale DNA dropped by ~ 4.5-fold 10 min after a dive (Székely et al., 2021), while for other cetacean species it was observed a decline in eDNA over the 15-min to 30-min intervals following the sighting (Alter et al., 2022). This can considerably reduce the possibility of molecularly identifying the passage of those marine mammals that spend a short time on the surface, such as those considered "deep divers" like the sperm whales and other odontocetes belonging to the Ziphiidae family. Deep-habitat cetaceans are generally difficult to study, as these animals regularly perform long dives and live in offshore habitats, leading to a limited knowledge of their population and demography (Torreblanca et al., 2022, Breck, 2006; Robbins et al., 2022).

The use of species-specific qPCR assays can enhance the efficiency of detecting the presence of the eDNA of a single target-species, as this approach is more sensitive than the multispecies PCR approach (metabarcoding) targeting at broad taxonomic groups (*e.g.* Neice and McRae, 2021; Plante et al., 2021; Valsecchi et al., 2022). Taxon-specific molecular assays have been developed for a number of marine mammal species, such as the harbor porpoise *Phocoena phocoena* (Foote et al., 2012), the Yangtze finless porpoise, *Neophocaena asiaeorientalis* (Ma et al., 2016), the killer whale, *Orcinus orca* (Baker et al., 2018; Pinfield et al., 2019), the bowhead whale, *Balaena mysticetus* (Székely et al., 2021), the Mediterranean monk seal, *Monachus monachus* (Valsecchi et al., 2022). Hunter et al. (2018) isolated also a genus-specific assay for detecting biological traces of the three manatee species of the Genus *Trichechus*. To the best of our knowledge no assay specific to any member of the Ziphiidae Family has so far been released.

Among deep divers, the Cuvier's beaked whale (*Ziphius cavirostris*) is the only beaked whale species commonly found in the Mediterranean Sea, listed as Data Deficient on the IUCN Red List at least until recently (Cañadas, 2012) when its status changed to Vulnerable (Cañadas and Notarbartolo di Sciara, 2018). This species is found both in the western and eastern basins of the Mediterranean Sea (Podestà et al., 2016). However, although the analysis of a massive long-term sightings dataset has recently better delineated the Mediterranean areas usually frequented by this species (Arcangeli et al., 2023; Gnone et al., 2023), information on the spatial ecology of the Cuvier's beaked whale is still limited and mainly restricted to certain areas (such as the Pelagos Sanctuary for Mediterranean Marine Mammals and Alboran Sea, *e.g.* Moulins et al., 2007; 2008; Tenan et al., 2023; Tepsich et al., 2014; Torreblanca et al., 2022), where the species is known to occur and where research efforts have been intense. With the advantage of more recent technologies and approaches, involving sound-and-orientation recording tags - DTAGs- (Alcázar-Treviño et al., 2021; Tyack et al., 2006), presence probability estimation based on spatial modeling (Arcangeli et al., 2016; Azzellino et al., 2011, Cañadas & Vazquez, 2014) and demographic inferences based on an integrated population model (Tenan et al., 2023), impressive progress has been achieved in the knowledge of this species. Nevertheless, knowledge gaps remain where the study effort is non-existent (Cañadas and Notarbartolo di Sciara, 2018; Podestà et al., 2016). Moreover, among cetaceans, the Cuvier's beaked whale is considered an extreme diver (Fig. 1), cryptic at the surface and with a strong preference for deep offshore waters (Moulins et al., 2007; Arcangeli et al., 2016), so obtaining robust knowledge on distribution and abundance presents unique challenges (Cañadas et al., 2018), especially considering the uneven distribution of research effort (Cañadas and Notarbartolo di Sciara, 2018) and possible temporal fluctuations in its distribution (Arcangeli et al., 2026). All these traits combined with the fact that in the marine realm, data collection is particularly difficult, resource intensive and expensive, moreso for studying highly mobile cetacean species (Richardson and Poloczanska, 2008), environmental DNA may be useful as a complementary approach

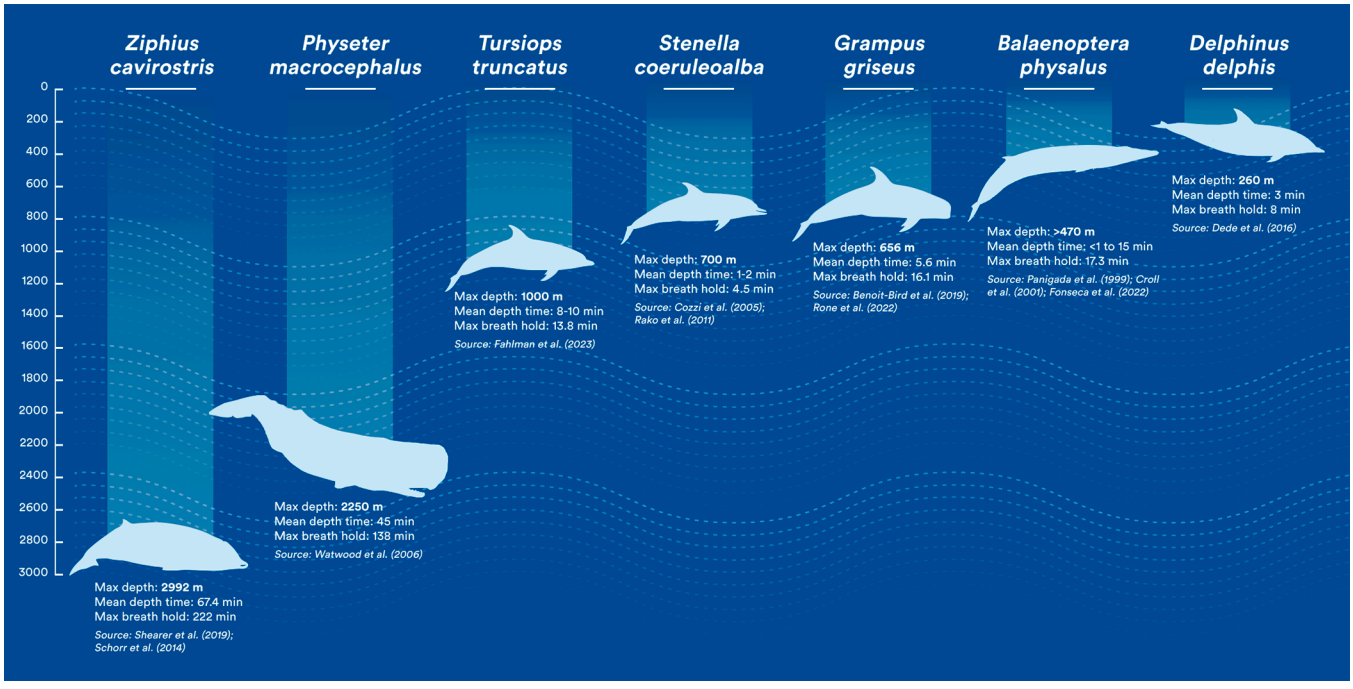


Fig. 1. Comparison between cetacean species in their diving profiles according to literature records. Maximum reached depth, mean diving time and maximum breath hold are reported.

to fill essential knowledge gaps and provide basic ecological information about presence and absence. Indeed, this technique has been already proved to be a fast and cheap methodology to detect rare and invasive species, including the elusive Mediterranean monk seal, one of the rarest pinnipeds at worldwide level, in offshore waters (Valsecchi et al., 2023). The utility of eDNA methodology is multifarious. For instance, it can overcome the cost of preliminary field monitoring, which might be expensive for offshore species like the Cuvier's beaked whale. Moreover, eDNA collection, not relying on visual observation, is a truly non-invasive approach (Thomsen and Willerslev, 2015) as it requires only water sampling without getting close to the target species, thus avoiding any activity that could potentially cause stress to the animal (Zhang et al., 2023). Finally, the simplicity in the acquisition of samples makes the approach well suited to the involvement of the general public (Citizen Science, *e.g.* Clarke et al., 2023), allowing for an extensive and synchronous (more samples collected simultaneously in different sites) sampling.

Considering the need to better understand the distribution coverage of Cuvier's beaked whale in the coming years, we designed and tested a taxon-specific primer set to infer the Cuvier's beaked whale's presence, with the following aims: 1) to examine the effectiveness of eDNA technique to detect the presence of deep-diving marine mammals in open waters, using the Cuvier's beaked whale as case study; 2) to provide data, within the framework of *The Caprera Canyon Project* carried out by One Ocean Foundation, on the spatiotemporal occurrence of this species within the Canyon of Caprera, a potential important area for the species life-history (Bittau and Manconi, 2016); and 3) to assess the species occurrence in the central northern Mediterranean Sea based on molecular traces. The results presented here are important not only for demonstrating the effectiveness of the eDNA approach for detecting traces of deep-diving cetacean species without observing them, but also furthering our understanding of the Cuvier's beaked whale geographical distribution in the Mediterranean Sea.

2. Methods

2.1. Sampling location

The study was carried out using three sets of samples. The first consisted of samples collected on a monthly basis, at three points along the rim of the Caprera Canyon (n = 18), an area known to be frequented by the Cuvier's beaked whale (Bittau and Manconi, 2016). Specifically, in the Caprera Canyon area (Supplementary Figure S1), the sampling activity took place once a month from May to October 2021, in 3 sites over the canyon: an inshore location, named Station 1 (41°20′04.1″ N, 9°46′27.9″ E) with a sea-bottom depth of approximately 550 m; a middle location, Station 2 (41°23′07.4″ N, 9°53′25.7″ E) with a depth of approximately 750 m; and an offshore location, Station 3 (41°25′11.2″ N, 10°04′24.4″ E) with an approximated depth of 1000 m. Station 1 and Station 2 do not encompass the range of bathymetries considered core habitat of the Cuvier's beaked whale Mediterranean population. The choice of these 3 points was made to cover the entire environment of the canyon from east to west, sampling at different depths and at progressive distances from the coastline. Overall, a total of 18 samples were collected over 6 monthly sampling activities. This group of samples was selected both in order to assess the efficiency of the developed species-specific molecular probe in detecting traces of the Cuvier's beaked whale eDNA and, secondly, to assess its occurrence in the Caprera Canyon area. The second set of samples included "control samples" collected along a line crossing perpendicularly the Caprera Canyon (n = 3), in order to verify whether Cuvier's beaked whales do really favor waters above underwater canyons. Finally, the study was complemented with a third and larger sample set (n = 32) collected in marine districts adjacent to the Caprera Canyon, (*i.e.* around Corsica, Sardinia and the Tuscan Archipelago) during the same period, within the project *Spot the Monk* (Valsecchi et al., 2023). All 53 samples were collected in 2021, from the 16th of May until the 12th of November and their full details are listed in Supplementary Table S1.

2.2. Sampling activities and seawater filtration

All samples were collected from the most superficial layer of the sea (0–30 cm below sea level) from the research vessel. For each sample, a total of 12L of marine water were collected by pumping in a resistant Flexmet made sterile Bags-in-Box containers, following Valsecchi et al. (2021). Once the containers were filled, they were stored in a dark and fresh place to avoid an elevated exposure to heat and UV light and to minimize the degradation of DNA traces. All filtering activities were carried out within 12 h from water collection for the Caprera Canyon samples, while for the remaining samples the time elapsing between collection and filtration could reach a maximum of 52 days (Valsecchi et al., 2023), with an average of 11.2 days. Each bag was divided into three 4-L aliquots, each filtered on a nitrocellulose filter with a porosity of 0.45 μm using the BioSart 100 filtration cylinders (Sartorius), resulting in filters A, B and C. For some of the samples collected outside the Caprera Canyon only one (A) or two (A and B) filters were obtained (Supplementary Table S1, see also Valsecchi et al., 2023). The water sample was forced to pass through the filter thanks to the negative pressure created by means of a vacuum pump (Fisherbrand FB70155, Fisher Scientific) applied to the water-collection vacuum flask.

After filtration the porous membranes were folded in two (filtrate side touching itself) inside an aluminum foil and accordingly labelled (sample id, location, date of sampling and filtration and filter number). Labelled filters were stored at −18 °C before the DNA extraction at the University of Milano-Bicocca - MaRHE Center Lab.

2.3. Molecular analyses

Environmental DNA was extracted using DNeasy PowerSoil Kit® (Qiagen), following the manufacturer's protocol. Candidate regions for designing the Cuvier's beaked whale specific primers were searched for within the mtDNA regions targeted by MarVer primers (12S-rDNA and 16S-rDNA), as this part of the mitogenome has proven to be highly polymorphic among vertebrates (Valsecchi et al., 2020). The candidate sets of primers were first tested on a panel of control tissue-extracted DNA templates (non eDNA) consisting of: *Ziphius cavirostris* DNA (positive control), and three mock templates containing of a mixture of a) fish DNAs (negative control), b) fish and cetaceans other than *Ziphius cavirostris* DNAs (negative control) and c) fish and cetaceans species DNAs including *Ziphius cavirostris* (positive control).

The 53 eDNA samples were screened using the best-performing set of newly designed primers for the detection of Cuvier's beaked whale DNA traces, through Real Time (RT) quantitative PCR (qPCR), using an Applied Biosystem AB 7500. For each reaction the following parameters were estimated: the amplification efficiency (E), the Limit of Detection (LOD) and the Limit of Quantification (LOQ) (Klymus et al. 2020). To standardize the Ct (cycle threshold) we purified and isolated the amplicon extracted from *Ziphius cavirostris* tissue sample obtaining a control template with a concentration of 284 ng/μl and we used it to run a seven-fold serial dilution series to standardize the curve. For the amplification reaction we used: 5.0 μl SsoFast EvaGreen Supermix with Low ROX (Bio-Rad), 0.1 μl each [10 μM] primer solution, 2 μl eDNA template and 2.8 μl of Milli-Q water Q-PCR. The thermocycler profiles consisted of the following steps: 10 min at 95 °C for the initial denaturation, followed by 40 cycles with denaturation at 95 °C for 15 s and 1 min of annealing-elongation at 52 °C and final dissociation stage. According to the LOQ calculated for this locus, qPCR DNA detection outcomes were divided in three classes: 1) No signal; 2) Cuvier's beaked whale eDNA detectable but not quantifiable (DBNQ); 3) positive quantifiable detection (PQD) of Cuvier's beaked whale eDNA.

2.4. Data analyses

Once obtained, the molecular data were divided into two groups, positive samples and negative samples, to investigate whether the incidence of positives was in any way related to the geographical characteristics of the sampled spots (*i.e.* depth of the seabed and distance from the coast), since the target species, being a deep-diver, has restraint requirements. Similarly, the two categories of results were investigated also to assess if the inhomogeneity in the processing times of the samples (namely time between sampling and filtering, that was immediate in the Caprera Canyon samples, while extremely variable and much longer in the remaining samples, collected through Citizen Science campaigns) could have affected the results.

3. Results

Three sets of primers, each including one Cuvier's-beaked-whale specific primer, were designed within the three hypervariable regions of the 12S-rDNA and 16S-rDNA mitochondrial genes described in Valsecchi et al. (2020), and tested. The set of primers identified within the 16S-rDNA region showed the best amplification yield (strong specific band and no amplification in related taxa) in PCR tests. The primers' pair was composed of the forward primer ZcaMV3F (5′CCCAAAAACTATAAATCTAAACCG3′), unique to the *Ziphius cavirostris* mitogenome (GenBank Accession Number NC_021435), and the reverse primer Ceto3R (5′TTGGATCAATAWGTGAT3′) conserved among most Cetaceans (Valsecchi et al. in prep.), amplifying in combination a 152 bp amplicon.

In the standardization-curve qPCR run the amplification efficiency reached 86,075 % LOQ corresponded to Ct = 34.44, while the melting temperature (TM) of the targeted amplicon was of 80.8 °C. A total of 444 qPCR reactions were run on the 53 eDNA samples. All negative controls (run in triplicates in each qPCR run) did not produce any signal indicating presence of *Ziphius cavirostris* genetic traces, excluding the possibility of cross-sample contamination. The qPCR results were divided in the three categories: No detection, Positive and Quantifiable Detection (PQD) and Detectable But Not Quantifiable (DBNQ), according to the LOQ classification established in the protocol. Detections (both PQD and DBNQ) were considered reliable when amplified products were supported by the specific melting temperatures recorded (*i.e.* 80.8 +/-0.3) in the dissociation test.

DNA traces (PQD and DBNQ detections combined) of *Ziphius cavirostris* were detected in 41 of the 53 samples (77.4 %), but in about half of these (n = 26, 49.1 %) the signal was intense enough to be quantifiable (PQD). The screening outcomes are summarized below, in Fig. 4.

Despite both PQD and DBNQ outcomes being indicative of the presence of Cuvier's beaked whale DNA in the sampled water column, the latter denotes the presence of traces so diluted that they could be residues of signals released far away from the sampling point (probably tens of km apart). Since one of the purposes of this study is to identify fine-scale differences of presence-absence in sampling stations whose reciprocal distance does not exceed 30 km (*e.g.* those of the Caprera Canyon), we have given more emphasis in the discussion to the PQD results, which identify stronger signals and therefore presumably were released by the animal near the sampling point.

The geographical distribution of all 53 samples is mapped in Fig. 2, which also highlights the samples that tested positive according to either of two criteria (PQD and DBNQ) described above. Fig. 3 focuses on the spatiotemporal distribution of positives in the 21 (18 plus 3 controls) samples collected monthly from the surface overlooking the Caprera

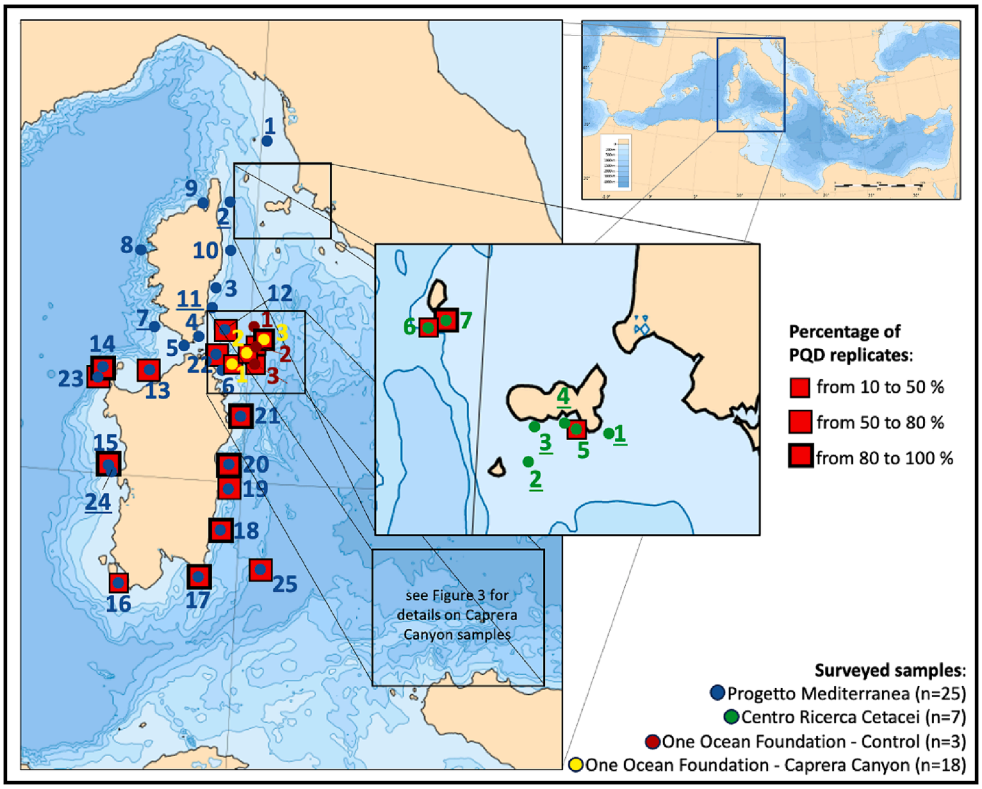


Fig. 2. Maps showing the 53 points sampled in 2021 in the Northern Tyrrhenian Sea and Sardinan Channel (Central Mediterranean Sea, see top right map). Red squares surround samples that returned a positive and quantifiable detection (PQD) of *Ziphius cavirostris* eDNA traces. Larger squares depict a stronger signal, with more than 50 % of the replicates returning a PQD detection (>80 % of replicates when squares are highlighted with a thicker outline). The numbers underlined indicate those points where a Detectable But Not Quantifiable (DBNQ) molecular signals referable to the Cuvier's beaked whale were found. Maps downloaded from https://commons.wikimedia.org/wiki/File:Mediterranean_Sea_Bathymetry_map.svg. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

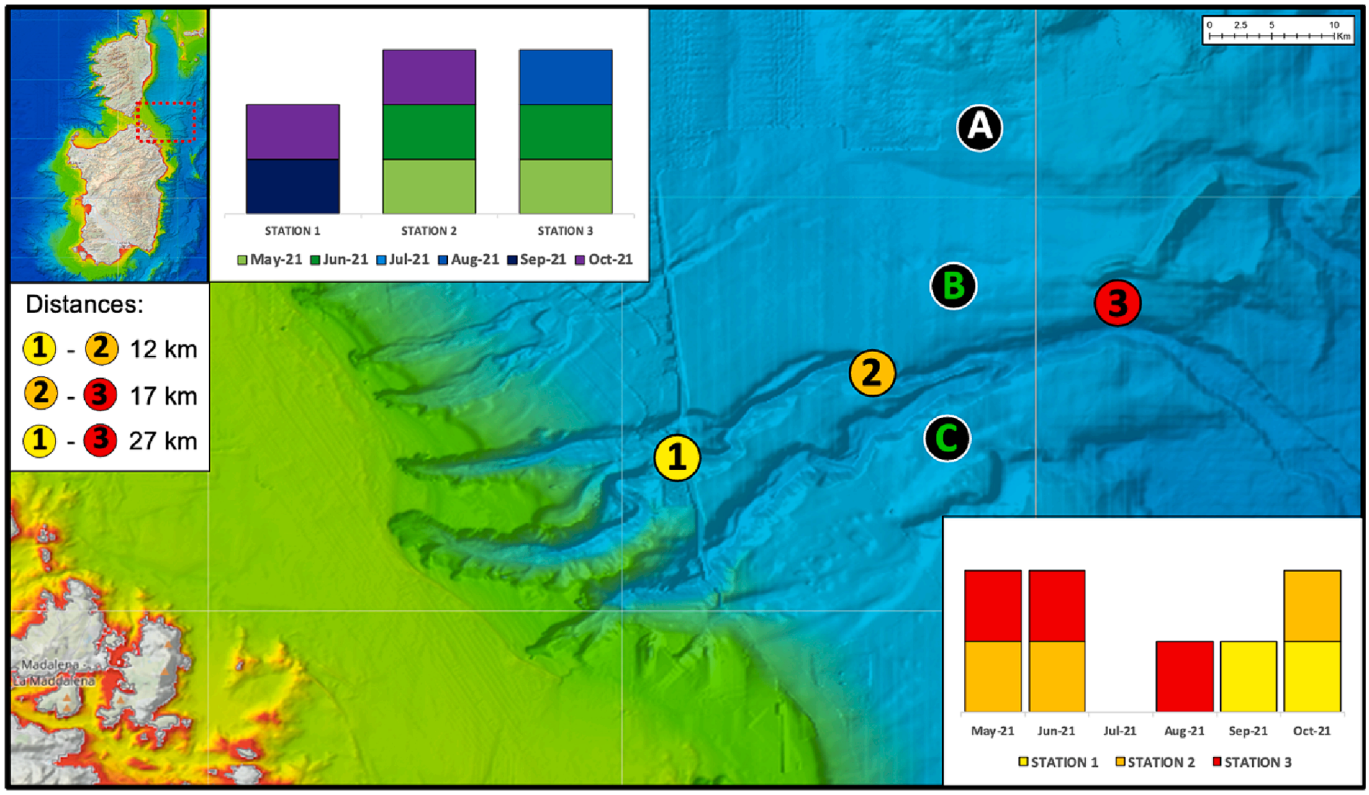


Fig. 3. Caprera Canyon sampling sites' map (area localised in the red dashed rectangular in the map in the left top corner). Coloured circles show the positioning of the three fix sampling stations sampled monthly from May to October 2021. The black circles indicate the 3 control samples surveyed in Nov 2021. The graph in the upper part of the figure shows the distribution of those samples returning a positive and quantifiable detection (PQD) in at least one of nine replicates, in the three fix stations over the six-month study period. While the graph below shows the monthly incidence of positive replicates in the three fix sampling stations. Only control samples B and C (shown in green) were positive to Cuvier's beaked whale eDNA. Maps retrieved from https://www.emodnet-bathymetry.eu/. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

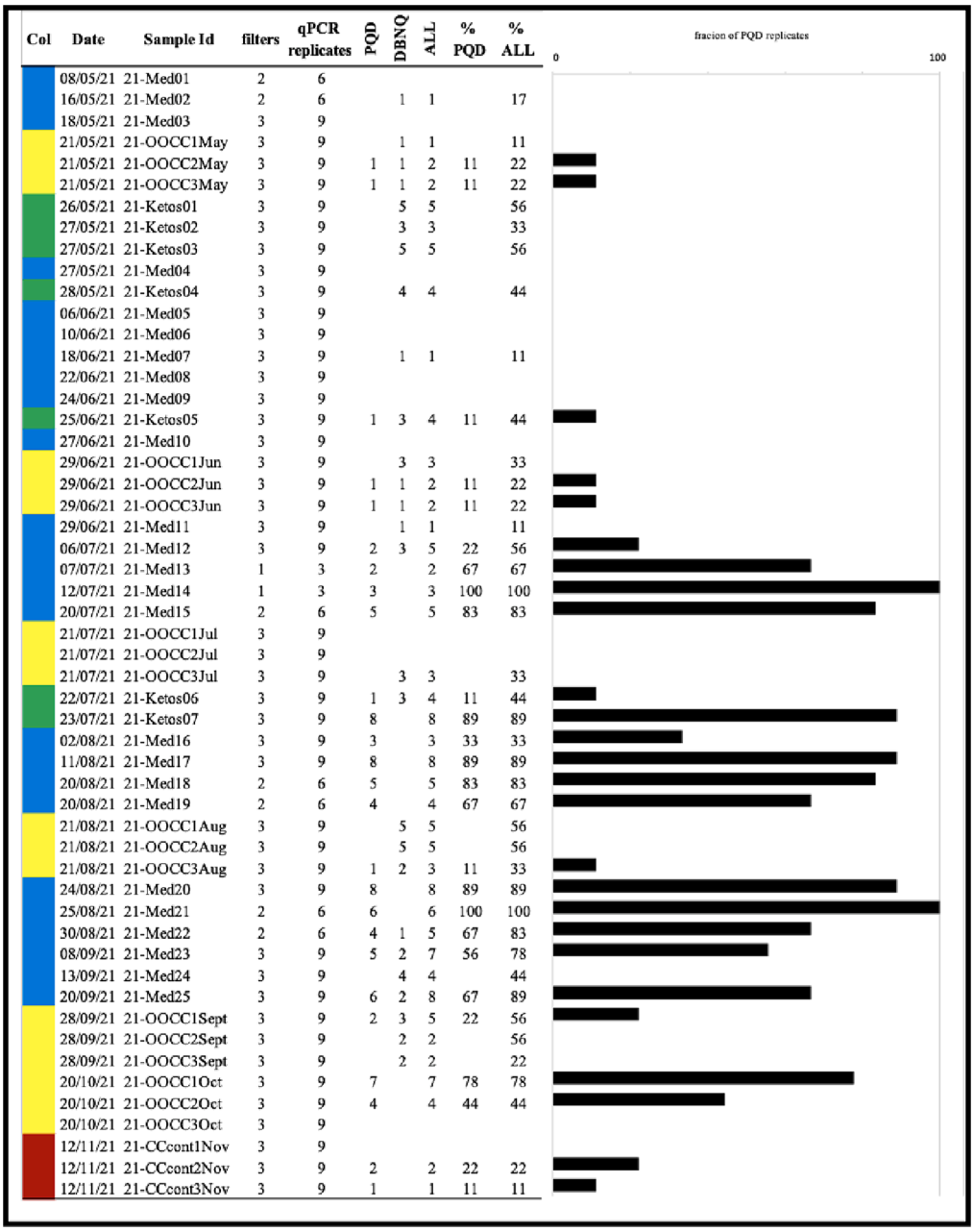
Canyon rim.

In order to better appreciate possible seasonal differences, positives' distribution was plotted in chronological order, highlighting a higher incidence of positives in the second half of the study period (Fig. 4). The 26 PQD positives, accounting for roughly half (49.1 %) of the total sample set, were found in samples collected, on average, in points with slightly higher bathymetry and further from the coast, compared to negative samples (as expected considering the pelagic habitat of the species, *e.g.* Arcangeli et al., 2016), however these differences were not significant (Supplementary Figure S2 A and B). Also, the long processing times of the Citizen Science samples did not seem to affect the incidence of positives (Supplementary Figure S2 C).

4. Discussion

In this study, a non-invasive, species-specific detection method, based on eDNA analysis, was developed to monitor the presence of the endangered and elusive Cuvier's beaked whale in the Canyon of Caprera, and in the wider Tyrrhenian Sea. The molecular assay successfully identified quantifiable traces of Cuvier's beaked whale DNA in about half of the 53 samples collected from both opportunist (citizen science) and dedicated sampling campaigns. For a quality check of molecular outcomes, a sub-sample of the sample set was re-analyzed months later, and the results obtained in the first screening were all confirmed, including unexpected positive (*e.g.* Capraia) and negative (*e.g.* Corsica) cases. This is a remarkable result if we consider that similar studies aiming at capturing cetacean species' eDNA have produced low detection rates even when the samples were collected in proximity of the target species or shortly after its immersion (Foote at al., 2012; Pinfield et al., 2019; Székely et al., 2021). This could be attributable to the fact that our sampling protocol involves the acquisition of a much larger volumes of seawater. We collected 12 L per sample, while the above-mentioned studies used samples obtained filtering 1 L of marine water or less (15 ml samples in Foote at al., 2012).

Although DNA metabarcoding is more useful and cost-efficient when detecting a large number of species at the same time (Gargan et al., 2022), cryptic species need highly specific and sensitive approaches to be identified (Vörös et al., 2017). Compared to other cetaceans, the Cuvier's beaked whale is one of the most difficult to study: these animals are able to dive at depth greater than 1000 m (Schorr et al., 2014; Shearer et al., 2019, see also Fig. 1) and for extended time period (1 h, or more). Cuvier's beaked whales alternate periods of surface intervals lasting from 2 to 8 min with a series of short and shallow dives, followed by deep foraging dives that can last over 100 min (Tyack et al., 2006; Baird et al., 2006; Schorr et al., 2014). These traits reduce their detectability by visual observers (Barlow et al., 2005). Indeed, field identification may be challenging, and prior experience is very important to an observer's ability to detect beaked whales (Barlow et al., 2005). Even so, our current knowledge on this and other cetacean species largely relies on the visual approach, which remains the most informative to date, especially if based on long-term data (Arcangeli et al., 2016; Cañadas et al., 2018; Gnone et al., 2023). Moreover, when dealing with Cuvier's beaked whales, traditional surveys that incorporate boat- and aerial-based surveys and offshore passive acoustic monitoring may result in very expensive monitoring programs. Collecting water samples for eDNA analysis overcomes the weather condition constraints imposed on visual survey methods, allows nocturnal surveys and requires less technical expertise than accurate taxonomic proficiency in morphological identification (Qu and Stewart, 2019; Suarez-Bregua et al., 2022; Valentini et al., 2016). Overall, the eDNA



Col	Date	Sample Id	filters	qPCR replicates	PQD	DBNQ	ALL	% PQD	% ALL
	08/05/21	21-Med01	2	6					
	16/05/21	21-Med02	2	6		1	1		17
	18/05/21	21-Med03	3	9					
	21/05/21	21-OOCC1May	3	9		1	1		11
	21/05/21	21-OOCC2May	3	9	1	1	2	11	22
	21/05/21	21-OOCC3May	3	9	1	1	2	11	22
	26/05/21	21-Ketos01	3	9		5	5		56
	27/05/21	21-Ketos02	3	9		3	3		33
	27/05/21	21-Ketos03	3	9		5	5		56
	27/05/21	21-Med04	3	9					
	28/05/21	21-Ketos04	3	9		4	4		44
	06/06/21	21-Med05	3	9					
	10/06/21	21-Med06	3	9					
	18/06/21	21-Med07	3	9		1	1		11
	22/06/21	21-Med08	3	9					
	24/06/21	21-Med09	3	9					
	25/06/21	21-Ketos05	3	9	1	3	4	11	44
	27/06/21	21-Med10	3	9					
	29/06/21	21-OOCC1Jun	3	9		3	3		33
	29/06/21	21-OOCC2Jun	3	9	1	1	2	11	22
	29/06/21	21-OOCC3Jun	3	9	1	1	2	11	22
	29/06/21	21-Med11	3	9		1	1		11
	06/07/21	21-Med12	3	9	2	3	5	22	56
	07/07/21	21-Med13	1	3	2		2	67	67
	12/07/21	21-Med14	1	3	3		3	100	100
	20/07/21	21-Med15	2	6	5		5	83	83
	21/07/21	21-OOCC1Jul	3	9					
	21/07/21	21-OOCC2Jul	3	9					
	21/07/21	21-OOCC3Jul	3	9		3	3		33
	22/07/21	21-Ketos06	3	9	1	3	4	11	44
	23/07/21	21-Ketos07	3	9	8		8	89	89
	02/08/21	21-Med16	3	9	3		3	33	33
	11/08/21	21-Med17	3	9	8		8	89	89
	20/08/21	21-Med18	2	6	5		5	83	83
	20/08/21	21-Med19	2	6	4		4	67	67
	21/08/21	21-OOCC1Aug	3	9		5	5		56
	21/08/21	21-OOCC2Aug	3	9		5	5		56
	21/08/21	21-OOCC3Aug	3	9	1	2	3	11	33
	24/08/21	21-Med20	3	9	8		8	89	89
	25/08/21	21-Med21	2	6	6		6	100	100
	30/08/21	21-Med22	2	6	4	1	5	67	83
	08/09/21	21-Med23	3	9	5	2	7	56	78
	13/09/21	21-Med24	3	9		4	4		44
	20/09/21	21-Med25	3	9	6	2	8	67	89
	28/09/21	21-OOCC1Sept	3	9	2	3	5	22	56
	28/09/21	21-OOCC2Sept	3	9		2	2		56
	28/09/21	21-OOCC3Sept	3	9		2	2		22
	20/10/21	21-OOCC1Oct	3	9	7		7	78	78
	20/10/21	21-OOCC2Oct	3	9	4		4	44	44
	20/10/21	21-OOCC3Oct	3	9					
	12/11/21	21-CCcont1Nov	3	9					
	12/11/21	21-CCcont2Nov	3	9	2		2	22	22
	12/11/21	21-CCcont3Nov	3	9	1		1	11	11

Fig. 4. Summary of results of the qPCR assay for Cuvier's beaked whale eDNA detection performed on the 53 samples, here ordered chronologically. From left to right are displayed: the color-code of samples as shown in Fig. 2; the date of sampling; the sample identification code; the number of filters obtained for each sample; the number of replicates (three per filter) tested in the qPCR assay. The next three columns (PQD, DBNQ, ALL) indicate the number of replicates in which either Positive and Quantifiable Detection (PQD), or Detectable But Not Quantifiable (DBNQ) or both were found, respectively. The final two columns indicate the percentage of replicates testing as positive for PQD (% PQD) or PQD and DBNQ (% ALL). Finally, the graph of the right depicts the distribution of PQD positive over the study period.

approach can provide a valid tool for identifying areas of interest where research efforts should be invested.

In this context, the Caprera Canyon has represented a perfect case scenario for detecting the presence of the Cuvier's beaked whale based on eDNA sampling. Indeed, this area is affected by dominant winds that determines strong weather events (Gerigny et al., 2011), thus limiting the possibility of traditional boat-based visual monitoring surveys (Dr Luca Bittau personal communication). Furthermore, besides the rarity of outstanding weather conditions, the Canyon of Caprera extends offshore approximately 15–30 nautical miles from the north-eastern coast of Sardinia, requiring a considerable effort in term of both time, trained operators, and costs. On the contrary, in this study, we have demonstrated that the eDNA approach has good potential for detecting elusive species in open-water conditions and carrying out long-term monitoring programs, complementary to more traditional approaches. As a matter of fact, positive detection showed a constant presence of the Cuvier's beaked whale in the Caprera Canyon (except in July) (Fig. 3), highlighting the importance of this area for this species. Preliminary results based on visual surveys carried out in 2011–2013 have speculated this area as a favorable habitat for the Cuvier's beaked whale (*e.g.* Bittau and Manconi, 2016; Gnone et al., 2023), and the results presented here, with data collected in recent years together with continuous monitoring, can only support this hypothesis. In addition, the control stations used in this study (Fig. 3) confirmed the result: the Cuvier's

beaked whale was detected only in stations B and C, which are located in proximity to the Canyon, but not in station A, located approximately 12 km north of it. However, a note of caution should be added regarding the interpretation of this result, given that one of the major limitations of the eDNA approach is linked to the effect of marine currents, as molecular traces might be drift away embedded in water masses. Therefore, any molecular detection should be interpreted by allowing a "buffer zone" of a few tens of kilometers around the sampling point, depending on the current regime.

Within the Caprera Canyon area, this species was mainly determined at station 2 and 3, which are characterized by a bathymetry of approximately 700–1000 m. This is line with what has been reported in previous studies from the Mediterranean Sea: for instance, most sightings from the Ligurian Sea were located between 756 and 1389 m (Moulins et al., 2007). The Cuvier's beaked whale is often associated with steep slope habitat and submarine canyons as its most common prey species in the Mediterranean are oceanic and meso- or bathypelagic cephalopods, inhabiting depths of approximately 1000 m (Azzellino et al., 2012; Blanco and Raga, 2000; Cañadas and Notarbartolo di Sciara, 2018; MacLeod, 2005). Therefore, the habitat distribution of the Cuvier's beaked whale in the Caprera Canyon likely reflect that of its prey. Interestingly, in Autumn (September and October), the Cuvier's beaked whale has been detected in the most inshore station (Fig. 3). This shift to shallower bathymetries might be linked to the inshore movements of its prey. Indeed, several squid species are known to undertake inshore–offshore movements (*e.g.* Agus, 2015; Arkhipkin, 2000; Pierce et al., 2008) and the Cuvier's beaked whales might follow their migration ending up in more inshore areas. Although the Cuvier's beaked whale diet appears to be mainly represented by histioteuthids (*e.g.* Carlini et al., 1992; Pedà et al., 2015), which are not known to make horizontal migrations, stomach content analyses also showed the presence of other cephalopod species belonging to different families (Carlini et al., 1992; Blanco and Raga, 2000; Kovačić et al., 2010). Moreover, the presence of mesopelagic fish was found to be a significant part of the stomach content of a specimen from the NW Mediterranean Sea, confirming direct consumption on fish (Garibaldi et al., 2015), indicating some degree of dietary generalism of the species. Therefore, the possibility of inshore movements of whales driven by different prey cannot be excluded. Alternatively, these movements might reflect seasonal changes in the preference for its prey items (Azzellino et al., 2008).

Consistently with what has been reported for the Canyon of Caprera monitoring, the opportunistic samples collected in the Tyrrhenian Sea further highlight the importance of submarine canyons for this species. Interestingly, positive detections, especially those with a stronger signal (>80 %, Figs. 2 and 4), were located in areas close to underwater canyons, confirming the species' preference for this habitat. As a matter of fact, stronger signals were reported in samples 21-Med13 and 21-Med14, collected in the Castelsardo Canyon area (northeastern Sardinia), in sample 21-Med15 gathered in proximity of Oristano Canyon, and in the four consecutive samples 21-Med18, 21-Med19, 21-Med20 and 21-Med21 collected in the area of Orosei, Gonone and Arbatax canyon systems in the same period (from the 20th to the 25th of August 2021), and finally sample 21-Med17, in the Simius Canyon (Fig. 2). However, contrary to the monitoring carried out at the Caprera Canyon, it is impossible to determine if in these Sardinian submarine canyon systems, the presence of the Cuvier's beaked whale is seasonal, year-round, or only transient. Future monitoring should be carried out to further investigate this aspect.

Interestingly, while this species is widely distributed around Sardinian's underwater canyon systems, it was never detected around Corsica Island (Figs. 2 and 4). The absence of detections might not be surprising when considering the eastern part of the island as this lacks of submarine canyons, but appears peculiar when looking at sites where samples 21-Med07, 21-Med08, 21-Med09 were collected: they are all in proximity of massive submarine canyon systems on the western side of Corsica. However, if we ignore the morphological characteristics of the Corsican coast, which would suggest the presence of a habitat congenial to the species, our results do not differ from what was observed by Cañadas et al. (2018) in a study based on stacked visual data collected over a 27-year period: sightings were scarce in the water surrounding Corsica despite the intense search effort in the area (see Fig. 1 in Cañadas et al., 2018). While considering that Corsican waters may lack suitable habitats for this species sounds an unrealistic hypothesis, the most immediate explanation for the lack of positive PQD detections in the Corsican samples is that those samples were collected too inshore; but so were the remaining opportunistic (Citizen Science) samples (mean distance from the coast 15.3 km, with a median value of 5.5 km), some of which tested positive in Sardinia and the Tuscan archipelago. Thus, a further consideration would be a "seasonality" effect. Our data show that positive samples were scarce in the months of May and June, not only in Corsican waters (all Corse samples were collected in May and June), but also in other marine districts. For example, in the Tuscan archipelago (Fig. 4), where the only two PQD positive samples (21-Ketos06 and 21-Ketos07) were collected towards the end of July, in close spatiotemporal proximity to each other (22nd and 23rd of July 2021, at 14.2 km from each other) not far from Capraia island. However, at the moment these can only be speculations since our sample size does not have the statistical power to confirm or refute the "seasonality" hypothesis. We recommend further investigation. For instance, a question that would deserve attention is as to whether it is possible that Cuvier's beaked whales come closer to the coast in the late summer months possibly due to seasonal movement of their prey. This still remains unknown due to insufficient research effort. What is certain is that, where we were able to sample systematically on a monthly basis (*i.e.* Caprera Canyon), the same trend was found: traces of *Ziphius*'s DNA were found in Station 1 (the most coastal one) only in the months of September and October. Inshore and offshore sampling should be carried out year-round in the western side of Corsica to clarify the results presented in this study.

As mentioned above, another element that cannot be overlooked is linked to one of the major limitations inherent in the use of the molecular approach. Marine eDNA is strongly affected by marine currents that may transport it away, embedded in the water mass. For this reason, we opted to focus solely on the interpretation of strong signals (PQD), likely released not too far from the sampling point. Nevertheless, until the dispersion dynamics of marine eDNA are clarified and modeled, we cannot exclude that the traces identified through such a sensitive methodology do not in fact come from afar. However, although in the Central Tyrrhenian Sea visual data suggests the species being predominantly pelagic (*e.g.* Arcangeli et al., 2016), the possibility of occasional incursions towards more coastal waters should be better investigated.

5. Conclusion

The study presents the first species-specific assay developed to attempt to capture molecular traces of a deep-diving cetacean species by mean of eDNA surveys not associated to its sighting. The approach proved its efficiency (providing the collection of large volumes of seawater) and its potential as a non-invasive molecular monitoring procedure not only for assessing the species presence or inferring seasonal movements but also for identifying areas where new research efforts, using complementary monitoring techniques, should be invested. Specifically, this study confirms evidence of the regular presence of the Cuvier's beaked whale, a threatened species, in the Canyon of Caprera, and, more widely, the importance of submarine canyons as elective habitats for this species, thus the pivotal priority to their conservation. As a matter of fact, our findings, in addition to previous studies that have been carried out in the area, indicate that the Caprera Canyon can be considered a hotspot area for the Cuvier's beaked whale. As conservation efforts are increasingly focusing at preserving critical habitats, rather than selective species, the Caprera Canyon should be considered at both national and international levels for strong protection.

Anthropogenic activities, including maritime traffic, fishing pressure, acoustic and chemical pollution may represent a threat for cetaceans living off north eastern Sardinia.

Ethical approval

Ethical approval is not relevant to this study, as all our samples consisted simply of marine water. Thus, all methods were carried out in accordance with relevant ethical guidelines and regulations.

Credit authorship contribution statement

Ginevra Boldrocchi: Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Investigation, Funding acquisition. **Livia Conte:** Visualization, Validation, Investigation, Formal analysis. **Paolo Galli:** Supervision, Project administration, Funding acquisition. **Roberta Bettinetti:** Supervision, Project administration, Funding acquisition. **Elena Valsecchi:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Primers' design, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2024.111966.

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