Science Advances

Supplementary Materials for

Deep-sea predator niche segregation revealed by combined cetacean biologging and eDNA analysis of cephalopod prey

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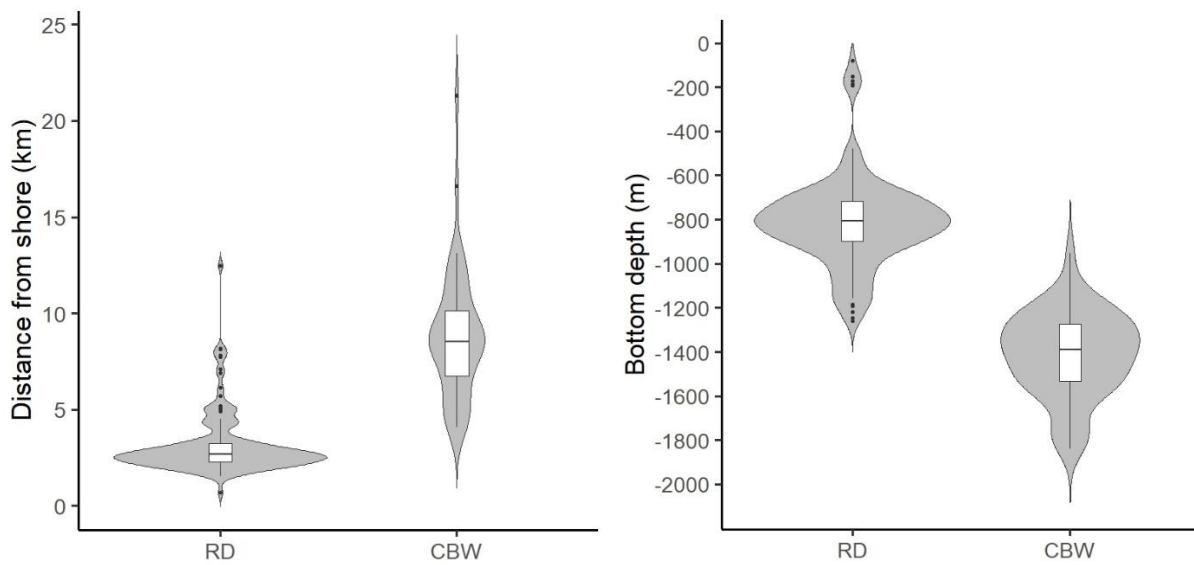
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Supplementary Figures and Tables

Supplementary Figure S1. Risso's dolphin and Cuvier's beaked whale foraging habitat.

Distance from shore (left) and bottom depth (right) at Risso's dolphin (*Grampus griseus*; RD)

foraging dive observations and sighting locations of Cuvier's beaked whale (*Ziphius cavirostris*;

CBW). Data represent group means.
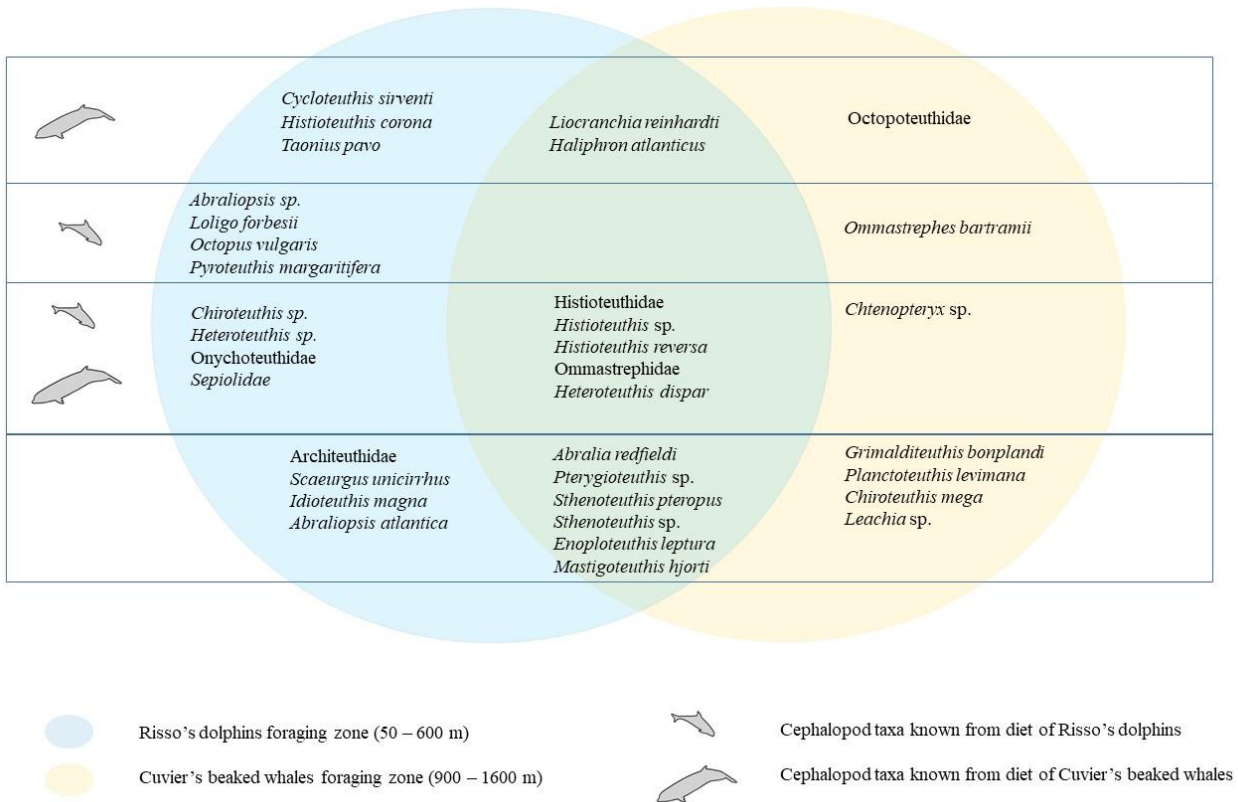
Supplementary Figure S2. Presence of cephalopod taxa preyed upon by one or both cetacean predators in their respective foraging zones. In total 35 cephalopod taxa (at taxonomic level of family or lower) were detected in the foraging zones for Risso's dolphins (*Grampus griseus*; RD) and Cuvier's beaked whales (*Ziphius cavirostris*; CBW) of which seven were only found in CBW zone, 15 were only found in RD zone and 13 taxa were found in both foraging zones.
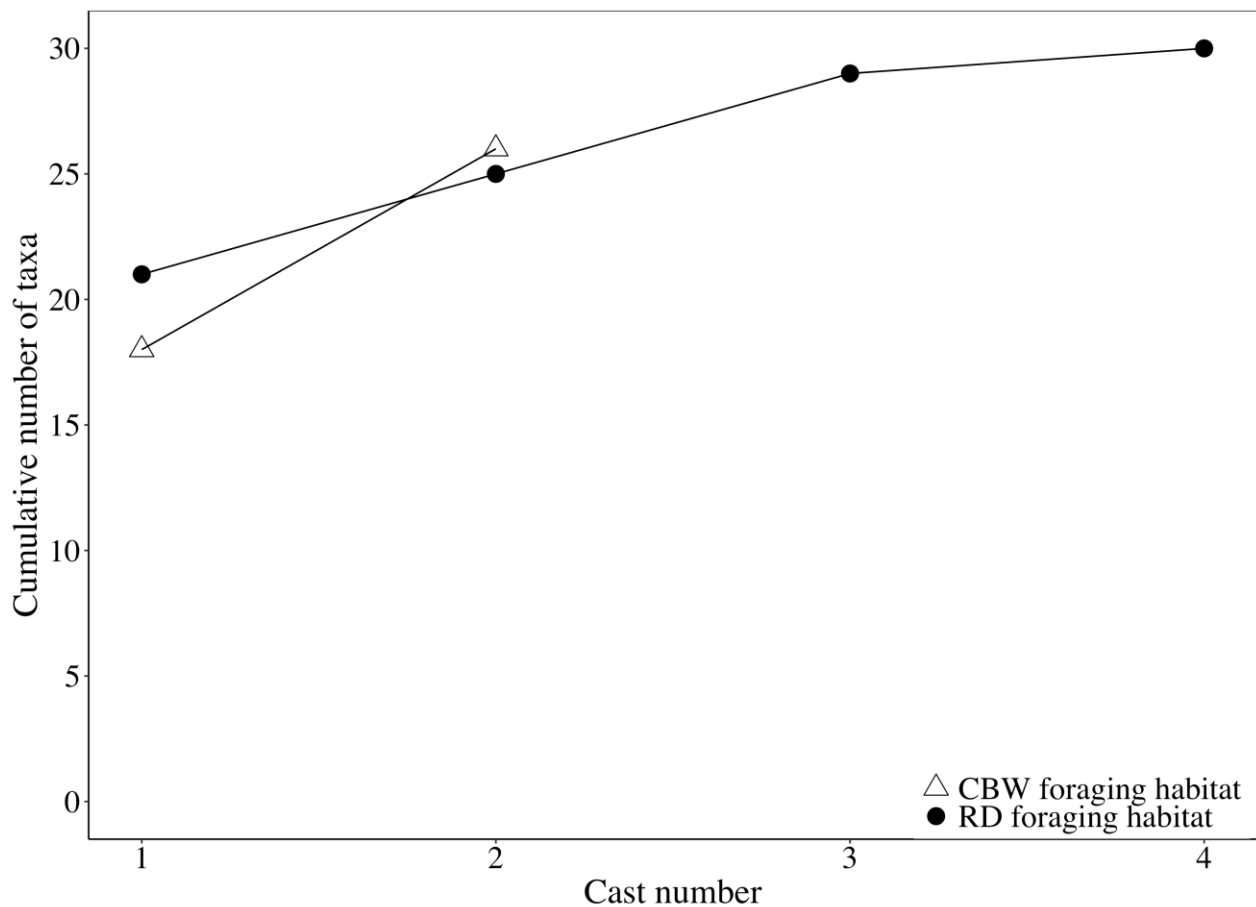
Supplementary Figure S3. Species accumulation curves of cephalopod eDNA taxa detections for Risso's dolphin and Cuvier's beaked whale foraging habitats. In total, 30 cephalopod taxa were detected in Risso's dolphin (*Grampus griseus*; RD) habitat (4 CTD casts) and the curve illustrates the nearing of the asymptote. In Cuvier's beaked whale (*Ziphius cavirostris;* CBW) habitat (2 CTD casts), 26 taxa were detected. Based on the Risso's dolphin habitat curve, a relatively small number of additional taxa (1-3 per cast), at relatively low eDNA density, is expected to be discovered with additional CTD casts.

Supplementary Table S1. Risso's dolphin (*Grampus griseus*) and Cuvier's beaked whale (*Ziphius cavirostris*) tag data record summary. Tag attachment and foraging dive characteristics of individuals tagged off Terceira Island, Azores. Ind. = individual. Dur = dive duration. Max. = maximum. Dive types performed: mesopelagic (MESO) and shallow (SH) by Risso's dolphin, Deep-Wide (DW) and Deep-Layer-Restricted (DLR) by Cuvier's beaked whale.

Ind.	Tag ID	Date	Local Time	Dur (h)	# Foraging Dives		Max. depth (m)	# buzzes
					total	MESO/SH	MESO / SH	
Risso's dolphin								
1	gg13_238a	26-8-2013	16:12	5.7	5	2/3	496/159	19
1	gg17_203a	22-7-2017	12:33	9.3	20	20/0	564/-	274
2	gg15_229a	17-8-2015	9:41	16.7	40	10/30	571/307	262
3	gg15_229c	17-8-2015	13:53	11.0	18	0 /18	-/413	133
4	gg16_169a	17-6-2016	14:12	4.9	11	8/3	249/559	62
5	gg16_171a	19-6-2016	8:51	11.7	4	4/0	623/-	16
6	gg17_200a	19-7-2017	11:04	15.9	26	25/1	534/146	267
7	gg18_214a	2-8-2018	15:37	10.3	21	6/15	364/244	155
Cuvier's beaked whale						DLR / DW	DLR / DW	
1	zc15_205a	24-7-2015	14:38	24.7	9	6/3	1138/1756	318
1	zc15_212a	31-7-2015	8:45	11.8	6	1/5	911/1625	237
2	zc15_212b	31-7-2015	8:46	12.3	5	0/5	-/1715	189
1	zc17_205a	24-7-2017	16:28	24.7	11	1/10	1092/1764	471
3	zc17_227a	15-8-2017	10:21	18.1	7	1/6	1067/1536	168
4	zc18_185a	4-7-2018	11:19	23.5	8	3/5	1101/1428	250
5	zc19_196a	15-7-2019	13:39	12.1	7	0/7	-/1605	220
6	zc19_197a	16-7-2019	16:26	23.5	7	0/7	-/1782	215
Total - Risso's dolphin				85.5	145	75/70		1188
Total – Cuvier's beaked whale				150.5	60	12/48		2068

Supplementary Table S2. Cuvier's beaked whale (*Ziphius cavirostris*; CBW) and Risso's dolphin (*Grampus griseus*; RD) Hidden Markov Model (HMM) dive type classification results. HMM classification of dive types based on maximum dive depth and width of the within-dive foraging zone (SD of buzz depth; CBW) and maximum dive depth (RD).

HMM SELECTION

Cuvier's beaked whale			Risso's dolphin		
	AIC			AIC	
3 states	1430.095	BEST MODEL	2 states	1731.197	BEST MODEL
4 states	1436.223		3 states	1746.782	
2 states	1439.224		4 states	1757.948	
1 state	1530.692		1 state	1801.410	

HMM SUMMARY - 3 STATES   **HMM SUMMARY - 2 STATES**

Maximum depth	State 1	State 2	State 3	State 1	State 2
Mean	1042.7	1299.1	1608.8	204.1	508.1
SD	66.1	148.8	109.2	81.1	51.8
SD Buzz depth (m)					
Mean	37.0	112.5	203.3		
SD	10.7	41.6	37.3		

Supplementary Table S3. Summary of CTD sampling records off Terceira Island, Azores.

Total number of samples obtained from a specific depth combining all sampling locations in Risso's dolphin (RD; *Grampus griseus*) foraging habitat (4 CTD casts; maximum bottom depth 800 m) and Cuvier's beaked whale (CBW; *Ziphius cavirostris*) foraging habitat (2 CTD casts; maximum bottom depth 1600 m). Each sample consisted of three biological replicates.

Depth (m)	RD foraging habitat 4 casts	CBW foraging habitat 2 casts
50	4	-
100	4	-
200	4	2
300	4	-
400	4	-
500	4	2
600	4	-
800	1	2
1100 - 1150		2
1150 - 1200		2
1250 - 1400		2
1475 - 1500		2
1550 - 1600		2

Supplementary Table S4. Cephalopod taxa detected using eDNA analysis in the foraging habitat of Risso's dolphins (*Grampus griseus*, RD) and Cuvier's beaked whales (*Ziphius cavirostris*, CBW) off Terceira Island, Azores. eDNA metabarcoding with two universal cephalopod primer pairs resulted in the detection of 39 cephalopod taxa. In total 29 and 16 sampling records were analyzed from respectively 4 and 2 casts in RD and CBW habitat. "x" indicates presence. % Presence: % of sampling records in which the species was detected. Diet: species previously recorded in the diet of RD, CBW or both, or not known from diet records. Diet data was derived from literature (stomach content analysis; Tables S5 and S6). eDNA records were identified to the nearest possible taxonomic level.

			RD habitat				CBW habitat			
			Sampling locations							
	Genus	Species	1	2	3	4	1	2	% Presence	Diet
Class										
Cephalopoda				x	x				4	Both
Order										
Teuthida			x	x	x	x	x	x	64	Both
Family										
Architeuthidae			x						2	Not known
Alloposidae	*Haliphron*	*atlanticus*	x		x	x		x	13	CBW
Chtenopterygidae	*Chtenopteryx*	sp.						x	2	Both
	Chtenopteryx	*sicula*						x	2	Both
Chiroteuthidae	*Chiroteuthis*	sp.		x					2	Both
	Chiroteuthis	*mega*						x	2	Not known
Grimalditeuthidae	*Grimalditeuthis*	*bonplandi*					x		2	Not known
	Planctoteuthis	*levimana*						x	2	Not known
Cranchiidae	*Bathothauma*	*lyromma*						x	2	Not known
	Liocranchia	*reinhardti*	x	x	x	x	x	x	38	CBW
	Leachia	sp.						x	2	Not known
	Taonius	*pavo*	x						2	CBW
Cycloteuthidae	*Cycloteuthis*	*sirventi*	x						2	CBW
Enoploteuthidae	*Abralia*	*redfieldi*	x	x	x	x	x	x	33	Not known
	Abraliopsis	*atlantica*	x	x	x				7	Not known
	Abraliopsis	sp.			x				2	RD
	Enoploteuthis	*leptura*	x	x	x	x	x	x	60	Not known

Table S4 – Continued

	Genus	Species	RD habitat				CBW habitat		% Presence	Diet
			Sampling locations							
			1	2	3	4	1	2		
Sepiolidae			x						2	Both
	Heteroteuthis	sp.	x	x	x	x	x	x	22	Both
	Heteroteuthis	*dispar*	x	x	x		x	x	22	Both
Histioteuthidae			x	x		x	x	x		Both
	Histioteuthis	sp.	x	x	x		x	x	20	Both
	Histioteuthis	*corona*				x			2	CBW
	Histioteuthis	*reversa*	x	x	x	x	x	x	33	Both
Loliginidae	*Loligo*	*forbesii*	x	x					11	RD
Mastigoteuthidae	*Idioteuthis*	*magna*	x			x	x		7	Not known
	Mastigoteuthis	*hjorti*		x	x	x	x		18	Not known
Octopodidae	*Octopus*	*vulgaris*	x						2	RD
	Scaeurgus	*unicirrhus*			x				2	Not known
Octopoteuthidae							x	x	4	CBW
Ommastrephidae			x	x			x		7	Both
	Sthenoteuthis	sp.		x	x	x	x		18	Not known
	Sthenoteuthis	*pteropus*			x	x	x	x	13	Not known
	Ommastrephes	*bartramii*					x		2	Risso
Onychoteuthidae						x			2	Both
Pyroteuthidae	*Pterygioteuthis*	sp.	x	x	x	x	x	x	36	Not known
	Pyroteuthis	*margaritifera*	x	x	x			x	13	RD
Total # of taxa			21	18	19	14	18	20		

Supplementary Table S5. Presence and contribution of cephalopods in the diet of Risso's dolphin (*Grampus griseus*) in the North Atlantic Ocean and the Mediterranean Sea.

Contribution indicated as % of individuals (N) and % biomass (B). Locations: MED = Mediterranean Sea, LIG = Ligurian Sea, AEG = North Aegean Sea, CEL = Celtic Sea, NEA = North-East Atlantic, NOR = Norwegian Sea. Diet data from supplementary references (*64–69*).

Family	MED N/B	LIG N/B	AEG N/B	CEL N/B	NEA N/B	NOR N/B	Source
Ancistrocheiridae	2.2						(*64*)
Argonautidae	45.5		0.8/2.98				(*64, 67*)
Brachioteuthidae	1.2		0.8/2.05				(*64, 67*)
Chiroteuthidae	0.4						(*64*)
Cranchiidae	0.7						(*64*)
	0.3						
Eledonidae	1.8			38/55		67.9/56.9	(*64–66*)
Enoploteuthidae	1.1						(*64*)
Histioteuthidae	8.4	3.6/1.2	29.6/61.4				(*64, 67–69*)
	5.1	78/80.6	5				
	60.9		14.4/33.52				
Loliginidae	0.3			7/8		7.9/19.5	(*64–66*)
Mastigoteuthidae	3.9						(*64*)
Octopodidae	0.3						(*64*)
	0.1						
	0.7						
Ocythoidae	1.5						(*64*)
Ommastrephidae	5.3	7.3/13.2		5/5		0.2/0.1	(*64–66, 69*)
	0.5			21/19		0.2/0.1	
	6.2						
	2.6						
Onychoteuthidae	7.3	9.1/4.9					(*64, 69*)
	3.5						
Sepiidae	0.7			28/13	85.7/94.6		(*64, 66, 70*)
Sepiolidae	0.3						(*64, 69*)
	0.3	1.8/0.04					

Supplementary Table S6. Presence and contribution of cephalopods in the diet of Cuvier's beaked whale (*Ziphius cavirostris*) in the North Atlantic Ocean and the Mediterranean Sea. Contribution indicated as % of individuals (N) and % biomass (B). Locations: CAN = Canary Islands, MED = Mediterranean Sea, NA = North Atlantic, NEA = North-East Atlantic, ADR = Adriatic Sea. Diet data derived from supplementary references (*70–74*).

Family	CAN N/B	MED N/B	NA N/B	NEA N/B	ADR N/B	Source
Alloposidae				10/10		(*71*)
Amphitretidae			0.02			(*73*)
Ancistrocheiridae	0.08/0.3	3.4/0.6 2.1/0.2				(*71, 74*)
Bathyteuthidae	0.01					(*74*)
Brachioteuthidae			0.3/0.02	1.2/0.1	0.3/0.03	(*70, 73*)
Chiroteuthidae	5.1/3.0	1.8/0.6 16.3/5.4	2.4/1.9		48.5/17.7 2.53/1.1 3.3/1.3	(*71–74*)
Chtenopterygidae		5.5/0.5 12.8/1.1	0.2/0.05			(*73, 74*)
Cranchiidae	2.5/2.4 9.4/1.3 24.8/3.1 1.3/2.1 24.3/10.9 5.92/2.4		9.5/7.2 0.04 0.3/0.8 10.8/9.4 22.2/13.9	9.5/8 1.6/1.4 1.4/1.2 26.6/22.7	9.1/8 1/0.2 4.9/3.7 48.9/4.3 17.2/9.7 35.5/24.8	(*70–74*)
Cycloteuthidae	0.3/2.1 0.4/1.0		0.04/0.4		0.1/0.7 0.4	(*73, 74*)
Gonatidae			11.2/26.3	2.6/7.6	4.6/9.0 3.56/5.8	(*70, 73*)
Histioteuthidae	0.8/6.6 1.7/1.2 14.7/7.3 5.0/9.6	7.5/34.6 12.1/40 63.1/24.4 12.8/4.3	0.4/2.9 1.5/5.3 0.4/0.8 4.3/2.2	2.1/4.4 32.6/26.6	9.1/14.6 12.1/20.1 0.9/1.7 0.3/0.4 27.6/15.8 0.4/0.5 0.4/2.1 0.1/0.1 3.03/1.2	(*70–74*)

Table S6 – Continued

Family	CAN N/B	MED N/B	NA N/B	NEA N/B	ADR N/B	Source
Mastigoteuthidae	13.8/7.4		9.5/8.1		5.2/4.1	(*71*, *73*)
			0.3/0.8		19.0/11.2	
Octopoteuthidae	7.3/15.2	2.6/1.8	0.7/1.7		19.2/39.1	(*71–74*)
	0.3/7.1	11.3/5.8			1.6/2.9	
					0.6/1.8	
Ommastrephidae		6.2/36.2	0.3/5.7	2.2/7.8	1.0/13.9	(*70*, *73*,
		6.4/39.9			1.0/12.3	*74*)
Onychoteuthidae	1.3/7.0	1.4/0.2	0.2/1.8			(*71*, *73*)
Pholidoteuthidae	0.4/5.1		0.2/4.3		1.3/19.5	(*71*, *73*)
					0.2/2.7	
Sepiolidae		0.5	0.02		1/0.2	(*72–74*)
Stauroteuthidae					0.1	(*73*)
					0.1	
Vampyroteuthidae	0.08/0.01				0.3/0.4	(*71*, *73*)

Supplementary Table S7. Overview of bioinformatic analysis of eDNA samples taken off Terceira Island, Azores. Number of sequencing reads kept for CephMLS targeting the mitochondrial 16S rRNA gene and Ceph18S targeting the nuclear 18S rRNA gene cephalopod primer pairs after each bioinformatic analysis step.

Bioinformatic analysis step	Ceph18S		CephMLS:16S	
	N Reads	Sequence variants	N Reads	Sequence variants
Sequencing reads from MiSeq run	5409847		4462367	
Quality filtering and trimming	2076862		4174222	
Merging of paired-end reads	2002720		4081555	
Removing of chimeras	1912785	2300	4003315	977
Remove sequences below e < 1e-20		409		123
Removal of unassigned sequence variants		230		88
Removal of non-cephalopod SV		219		51

Supplementary Methods – eDNA Processing

Contamination reduction measurements

To prevent contamination, single-capped PCR-tubes, single-use consumables and disposable gloves were used and sampling devices, laboratory equipment and surfaces were cleaned with 50% bleach regularly. eDNA extractions, pre- and post-PCRs were physically separated.

DNA extractions

Each filter was cleaned with 50% bleach from the outside to remove any DNA contamination. 720 µl ATL-buffer and 80 µl proteinase K were added directly into the filter with 5 ml single-use syringes. The filters were incubated for at least 2 hours at 56 °C with agitation. After incubation, the buffer mix from inside the filter was transferred to a sterile 2 ml Eppendorf tube using 5 ml syringes. From each sample, 600 µl was pipetted to a new 2 ml Eppendorf tube to obtain equal volumes for all samples. After adding 600 µl AL-Buffer and 600 µl 99% ethanol high grade, the normal DNeasy Blood and Tissue protocol was used. DNA was diluted in 2x 25 µl AE-Buffer from the kit.

Metabarcoding PCRs - Controls, replicates and sequencing

For the CephMLS primer, tissue-derived DNA from three cephalopod species that do not occur in the central Atlantic: *Alloteuthis media, Pareledone* sp. and *Megaleledone setebos* and a mock community control including 3 ng/µl of each cephalopod species mentioned above have been used. The same were used for the Ceph18S primer, but after the first PCR it was noted that the positive controls did not work well with the Ceph18S primer pair according to a gel picture. Four extra positive controls were added for samples processed with the Ceph18S primer pair: *Bathyteuthis abyssicola*, *Mastigopsis hjorti* and *Histioteuthis reversa*, and a mock community control with these three species (~3 ng/µl per species).

These species do occur in the Azores area, but because they were added to the samples after the first PCR the possibility of contamination was minimal. Final concentrations of the positive controls were measured with a Qubit fluorometer and ranged between 10 – 19 ng/µl. Negative controls included filtration blanks with MilliQ, extraction blanks and PCR negative controls. In eight extraction blanks, one filtration blank and five PCR blanks (including replicates) from 30 blanks in total, contamination was found ranging from 11 to 16,188 reads. The positive controls worked on all plates, except on one plate processed with the primer CephMLS. This plate including all samples was excluded from further analysis. Of all detected taxa, 47% were found in PCR duplicate and 60% in biological replicates. This is consistent with other studies (75), showing that a large percentage of operational taxonomic units or sequence variants is only found in one of the biological replicates.

The two sequencing runs of all 313 samples (incl. positive controls, blanks and replicates) produced 5,409,847 and 4,462,367 input reads for Ceph18S and CephMLS, respectively. After DADA2 analysis, 1,912,785 and 4,003,315 output reads were obtained resulting in 2,327 and 978 sequence variants for Ceph18S and CephMLS, respectively. Sequences were only kept when they could be aligned to at least order level resulting in 241 and 54 sequence variants for Ceph18S and CephMLS, respectively, that were discarded. After discarding all sequence variants with less than ten reads, 282 assigned sequence variants were retained. The mean (SD) sequencing depth across samples was 3726.13+/- 16161.73.

Bioinformatic analysis with IDTAXA

The taxonomic assignment of the environmental samples and all controls against the training set was performed by IDTAXA with the R package DECIPHER version 2.6.0. The confidence threshold for accepting a classification was set to the default of 60% which is a very high

confidence and provides a conservative classification with relatively few misclassifications and over classifications. In addition to the IDTAXA algorithm, we applied the BLAST classifier which assigns a sequence based on its nearest neighbor in a training set. Sequences were searched against the NCBI nucleotide (nt) database (retrieved in June 2020) using the blastn program. Due to the reasons stated above, IDTAXA was used as baseline analysis. Differing taxonomic assignments made by BLAST resulting in higher taxonomic level classifications compared to IDTAXA where checked and added to the final results when they met the following criteria: a) 100% match of the whole query sequence against the reference sequence with no other species with 100% match: accepted at species level, b) 97-99% match of the query sequence against the reference sequence accepted at genus level, c) 95 – 97% match of the query sequence against the reference sequence: accepted at family level.

Several sequence variants occurring in one sample but assigning to the same species were combined and the total number of reads and the number of merged sequence variants were kept. Sequence variants with less than ten reads were removed to reduce the possibility of contamination or sequencing error. All species found in the negative controls were removed from the corresponding plates. That is, all taxa found in the PCR negative control were removed from the corresponding plate and taxa found in the filtration and extraction blanks were removed from the corresponding batch of samples that were filtered or extracted together with that control.

Supplementary Discussion – eDNA analysis

The need for replication in eDNA studies is related to the patchy distribution of eDNA and PCR stochasticity (*76*). False positives may originate from contamination (either from the field, lab or cross-contamination between samples or the positive controls) or from sequences being assigned to the wrong taxa. This may result from the limited number of sequences available in Genbank which are sometimes associated with a wrongly identified taxa. Alternatively, one gene may not be sufficient to differentiate between closely related species. False negatives may occur when species are known to occur in the region and are abundant prey in cetacean diets but were not detected by eDNA. Examples are *Taningia danae* and *Ancistrocheirus lesueurii* (*77*, *78*). The fact that these two species have not been found with eDNA in this study may be a consequence of an insufficient reference database, as *T. danae* and *A. lesueurii* sequences are not well represented in GenBank, but are known from sperm whales hunting off the Azores. Other possibilities for false negatives are patchiness of eDNA, insufficient primer specificity for certain taxa, PCR stochasticity and also species-specific eDNA shedding rates. *Taningia danae* is a very large squid (*78*) and one would expect enough eDNA to be present for detection. The same question arises for *Architeuthis*, the longest squid species on earth, which was not found frequently in our eDNA data. However, previous studies show that the size of an animal does not always reflect the amount of eDNA that is being shed (*79*). Our findings confirm that replication as well as positive and negative controls are indispensable for obtaining a representative number of species in a sampled water (*75*). Several factors such as weather conditions, temperature, behavior, body size, age, density, habitat use and skin texture together with varying oceanographic properties influence the production and degradation of eDNA. Therefore, detection rates with eDNA may be species-specific.

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