DPLUS168: Diet assessment of Falklands fur seals through DNA metabarcoding

COMPILED BY DR SÍLVIA PÉREZ-ESPONA

01/05/2025 VERSION 1.0

DPLUS168: DIET ASSESSMENT OF FALKLANDS FUR SEALS THROUGH DNA METABARCODING

VERSION CONTROL

VERSION	REVIEWED BY	DATE
1	Alastair Baylis	08 May 2025

This research was funded by the UK Government through Darwin Plus

For more information, please contact The South Atlantic Environmental Research Institute (SAERI) at: info@saeri.ac.fk or visit www.south-atlantic-research.org

Stanley Cottage North
Ross Road
Stanley
FIQQ 1ZZ
Falkland Islands
+500 27374

Falklands House
14 Broadway
London
SW1H 0BH
United Kingdom
+44 (0)203 745 1731

SAERI is a charity registered at the Charities Commission of England and Wales under charity number 1173105 and acknowledged on the Register of Charities in the Falkland Islands under number C47.

Cover photo © SAERI, 2023. Falkland Islands South American Fur seals.

DPLUS168: DIET ASSESSMENT OF FALKLANDS FUR SEALS THROUGH DNA METABARCODING

ABOUT THE SOUTH ATLANTIC ENVIRONMENTAL RESEARCH INSTITUTE (SAERI)

The South Atlantic Environmental Research Institute (SAERI) is an academic organisation conducting research in the South Atlantic from the tropics down to the ice in Antarctica. SAERI's remit includes the natural and physical sciences. It aims to conduct world class research, teach students, and build capacity within and between the United Kingdom's South Atlantic Overseas Territories. Its mission is to advance environmental understanding in the South Atlantic through research excellence and innovative scientific leadership. SAERI was a Falkland Islands Government initiative and operated as an arm's length government department from 2012 in July 2017.

Our vision is to be an internationally recognised academic institute with its main base in the Falkland Islands, operating in the South Atlantic from the equator down to the ice in Antarctica, conducting world class natural and physical science research, teaching students, and building capacity within and between the UK South Atlantic Overseas Territories.

Strategically, SAERI aims to be a world-class research institute that teaches students and builds capacity within and between the South Atlantic Overseas Territories. In order to achieve that it must be:

1. Project optimised – by operating as a streamlined and efficient organisation through the Focal Areas;

2. Fully funded – Falklands registered limited company is able to fund SAERI overheads, ensuring SAERI ultimately becomes fully financially independent from Falkland Islands Government and by ensuring that all grant applications (where possible) contain cost of seat coverage; and

3. The holder of proprietary environmental knowledge of the South Atlantic – by continuing to provide the research expertise offered to date.

TABLE OF CONTENTS

DPLUS168: DIET ASSESSMENT OF FALKLANDS FUR SEALS THROUGH DNA METABARCODING

1. PROJECT SUMMARY

This report summarises results obtained from a diet assessment of South American fur seals on Bird Island (Falkland Islands) through DNA metabarcoding of scat samples collected by SAERI from the 16th to the 21st of July 2023. After homogenisation of the samples, genomic DNA was extracted from all scats using a faecal DNA extraction kit. The genetic marker Zfx/Zfy was used to assign sex to all samples, with 37 samples determined as males and 12 females with confidence. Two DNA Cox1 barcodes designed to preferentially amplify krill and fish and squid, respectively, were tested using the SAFS extracted genomic DNA. The fish/squid Cox1 DNA barcode was successfully amplified in SAFS samples; however, no amplification for the krill Cox1 DNA barcode was observed with SAFS samples. Inspection of the scat samples indicated that crustacean was likely lobster krill. Therefore, it was decided to amplify an additional, more generic, DNA barcode within 18S rRNA as an attempt to amplify further targeted prey. Due to the low levels of amplification of the krill Cox1 DNA barcode, only amplicons obtained with the fish/squid DNA barcode and the 18S rRNA DNA barcode were sent for x300 bp paired-end Illumina Miseq sequencing.

Despite that the Cox1 primer pair was designed to preferentially amplify fish and squid DNA sequences, the amplicons also included pinniped sequences and other non-targeted taxonomic groups representing prey of prey, parasites, and bacteria and fungi from the environment. The amplification of non-targeted DNA sequences is a common challenge encountered in DNA metabarcoding projects. Some measures that could be implemented in future projects, such as the incorporation of synthetic nucleic acids blocking the amplification of host DNA or/and increasing the stringency of the PCR conditions. However, these protocol optimisations require considerable time and would not have been feasible within the time constraints of this project. However, the results from the sequencing provided very useful information about potential misidentification of scats in the field or potential labelling mistakes during sample collection, as two samples were identified as South American sea lion. The data also provide useful information about the levels of environmental contamination

during scat collection in terms of presence of DNA sequences from other pinniped species that would help inform future field sample collection protocols.

Nonetheless, the fish/squid Cox1 DNA barcode was very successful to identify eight fish taxa, seven of them at the species level, Magellanic rockcod (*Paranotothenia magellanica*), Marbled rockcod (*Nothenia rossii*), Southern blue whiting (*Micromesistius australis*), Butterfly kingfish (*Garterochisma malampus*), Pink cusk-eel (*Genypterus blacodes*), Falkland spratt (*Sprattus fuegensis*), and Broadnose skate (*Bathyraja brachyurops*) and one of them at the genus level as *Patagonotothen sp.,* as the same DNA sequence was found to be assigned to two different cods, Wilton's Patagonia rockcod (*Patagonotothen wiltoni*) and Longtail southern cod (*Patagonotothen ramsayi*). In addition to these fish taxa, two bird taxa were identified in the DNA sequences, one identified at the genus level *Leococarbo* sp, as the sequences were found to be 100% identical to sequences in databases that were labelled as Imperial shag (*Leucocarbo atriceps / Leucocarbo albiventer*), Antarctic shag (*L. brandsfieldensis*) and Crozet shag (*L. melanogenis*). The second bird taxon identified was at the species level, Striated caracara (*Phalcobenus australis*). Although the presence of DNA samples from these two bird species could potentially be due to environmental contamination, it would be worth inspecting the scat sample for which a high relative frequency of DNA sequences of Striated caracara was found in case there were any feathers in the sample.

Inspections of the relative percentage of sequences of fish taxa found in the SAFS scat samples indicated a predominance of Magellanic rockcod in the samples over the collection period. Initial comparisons of this data between males and females indicate a more diverse diet for males than females within the collection period; however, this data should be interpreted cautiously as the number of females analysed was smaller than males.

The analyses of sequencing results from the 18S rRNA barcode also indicated, as expected from a generic metazoan barcode, co-amplification of DNA sequences of pinniped but also other taxa (prey of prey, parasites, bacteria and fungi) alongside DNA sequences of targeted prey items. However, a large proportion of the sequences were targeted prey in comparison to the Cox1 DNA barcode. The 18S rRNA sequencing results proved complementary to the Cox1 DNA barcode as other targeted prey were identified, in particular Gregarious squat lobster (*Grimothea gregaria*), squid (Loligo sp.) and Antarctic krill (*Euphasia superba*); although the latter is more likely to have been prey of prey as only a reduced number of DNA sequences were found in one SAFS sample. In addition to Gregarious squat lobster and squid, fish DNA sequences belonging to different fish clades were identified and corresponded to the ones identified with the Cox1 DNA barcode; Percomorphaceae were Magellanic rockcod (*Paranotothenia magellanica*), Marbled rockcod (*Nothenia rossii*), Patagonian rockcod (*Patagonotothen* sp.), Butterfly kingfish (*Garterochisma malampus*) and Pink cusk-eel (*Genypterus blacodes*); Gadiformes was the Southern blue whiting (*Micromesistius australis*), Clupeiformes the Falkland spratt (*Sprattus fuegensis*), and Rajiformes the Broadnose skate (*Bathyraja brachyurops*).

Overall trends of the 18S rRNA indicated a predominant presence of DNA sequences of Gregarious squat lobster in the SAFS samples, with an apparent increase of this taxon during the last collection days. Comparison of trends between male and female samples suggested a predominance of Gregarious squat lobster in the diet across the collection period, but an increase of it in males during the last collection days. However, as indicated above, these comparisons should be interpreted cautiously until robust statistical analyses addressing differences in sample size are conducted.

2. DIET ASSESSMENT THROUGH DNA METABARCODING

2.1. SAMPLES AND PERMITS

A DNA metabarcoding approach was taken to assess diet in 95 scat samples of South American fur seal (*Arctocephalus australis*) collected between 16th -20th October 2023 on Bird Island (Falkland Islands) under the Research Licence No: R09/223. Following collection, the scat samples were stored at -20°C and shipped in dry ice to The Roslin Institute (Edinburgh, Scotland) under the export permit from the Falkland Islands Government R09/223 and import permit from the Animal Plant Health Agency (APHA) from the UK, ITIMP24.0459. Ethical approval for the research was obtained from the Veterinary Ethical Review Committee at The Royal (Dick) School of Veterinary Studies – R(D)SVS, VERC Reference: 59.23. A biological agents and materials risk assessment was also conducted for this research at The Roslin Institute, local reference number BA 0223.V2. All samples were stored in a 20°C walk-in freezer upon arrival.

2.2. SAMPLE PREPARATION

Subsets of working samples were thawed in a flow hood cabinet and homogenised manually within the individual sampling plastic zip lock bag to avoid any contamination (Figure 1).

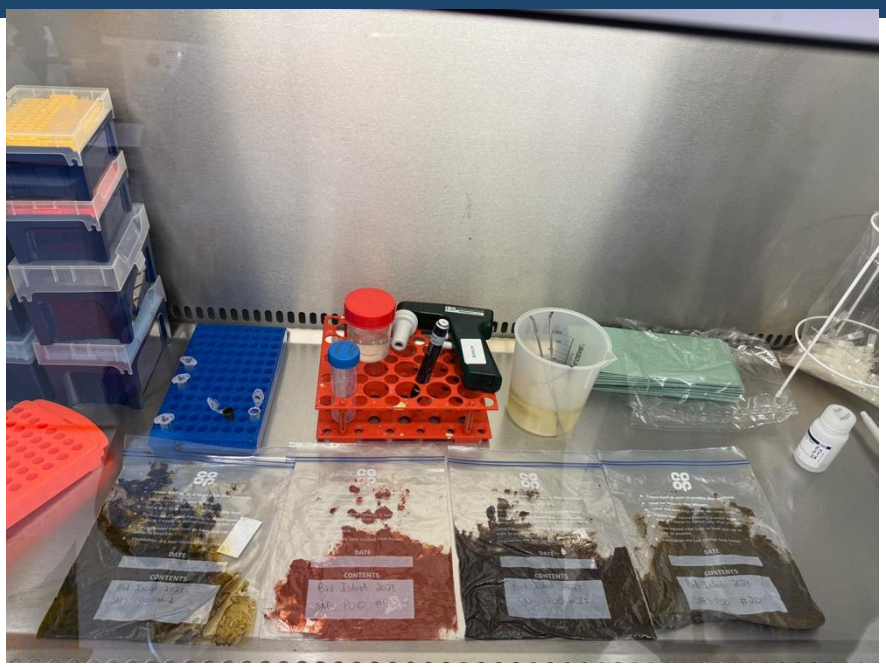


***Figure 1**. Photograph illustrating homogenised scat samples when subsampling for genomic DNA extraction.*

2.3. GENOMIC DNA EXTRACTION

Genomic DNA was extracted using the Quick-DNA Fecal/Soil Microbe Microprep kit (Zymo Research) following the manufacturer's instructions. Approximately 50 mg from the homogenate was used for the DNA extraction of each sample, and DNA was eluted in a total of 40 µL. A subset of the first few samples extracted was run in a 1% agarose gel to check that the overall procedure was successful. A total of 5µL of PCR product was combined with 3µL of diluted TriTrack DNA Loading Dye (6X) and loaded into the wells for electrophoresis. Genomic DNA visual quantification was conducted through comparison to a 1Kb Hyperladder (Meridian). The gel was run initially at 50V for a few minutes until the product left the well, and then at 90V for 45 minutes. This test indicated that genomic DNA extractions from the scats were successful using the established protocol, and although the amount of DNA varied between the tested samples, quality DNA was obtained in sufficient amounts to conduct PCR (Figure 2).

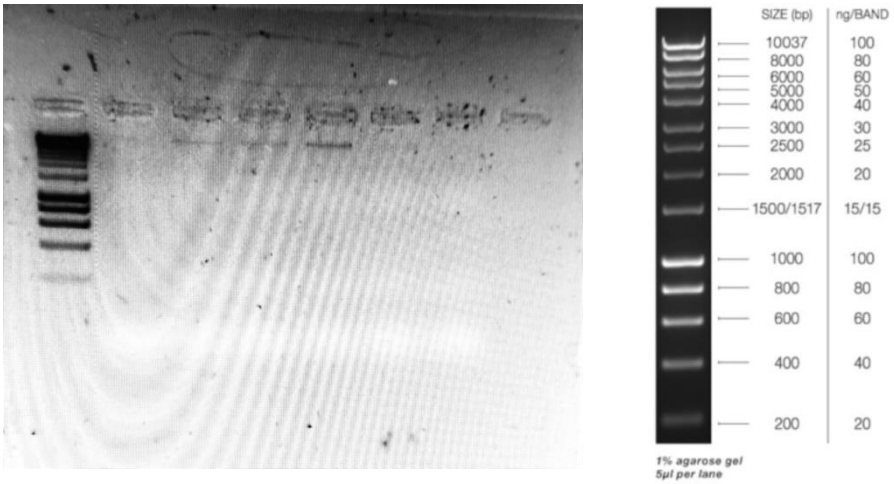


***Figure 2**. Agarose gel illustrating genomic DNA extracted from South America fur seal scat samples. Hyperlader 1Kb was used as standard in this gel. 5uL of genomic DNA loaded in the gel.*

2.4. GENETIC SEXING OF SAMPLES

Genetic sexing of all samples was conducted by amplifying an intron in the Zinc finger gene of the X and Y chromosomes, Zfx/Zfy marker. Amplification of these introns was conducted via Polymerase Chain Reaction (PCR) using the primers LGL331 and LGL335 as these have been previously successfully used in seals. The PCRs were conducted in a total volume of 15uL, with 7.5uL of Meridian MyTaq HS Mix, 0.3µL of each primer (at a concentration of 0.2 µM in the reaction), 1.5uL of DNA (1-25ng/µL) and 5.4 µL of nuclease-free water. PCR cycling conditions were as follows: Initial denaturation at 95°C for 1 min, 38 cycles of denaturation at 95°C for 20 sec, annealing at 55°C for 20s, extension at to 72 °C for 1 min (with ramp from annealing to extension set at 0.3°C/second), and a final extension at to 72 °C for 2 min.

Visualisation of the PCR products was conducted using a 2% agarose gel with sizing of PCR products through comparison to a 1Kb Hyperladder (Meridian). A total of 5uL of PCR product was combined with 3uL of diluted TriTrack DNA Loading Dye (6X) and loaded into the wells for electrophoresis. Gels were run initially at 50 V for a few minutes until the product left the well and then 90V until clear separation of bands of the X and Y introns was achieved (generally more than 1 hour). Samples presenting only one band slightly below the 1,000bp target size were scored as females (due to the introns in both sex chromosomes being of the same size) and samples presenting two bands at expected sizes between the 800-1,000bp

target sizes were scored as males (due to introns in the X and Y chromosomes differing on size, with the Y chromosome intron being of smaller size). See Figure 3 for an example of genetic sexing of SAFS samples. For those with initial unsuccessful amplification or that presented unclear banding patterns, amplifications and gel electrophoresis were repeated.

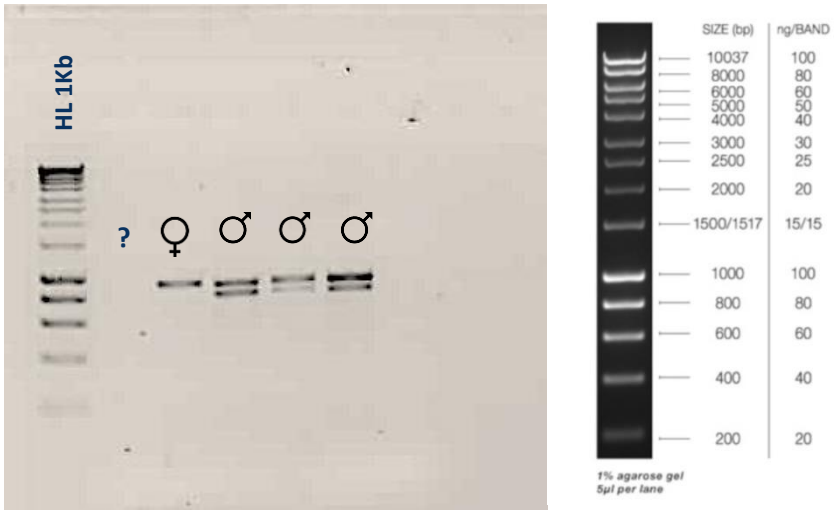


***Figure 3**: Photograph illustrating SAFS samples sexed with confidence using the Zfx/Zfy marker.*

2.5. DIET ASSESSMENT

Identification of prey items as a diet assessment of the South American fur seal samples was conducted through DNA metabarcoding using the mitochondrial Cytochrome Oxidase subunit 1 (Cox1) and the ribosomal RNA 18S (18S rRNA) molecular markers. Two sets of novel and unpublished primers were used to amplify Cox1 fragments, one set of primers targeting krill (Cox1KrF/Cox1KrR) and the other set targeting fish and squid (Cox1FishCephF/Cox1FishCephR). The 18S rRNA v8-v9 fragment was amplified with the already available universal primers 18S 4F/ER. All the primer sets used also included adaptor sequences for individual barcoding and Illumina pair end sequencing (MiSeq , 2 x 300 bp). Amplification of the three DNA barcode markers was conducted in separate triplicate PCRs to minimise effects of PCR stochasticity on prey barcode amplification.

A small subset of SAFS samples was initially used for amplification optimisation of the three DNA barcodes (Figure 4 and 5). A scat sample of Antarctic fur seal (*Arctocephalus gazella*) that was successfully amplified in a previous experiment was used as a positive control. Nuclease-free water (QIAGEN) was used as negative control. After optimisation of primer concentrations, annealing temperatures and cycling conditions, final PCRs were conducted in a total volume of 15µL, with 7.5µL of Meridian MyTaq HS Mix, with 0.45 µL of each primer (at a concentration of 0.3µM in the reaction), 1.5uL of DNA (1-25ng/uL) and 1.5 uL of QIAGEN nuclease-free water. PCR cycling conditions were as follows: Initial denaturation at 95°C for 1 min, denaturation at 95°C for 20 sec, annealing at 48°C for the krill primer set, 52°C for the fish/squid primer set, and 56°C for the 18S rRNA primer set, extension was at to 72 °C for 1 min (with ramp from annealing to extension set at 0.3°C/second). The number of cycles denaturation-annealing-extension varied between the markers, with 42 cycles for the krill primer set, 40 cycles for the fish/squid primer set and 30 cycles for the 18rRNA.

Evaluation of amplification success and approximate sizing of products was conducted through gel electrophoresis in a 2% agarose gel. A total of 5µL of PCR product was combined with 3µL of diluted TriTrack DNA Loading Dye (6X) and loaded into the wells for electrophoresis. Sizing of PCR products were conducted through comparison to a 1Kb Hyperladder (Meridian). Gels were run initially at 50 V for a few minutes until the product left the well and then 90V for 1 hour.

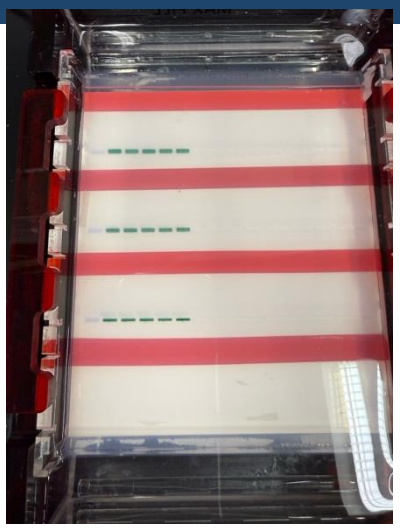


Figure 4. *Photograph of samples loaded for gel electrophoresis during PCR optimisation tests.*

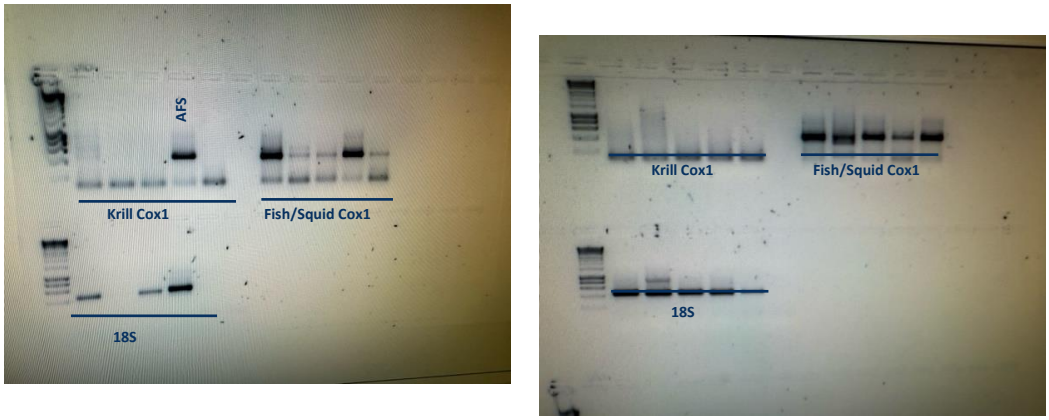


Figure 5. *Gel electrophoresis illustrating amplification of the three DNA barcodes in SAFS samples. Left - Gel including an Antarctic fur seal sample (AFS) with successful amplification with the krill Cox1 DNA barcode. Right - Gel illustrating amplification of the three DNA barcodes in further SAFS samples illustrating that amplification of the krill Cox1 DNA barcode was not successful even after PCR optimisation.*

Three SAFS samples with successful amplification of the three DNA barcodes were selected for a sequencing test to confirm that prey items were amplified with our protocol. In addition, a sample of Antarctic fur seal (*Arctocephalus gazella*) for which a strong amplification of the krill DNA barcode was obtained was also sent for sequencing. After visual evaluation of the PCR products across the four samples, PCR products were pooled to achieve approximate equimolarity and purified using ExoSAP-IT™ (ThermoFisher) following the manufacturer's instructions. After purification, an aliquot of c. 28µL of each pooled sample was sent for outsourced MiSeq sequencing (Figure 6).

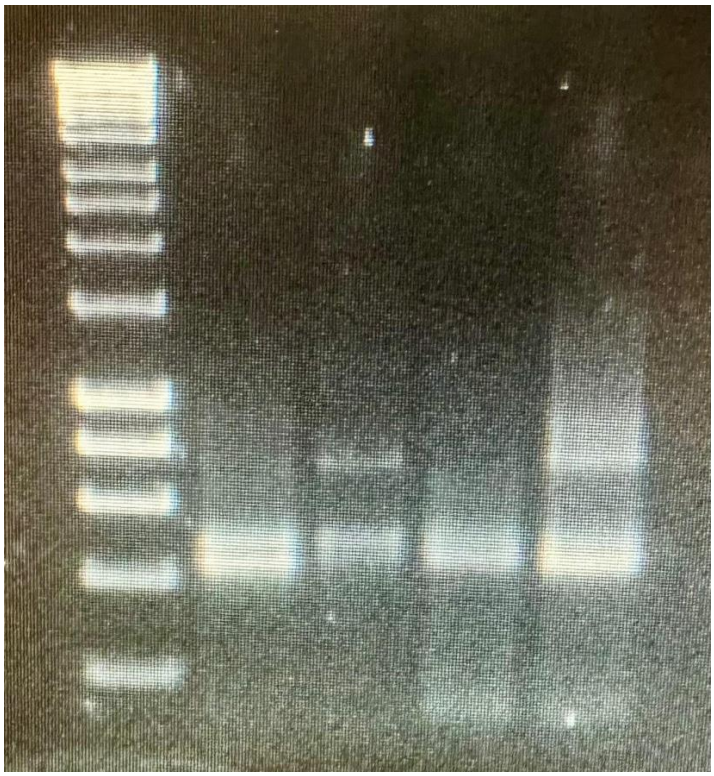


***Figure 6**. Gel electrophoresis illustrating pooled PCR products of the three DNA barcodes of the 4 samples test.*

Genomic DNA extractions and amplification of the three DNA barcodes in the remainder of the samples was conducted following the protocol described above. For samples for which amplification of the barcodes was not optimal, further triplicate PCRs were conducted in an attempt to obtain enough PCR product for sequencing (see Results section).

2.6. BIOINFORMATICS

Preliminary diet assessment analyses were conducted using the demultiplexed sequences for each sample provided by the outsourcing company. Barcode primers were trimmed from all the sequences for each sample using the Cutadapt tool in Python 3.5.5. Sequence quality filtering, amplicon sequence variant (ASV) inference and taxon assignment was conducted using the DADA2 package in the R statistical environment. The quality filtering applied removed all sequences with ambiguous bases (trncQ=11), >2 expected errors and lengths <100 bp. Error rates were estimated for the forward and reverse sequences obtained from

each individual and sequence variants were inferred before merging sequences with a minimum overlap of 30 bp. Putative chimeras were removed using the consensus method. Taxonomies were assigned to the resulting amplicon sequence variants using the RDP classifier, with a general eukaryote 18S rRNA reference sequence database from SILVA, MIDORI and a selection of anticipated seal and prey sequences obtained from BOLD Systems. ASVs that were abundant but lacked taxonomic assignments were further, individually, investigated using BLAST to determine if a taxonomy could be assigned using the most up-to-date records held on the NCBI-GenBank database. The taxonomy assignments and ASV sequence read counts were imported into Microsoft Excel for a preliminary assessment of the relative proportions of prey items in addition to assessing the host species identity of the samples. After determining which prey species were present in the sample, the next data analyses focused on thoroughly interrogating the original sequence data through directly targeted prey item sequence mapping (counting of sequences of a particular prey) using the Geneious software package. This approach involves less data pre-processing and allows for more realistic taxonomy assignments than the default DADA2 method described above. Therefore, it will provide a more complete assessment of the proportions and identities of the prey item DNA sequences.

3. RESULTS

3.1. GENETIC SEXING

Amplification of the Zfx/Zfy marker was successful for genetic sexing of scats from SAFS despite the large intron fragments (800-100bp) to be amplified from the scat genomic DNA extractions. However, due to variation in the amount of genomic DNA extracted from samples and potentially also degradation effects, amplification of the genetic sex marker varied between samples (Figure 7).

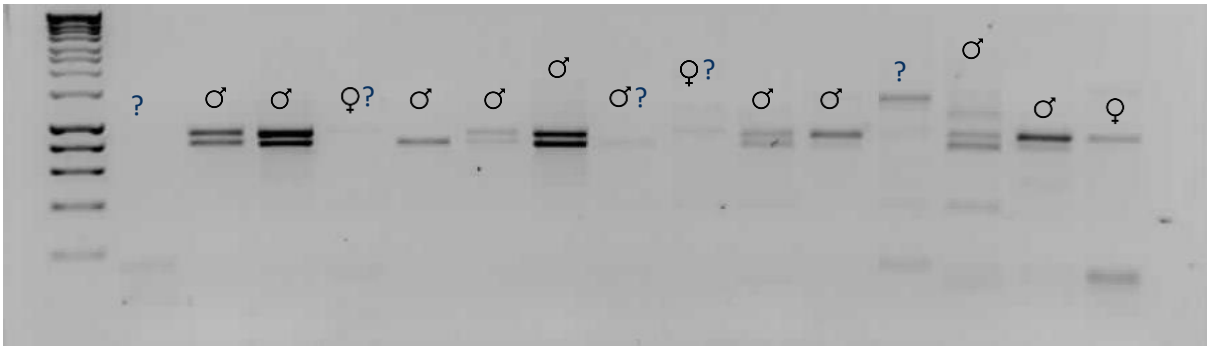


Figure 7. Gel electrophoresis photograph illustrating amplification success variation between samples, with some not being able to be genetically sexed with confidence (indicated by sex symbol and question mark).

In total, 49 out of the 95 SAFS samples were confidently sexed using the Zfx/Zfy marker, 37 males and 12 females. In addition, 4 samples were assigned as possibly males and 2 samples as possibly males; the remaining 40 samples could not be genetically sexed even after repeating the Zfx/Zfy amplification (see Table 1). A further attempt to genetically sex these samples will be conducted by varying the amplification conditions and failing that, by re-extracting the DNA if the same sample also failed to optimally amplify the DNA barcodes for the diet assessment.

Table 1. *Genetic sexing of 96 SAFS samples using the genetic sex marker Zfx/Zfy, M (males), F (females), M? (possibly male), F? (possibly F), ? (not clear from amplification or failed to amplify).*

Sample name	Collection date	Sex
SAFS_POO_01	16/10/2023	?
SAFS_POO_02	16/10/2023	M
SAFS_POO_03	16/10/2023	?
SAFS_POO_04	16/10/2023	?
SAFS_POO_05	16/10/2023	F
SAFS_POO_06	16/10/2023	?
SAFS_POO_07	16/10/2023	M
SAFS_POO_08	16/10/2023	?
SAFS_POO_09	16/10/2023	?
SAFS_POO_10	16/10/2023	F
SAFS_POO_11	16/10/2023	?
SAFS_POO_12	16/10/2023	?
SAFS_POO_13	16/10/2023	M
SAFS_POO_14	16/10/2023	F
SAFS_POO_15	16/10/2023	M
SAFS_POO_16	16/10/2023	M
SAFS_POO_17	16/10/2023	?
SAFS_POO_18	16/10/2023	M
SAFS_POO_19	16/10/2023	?
SAFS_POO_20	17/10/2023	F
SAFS_POO_21	17/10/2023	M
Sample name	**Collection date**	**Sex**
SAFS_POO_22	17/10/2023	M
SAFS_POO_23	17/10/2023	M
SAFS_POO_24	17/10/2023	M
SAFS_POO_25	17/10/2023	M
SAFS_POO_26	17/10/2023	M
SAFS_POO_27	17/10/2023	?
SAFS_POO_28	17/10/2023	F?
SAFS_POO_29	17/10/2023	F
SAFS_POO_30	17/10/2023	?
SAFS_POO_31	17/10/2023	?
SAFS_POO_32	17/10/2023	M
SAFS_POO_33	17/10/2023	M
SAFS_POO_34	17/10/2023	M
SAFS_POO_35	17/10/2023	?
SAFS_POO_36	17/10/2023	?
SAFS_POO_37	17/10/2023	?
SAFS_POO_38	17/10/2023	F
SAFS_POO_39	17/10/2023	F
SAFS_POO_40	18/10/2023	?
SAFS_POO_41	18/10/2023	F
SAFS_POO_42	18/10/2023	?
Sample name	**Collection date**	**Sex**
SAFS_POO_43	18/10/2023	?
SAFS_POO_44	18/10/2023	M
SAFS_POO_45	18/10/2023	M
SAFS_POO_46	18/10/2023	M
SAFS_POO_47	18/10/2023	?

SAFS_POO_48	18/10/2023	?
SAFS_POO_49	18/10/2023	?
SAFS_POO_50	18/10/2023	F
SAFS_POO_51	18/10/2023	M
SAFS_POO_52	18/10/2023	F
SAFS_POO_53	18/10/2023	F
SAFS_POO_54	18/10/2023	F
SAFS_POO_55	18/10/2023	?
Sample name	**Collection date**	**Sex**
SAFS_POO_56	18/10/2023	?
SAFS_POO_57	18/10/2023	M

SAFS_POO_58	18/10/2023	M
SAFS_POO_59	18/10/2023	?
SAFS_POO_60	19/10/2023	?
SAFS_POO_61	19/10/2023	?
SAFS_POO_62	19/10/2023	?
SAFS_POO_63	19/10/2023	?
SAFS_POO_64	19/10/2023	?
SAFS_POO_65	19/10/2023	?
SAFS_POO_66	19/10/2023	M
SAFS_POO_67	19/10/2023	M
SAFS_POO_68	19/10/2023	?

SAFS_POO_69	19/10/2023	M
SAFS_POO_70	19/10/2023	M
SAFS_POO_71	19/10/2023	F
SAFS_POO_72	19/10/2023	?
SAFS_POO_73	19/10/2023	M
SAFS_POO_74	19/10/2023	M
SAFS_POO_75	19/10/2023	?
SAFS_POO_76	19/10/2023	?
SAFS_POO_77	19/10/2023	M
SAFS_POO_78	19/10/2023	?
SAFS_POO_79	19/10/2023	M
SAFS_POO_80	20/10/2023	M
SAFS_POO_81	20/10/2023	F?
SAFS_POO_82	20/10/2023	M?
SAFS_POO_83	20/10/2023	M
SAFS_POO_84	20/10/2023	M
SAFS_POO_85	20/10/2023	M?
SAFS_POO_86	20/10/2023	F?
SAFS_POO_87	20/10/2023	M
SAFS_POO_88	20/10/2023	M
SAFS_POO_89	20/10/2023	F?
SAFS_POO_90	20/10/2023	M
SAFS_POO_91	20/10/2023	M
SAFS_POO_92	20/10/2023	F
SAFS_POO_93	20/10/2023	M
SAFS_POO_94	20/10/2023	?
SAFS_POO_95	20/10/2023	?

3.2. DIET ASSESSMENT THROUGH DNA METABARCODING

DNA barcodes amplification

Amplification of the DNA barcodes (Cox1 and 18S rRNA) across the 95 samples indicated that fragments of the expected size for each marker were obtained, albeit with instances of multiple fragment sizes and low amplification or unsuccessful amplification for some samples (Figures 8-10).

The krill Cox 1 DNA barcode amplification was largely unsuccessful in SAFS samples, in comparison to a previous experiment with scat samples from Antarctic fur seals. Only a reduced number of SAFS samples presented a krill DNA barcode PCR product of the expected target size (Figure 8). SAFS scat samples with red colouration that could indicate potential ingestion of krill but that did not provide a successful amplification with the krill Cox1 barcode were further visually inspected for remaining hard part features that could shed some light on the identity of the source of the red colouration if it was not krill. These inspections indicated that hard parts in those samples presented cephalothorax morphological features not consistent with krill. Repeats for some of the samples were conducted to exclude any potential problems with the krill Cox1 DNA barcode amplifications. The repeats also led to unsuccessful amplifications, except for those individuals that presented a band of the right size in previous experiments. Therefore, due to the low success rate of amplification of the krill Cox1 DNA barcode in the SAFS samples and visual inspections of the scats samples indicating that the majority of visible hard parts might not be krill, it was decided not to send the krill Cox 1 PCR products for sequencing as the multiple bands found in some samples and the large amount of primer dimer of the unsuccessful PCRs would have negatively interfered with the sequencing of the other DNA barcodes. Furthermore, as we were sequencing all SAFS samples with the 18S rRNA, potential krill presence in the SAFS scat samples, albeit in small amount, could also be detected with this DNA barcode.

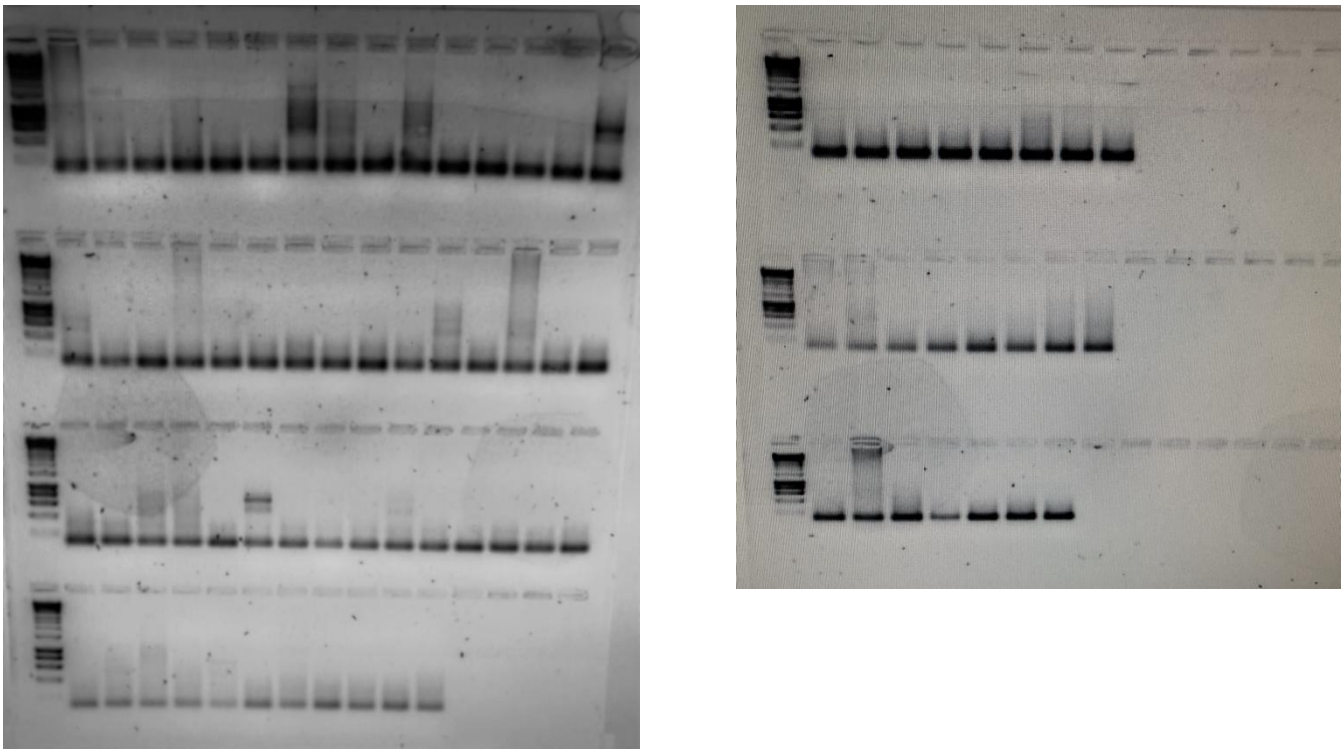


Figure 8. *Gel electrophoresis illustrating amplification of the krill Cox1 DNA barcode (triplicates pooled) in the remaining SAFS samples (i.e., samples not used in previous tests and for which PCR products were already available).*

Amplification of the Fish/squid Cox1 barcode was largely successful in the SAFS samples (Figure 9). However, some samples presented a faint band for the targeted fragment, of similar or lower strength to the primer dimer band. Therefore, amplification for all these samples was repeated to obtain optimal amounts of PCR products for MiSeq sequencing.

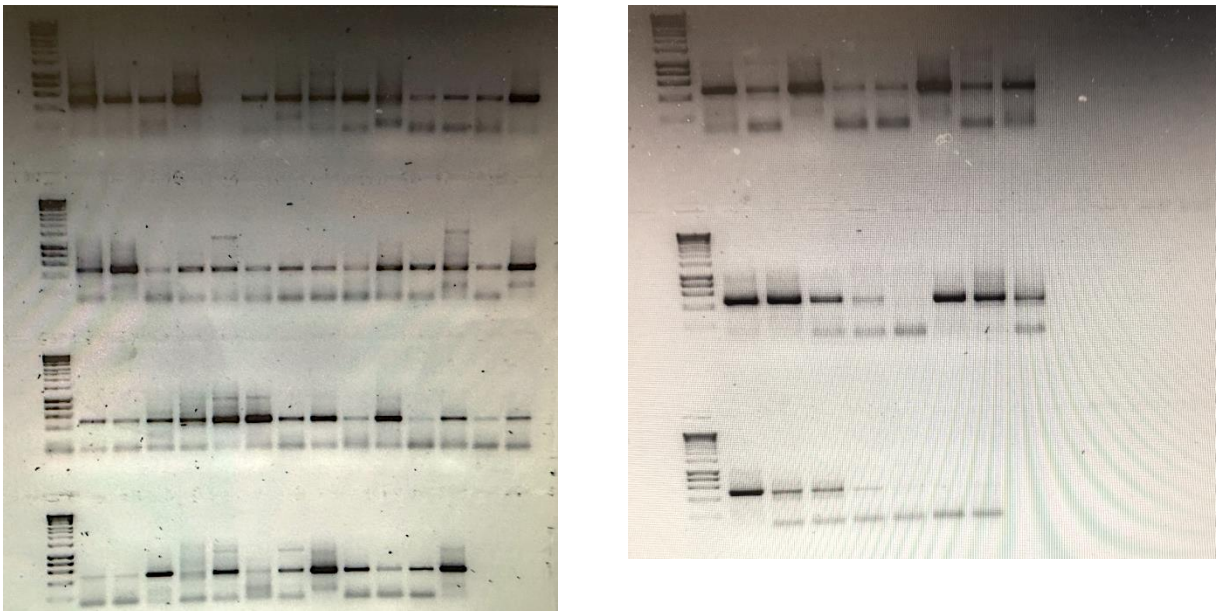


Figure 9. *Gel electrophoresis illustrating amplification of the fish/squid Cox1 DNA barcode (triplicates pooled) in the remaining SAFS samples (i.e., samples not used in previous tests and for which PCR products were already available).*

Amplification of the 18S rRNA DNA barcode was also largely successful in some SAFS samples (Figure 10). Similar to the Fish/squid Cox1 DNA barcode, some samples presented faint bands for the targeted fragment and, therefore, amplification was repeated to achieve optimal amount of PCR product for MiSeq sequencing.

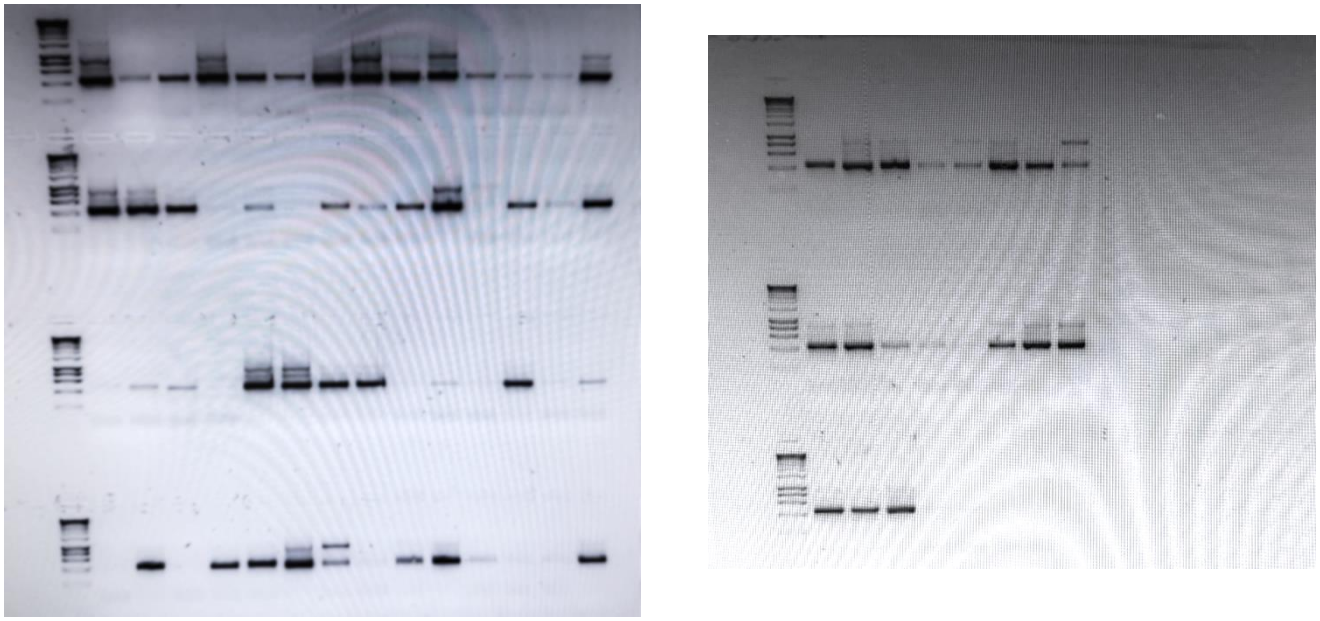


Figure 10. *Gel electrophoresis illustrating amplification of the 18S rRNA DNA barcode (triplicates pooled) in the remaining SAFS samples (i.e., samples not used in previous tests and for which PCR products were already available).*

After conducting all the repeats, a total of 73 samples were considered to contain strong enough PCR product bands for the Fish/squid Cox1 and 18S rRNA DNA barcodes and, hence, were prepared to be sent for MiSeq sequencing. Visual quantification through examination of the electrophoresis gels was conducted to inform pooling ratios of the resulting DNA barcodes to achieve approximate equamolarity. Purification of PCR products was conducted with ExoSAP-IT™ following the manufacturer's instructions. and a total of 28µL for each of the 73 samples was sent for outsourced MiSeq sequencing (Figure 11).

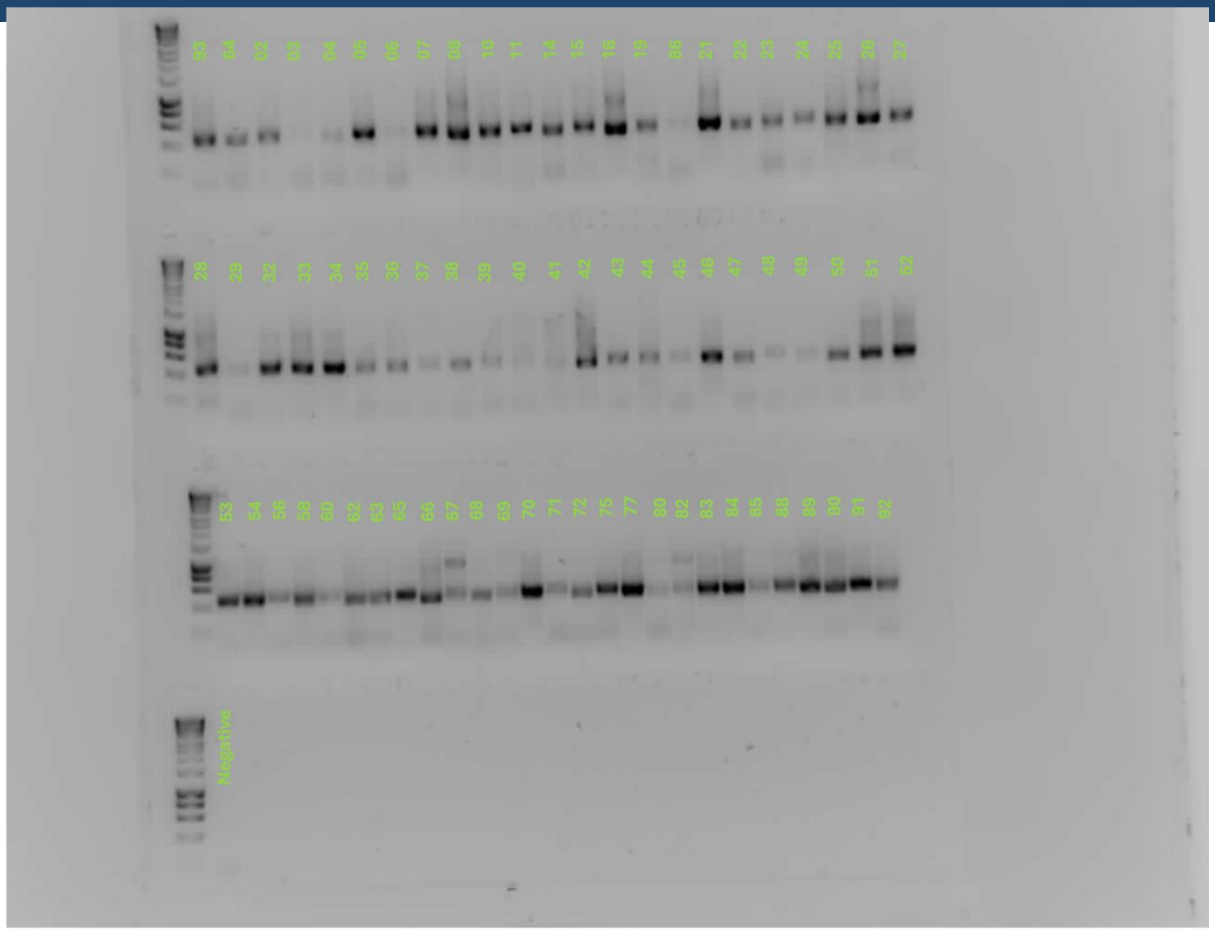


Figure 11. *Gel electrophoresis of the pooled PCR products from the Fish/squid Cox1 and 18S rRNA DNA barcodes sent for MiSeq sequencing, numbers relate to the sample code (e.g., 93 is SAFS_POO_93).*

Three samples (SAFS POO 29, SAFS POO 40, SAFS POO 80) could not be sequenced and, hence, will be sent to sequence again with samples for which we did not obtain sufficient amplification of the DNA barcodes. Therefore, this report presents data for 71 samples out of the 95 collected samples. Genomic DNA for the samples that did not provide sufficient quality DNA for the amplification of the fish/squid Cox1 and 18S markers will be re-extracted and repeated to try to complete the data set.

3.3. DNA BARCODE SEQUENCE DATA

Cox1 DNA barcode

A total of 7,271,820 Cox1 sequences were obtained for the 71 successfully sequenced samples, with the of sequences per sample ranging from 32,396 (SAFS_POO_62) to 287,238 (SAFS_POO_45) and an average number of sequences per sample of 102,420. Although the Cox1 DNA barcode primer set theoretically was designed to target fish and squid species, sequence analyses revealed that the primer set also amplified other taxa, including mammal DNA, prey of prey, parasites, and environmental fungi and bacteria (Figure 12). This amplification of non-targeted DNA is common in DNA metabarcoding analyses, in particular when using scats as a source, as less stringent PCR conditions are required for DNA amplification of the mix sample. Although synthetic Peptide Nucleic Acids (PNAs) can be incorporated in the PCRs to act as clamps and reduce co-amplification of host DNA, this protocol modification would have required significant additional time for optimisation in the SAFS samples. Furthermore, as an initial DNA metabarcoding for SAFS, the sequencing of pinniped DNA provided further information about host provenance of samples and valuable information about potential host parasites and other organisms found in the sampling location.

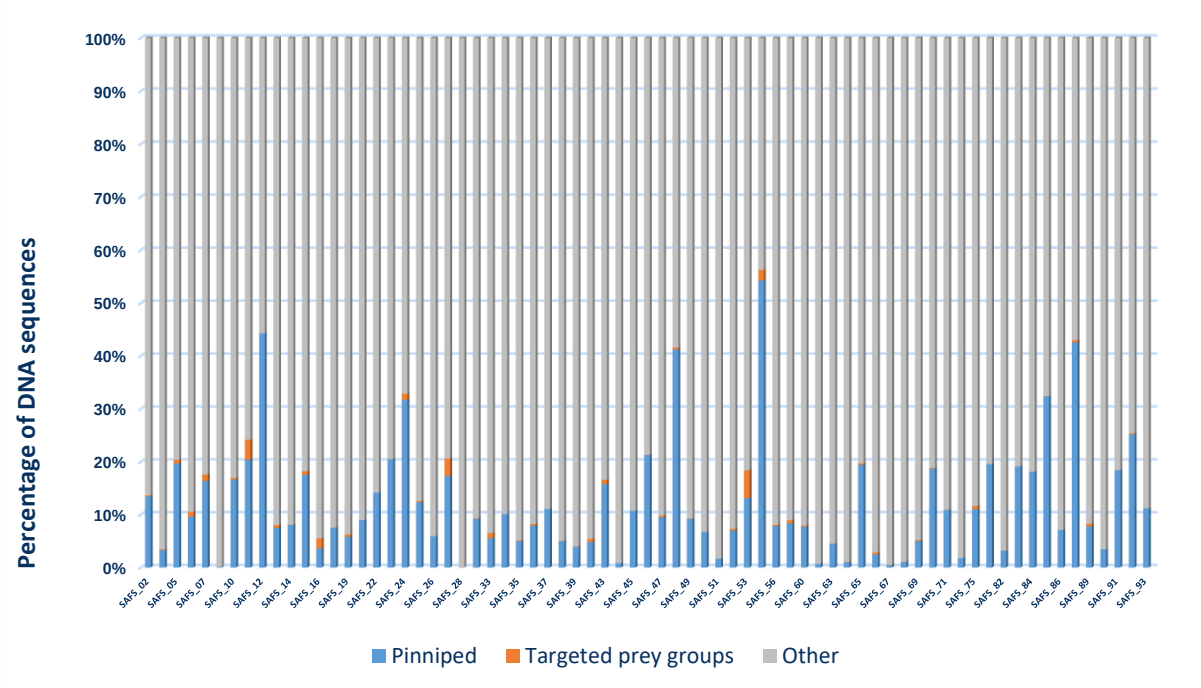


***Figure 12**. Percentage of DNA sequences identified as pinniped, targeted prey groups and other taxonomic groups.*

Pinniped DNA sequences obtained in higher percentages were assumed to confirm host species, 65 scat samples were assigned as SAFS; however, two samples, SAFS POO 15 and SAFS POO 22, contained a higher frequency of DNA sequences characteristic of South American sea lion (*Otaria byronia*) – SASL (Figure 13). The presence of SASL DNA sequences in the sample of SAFS POO 22 can be attributed to a misidentification of the scat during the field collection or potential error when labelling the sampling bag. For SAFS POO 15, the relative frequency of SASL DNA sequences was smaller than for SAFS POO 22 but still over 60% of the pinniped DNA sequences found in that sample; therefore, indicating a potential mix of scats from both species. A third sample, SAFS POO 28 presented over 20% of pinniped sequences characteristic of SASL, potentially indicating also a mix of sample or strong environmental contamination. As indicated in Figure 13, SASL DNA sequences were also identified in additional samples, but the relative frequency was of < 10% so these could be considered potential environmental contamination due to shared spaces between species.

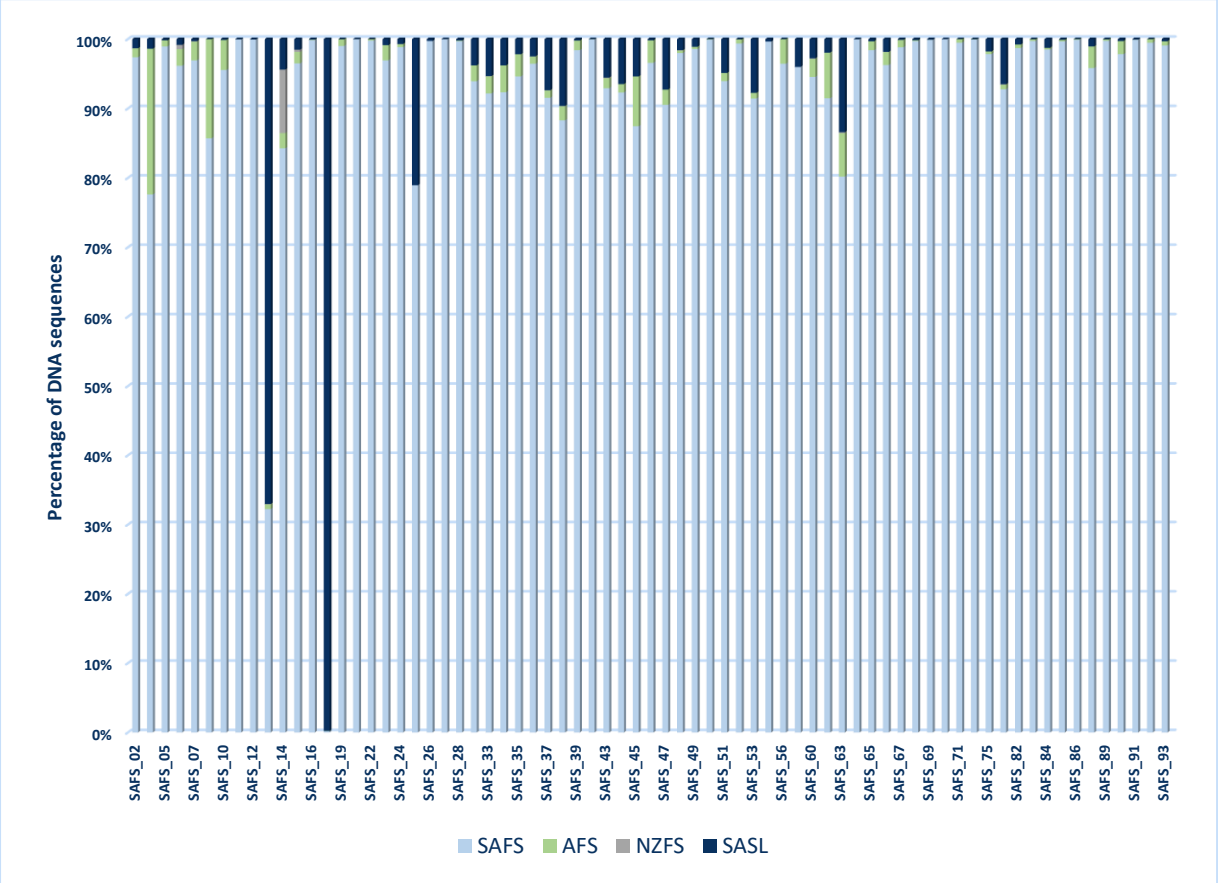


Figure 13. *Percentage of DNA sequences from pinniped assigned to each taxa, SAFS (South American sea lion), AFS (Antarctic fur seal), NZFS (New Zealand fur seal) and SASL (South America sea lion).*

DNA sequences from a third seal species, Antarctic fur seal (*Arctocephallus gazella*) - AFS, were also identified in a number of samples, which could be also interpreted as environmental contamination (Figure 13). However, in two samples, SAFS POO 04 and SAFS POO 08, the frequency of AFS DNA sequences was > 10%. Considering that the AFS pups in Bird Island are born in mid-November, potential predation of AFS pups by SAFS adults could be discarded but these samples will be further inspected for presence of AFS hairs, in case the presence of DNA sequences could be due to contamination of the samples due to moulting of AFS. DNA from a fourth seal species, New Zealand fur seal (*Arctocephallus forsteri*) – NZFS, was identified in 1 sample (SAFS POO 16). The identification of NZFS DNA in a SAFS sample is intriguing, as Bird Island is outside the reported distribution of this species.

Analyses of the DNA sequences for targeted prey taxonomic groups indicated assignment of DNA sequences to eight different fish taxa (Figure 14). For seven of those taxa, identification was to the species level: Magellanic rockcod (*Paranotothenia magellanica*), Marbled rockcod (*Nothenia rossii*), Southern blue whiting (*Micromesistius australis*), Butterfly kingfish (*Garterochisma malampus*), Pink cusk-eel (*Genypterus blacodes*), Falkland spratt (*Sprattus fuegensis*), and Broadnose skate (*Bathyraja brachyurops*). For the eight taxa, the identified sequence variant matched 100% to Wilton's Patagonia rockcod (*Patagonotothen wiltoni*) and Longtail southern cod (*Patagonotothen ramsayi*); therefore, the identification was annotated as *Patagonotothen sp.*

No other targeted prey taxonomic groups were identified with the Cox1 primers; however, in a few samples it was identified the presence of DNA sequences that could be assigned to *Leococarbo* sp, as the sequences were found to be 100% identical to sequences in databases that were labelled as Imperial shag (*Leucocarbo atriceps / Leucocarbo albiventer*), Antarctic shag (*L. brandsfieldensis*) and Crozet shag (*L. melanogenis*). It is important to note that the DNA sequence that match with the above taxa it was only one 1bp different to the South Georgia shag (*Leucocarbo georgianus*). Furthermore, another bird species was identified in one sample (SAFS POO 46) for which DNA sequences were identified as Striated caracara (*Phalcobenus australis*). The identification of *Leucocarbo* sp. could be explained as environmental contamination although the relative proportion of *Leucocarbo* sp. in some samples was > 10% (e.g., SAFS POO 21, 42 and 43). The presence of Striated caracara DNA sequences in sample SAFS POO 46 is more difficult to be attributed to environmental contamination as it was the only taxa identified in that sample besides DNA sequences of pinniped and other taxa (prey of prey, bacteria, fungi, parasites).

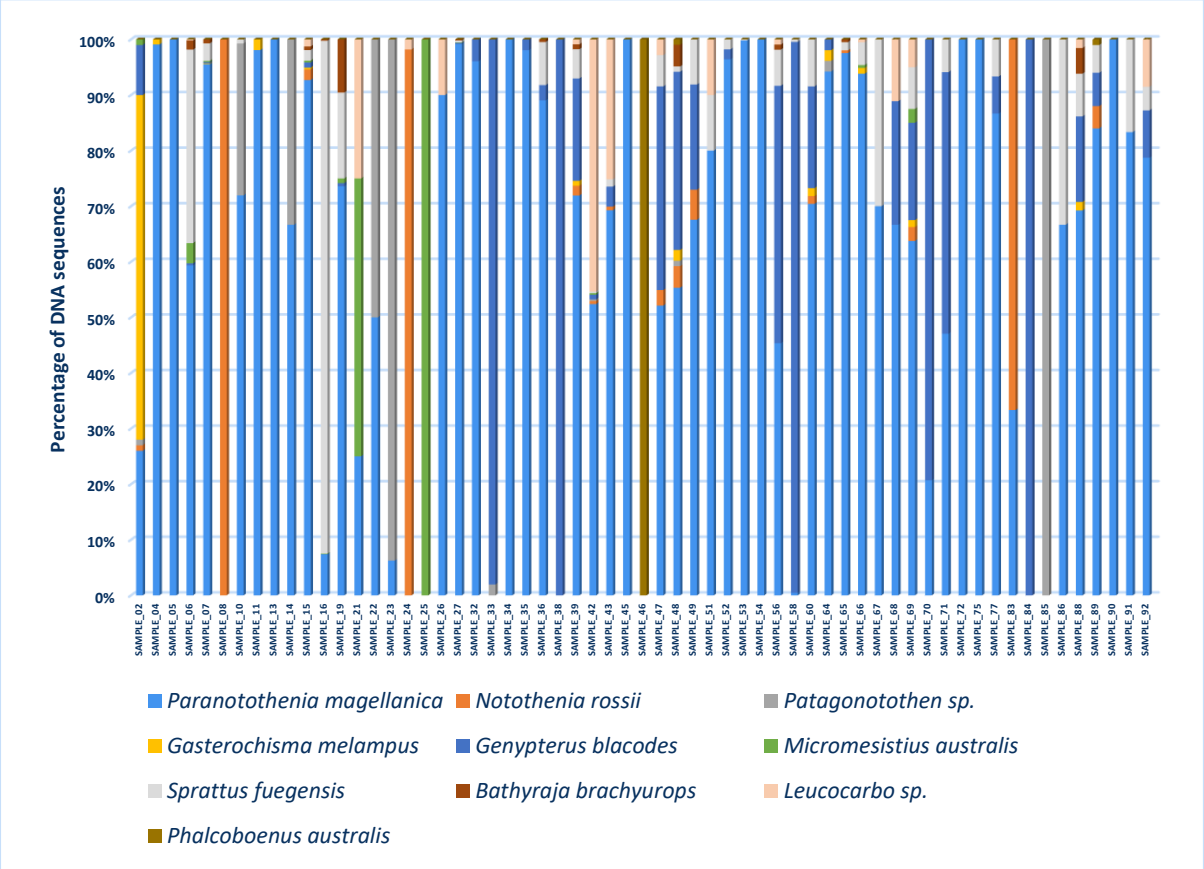


Figure 14. *Relative percentage of DNA sequences to the different identified fish and bird taxa using the Cox1 DNA barcode, after excluding the number of DNA sequences identified as pinniped or other taxa such as prey of prey, bacteria, fungi and parasites.*

Relative percentages of fish taxa DNA sequences for SAFS samples (i.e., excluding samples SAFS POO 15 AND SAFS POO 22, both identified as SASL males) we also assessed by grouping samples according to the identified sex (Figure 15). Visual inspection of the plots would indicate an apparent more diverse diet in males than females; however, this data should be interpreted cautiously as the number of scats identified as females was smaller than males, 12 females and 37 males.

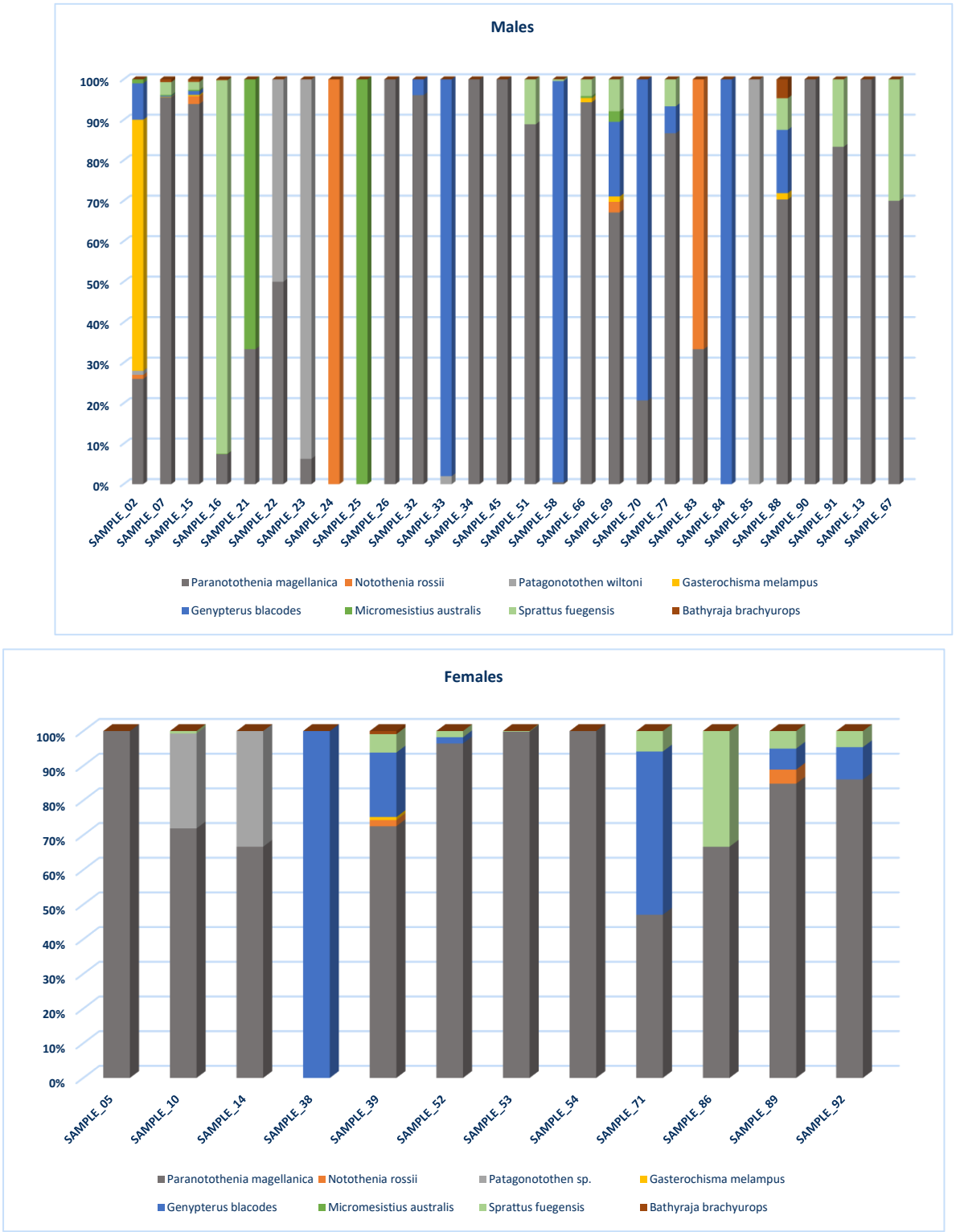


Figure 15. *Relative percentage of sequences identified as fisu taxa in SAFS scats identified as females (left) and males (right).*

To assess potential trends of fish consumption during the collection period, the average relative percentage of DNA sequences of a particular taxon was plot by collection date (Figure 16). Inspection of trends by collection date indicated some variation in terms of the number of different taxa identified in the scats but a clear overall predominance of Magellanic rockcod DNA sequences in the scats during the collection period. Samples SAFS POO 15 and SAFS POO 22, both samples originated from male SASL individuals were not included in the plot.

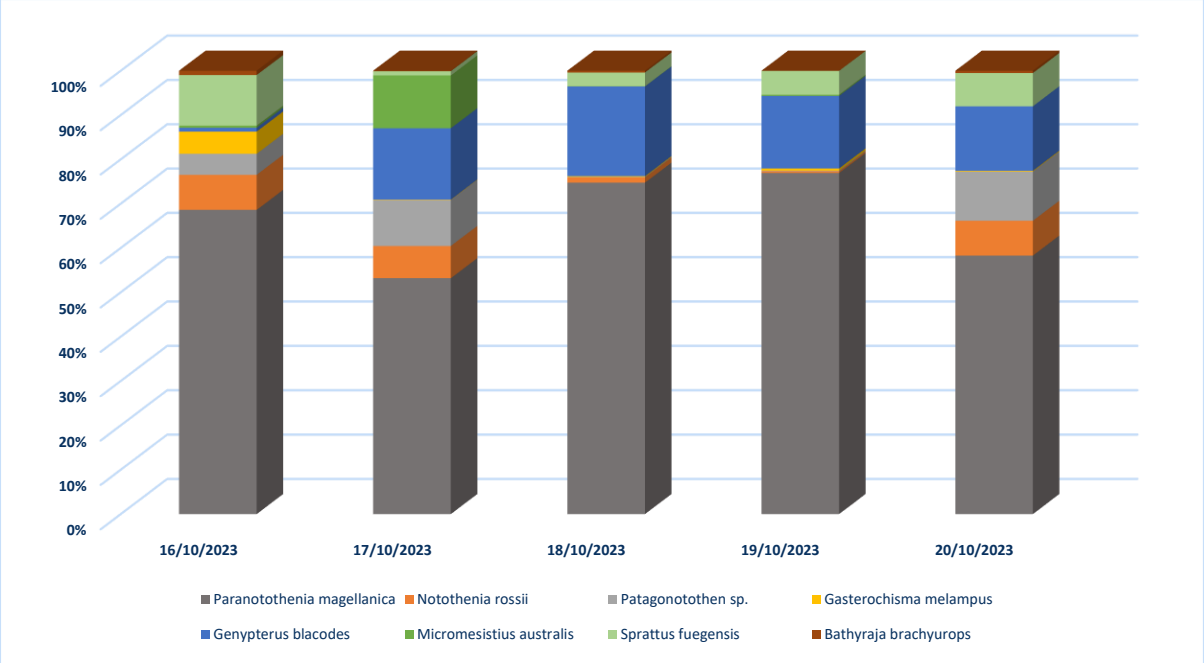


Figure 16. *Relative percentage of DNA sequences identified as fish taxa grouped by collection date.*

Difference in trends of identification of fish taxa in the SAFS scats across the collection period was also assessed by plotting the average relative percentage of sequences of those scats identified with confidence to have originated from females (n = 12) and those from males (n = 37). Visual inspection of the plots would indicate a difference in the diet of SAFS males and females, with a more diverse diet observed in males; however, these are just preliminary analyses and should be interpreted cautiously as the number of samples identified as females was smaller than males. Nonetheless, it was apparent that Magellanic rockcod was an important prey item in both female and male SAFS samples analysed in this project (Figure 17).

***Figure 17**. Relative percentage of DNA sequences identified as fish taxa grouped by collection date for SAFS samples identified as females (left) and males (right). Number of scats identified as females was n = 12 and number of scats identified as males was n =37.*

3.4. 18S rRNA DNA BARCODE

The total of sequence reads for the 18S rRNA barcode ranged from 14,770 to 170,812, with an average of 76,404 sequences per sample. Analyses of the sequencing obtained with the 18S rRNA barcode indicated as expected of a wider range of taxa amplified with this more generic metazoan barcode (Figure 18). However, larger relative percentages of sequences for potential prey taxonomic groups were obtained with the18S rRNA than the Cox1 barcode.

The taxa identified from the different amplicon sequence variants (ASVs) obtained with the 18S rRNA DNA barcode were the following: Gregarious squat lobster (*Grimothea gregaria*), squid (Loligo sp.), fish taxa belonging to belonging to the clades Percomorphaceae, Gadiformes, Clupeiformes, and Rajiformes, and Antarctic krill (*Euphasia superba*). Comparison of the ASVs obtained for fish with the Cox1 data the fish identified as Percomorphaceae were Magellanic rockcod (*Paranotothenia magellanica*), Marbled rockcod (*Nothenia rossii*), Patagonian rockcod (*Patagonotothen* sp.), Butterfly kingfish (*Garterochisma malampus*) and Pink cusk-eel (*Genypterus blacodes*); Gadiformes was the Southern blue whiting (*Micromesistius australis*), Clupeiformes the Falkland spratt (*Sprattus fuegensis*), and Rajiformes the Broadnose skate (*Bathyraja brachyurops*).

Inspection of the relative percentage of sequences identified as potential taxonomic prey with the 18S rRNA DNA barcode indicated an overall predominance of sequences identified as Gregrarious squat lobster in a large number of samples (Figure 19), in particular for those samples collected in the last days of the collection period. In this plot, data for the two samples identified as SASL (SAFS POO 15 and SAFS POO 22) were also included, as well as one sample of AFS collected by the British Antarctic Survey (BAS) that was included in a previous test. This AFS sample (BAS) is included to illustrate to indicate the resolution of the DNA barcode to identify Antarctic krill, which was only identified in one SAFS sample (SAFS POO 04) at very low frequency (< 1%); therefore, likely indicating that it was prey of prey rather than direct SAFS prey.

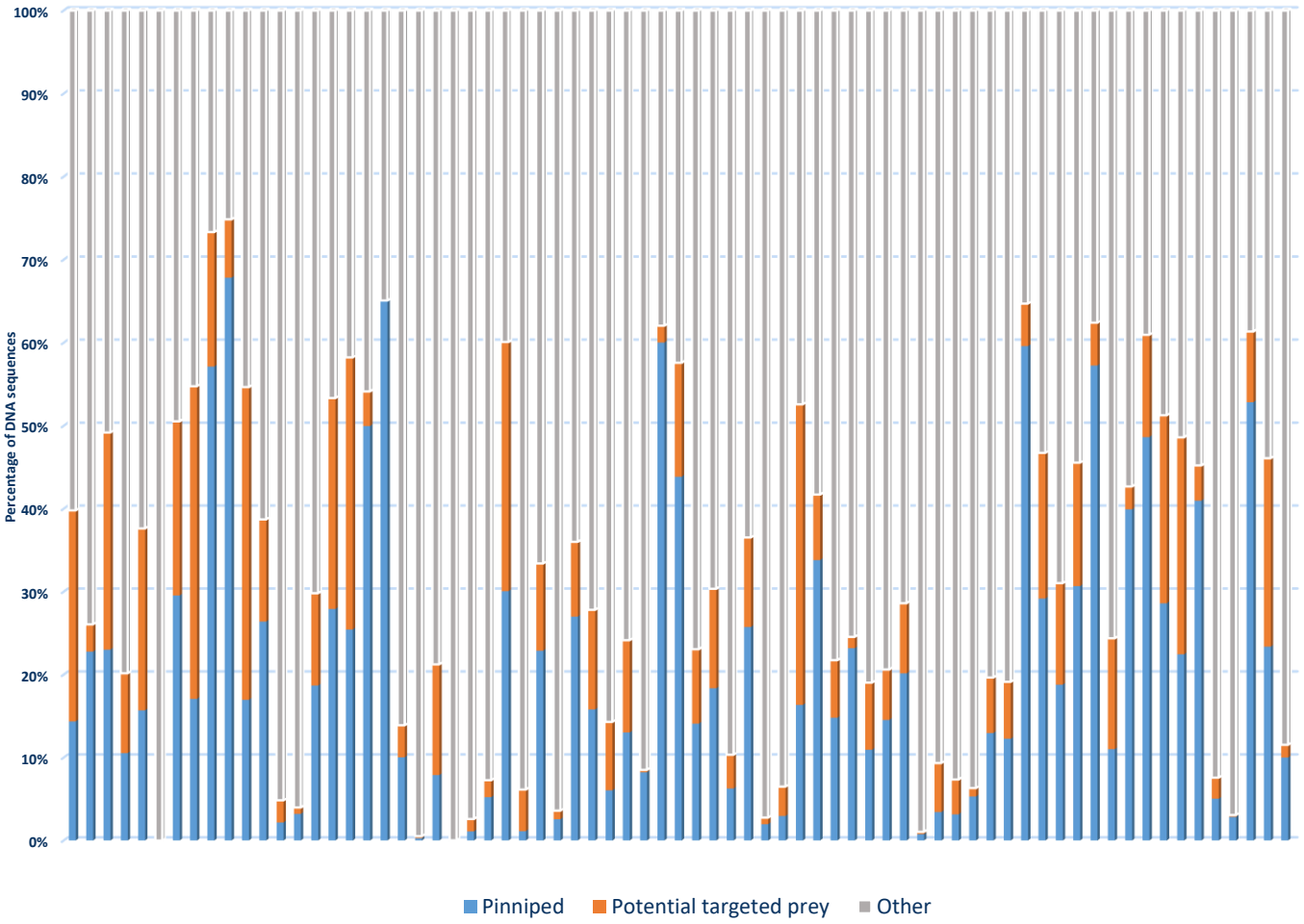


Figure 18. *Relative percentage of DNA sequences identified as pinniped, potential targeted prey and other (prey of prey, parasites, fungi and bacteria).*

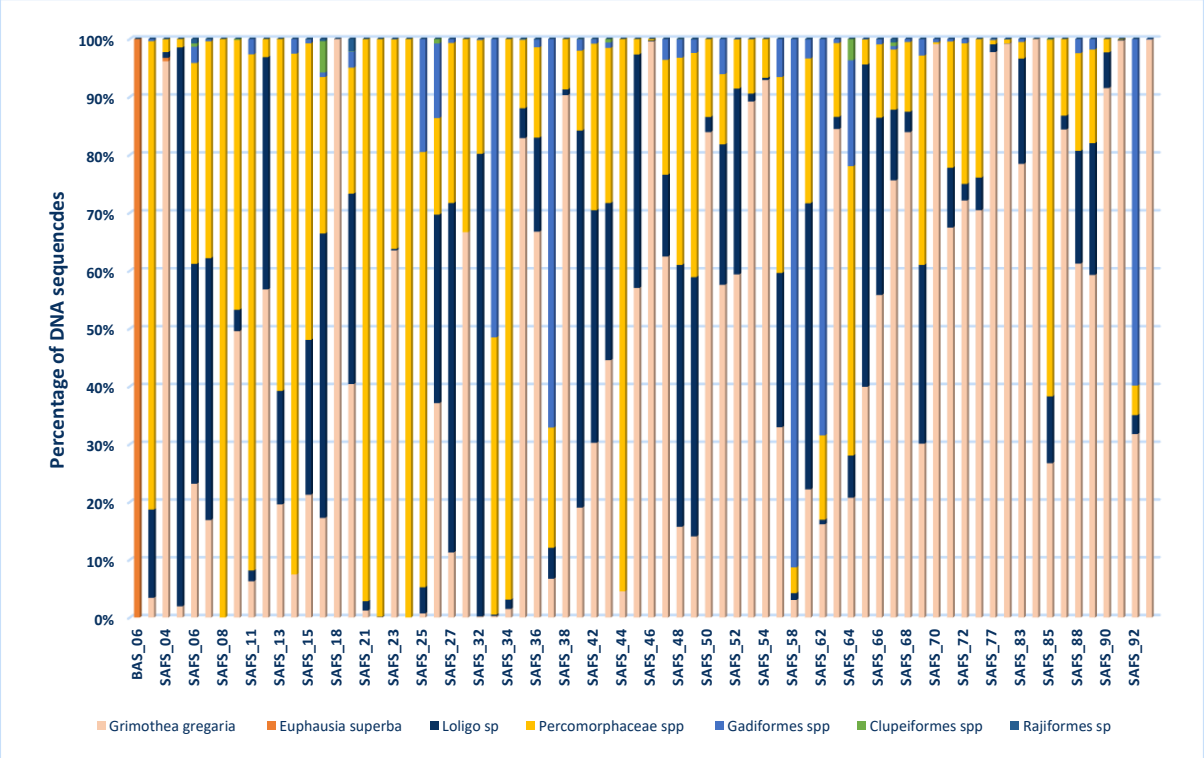
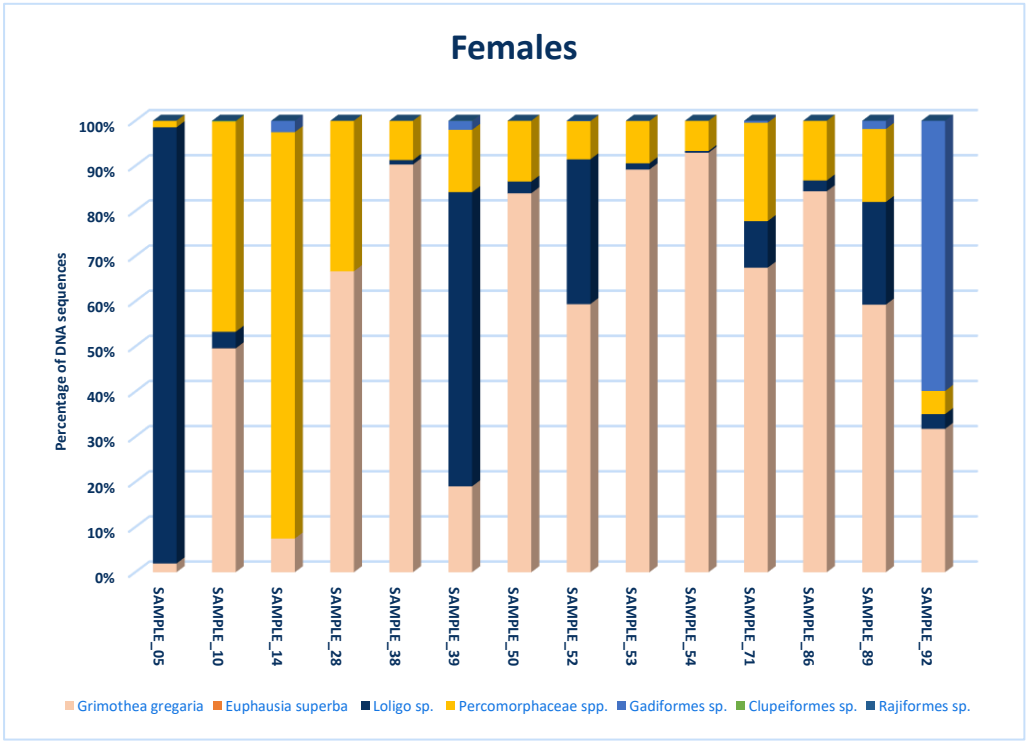


Figure 19. Relative percentage of DNA sequences identified as a particular prey item taxon. This plot includes the two samples identified as SASL (SAFS POO 15 and SAFS POO 22) and one AFS sample (BAS 06).

Data for confirmed SAFS samples was also plotted according to the assigned sex (Figure 20). Visual inspections of the plot would indicate an overall predominance of DNA sequences of Gregarious squat lobster DNA sequences in females in comparison to males, although the predominance of Gregarious squat lobster in male samples was evident for samples collected at the end of the collection period.



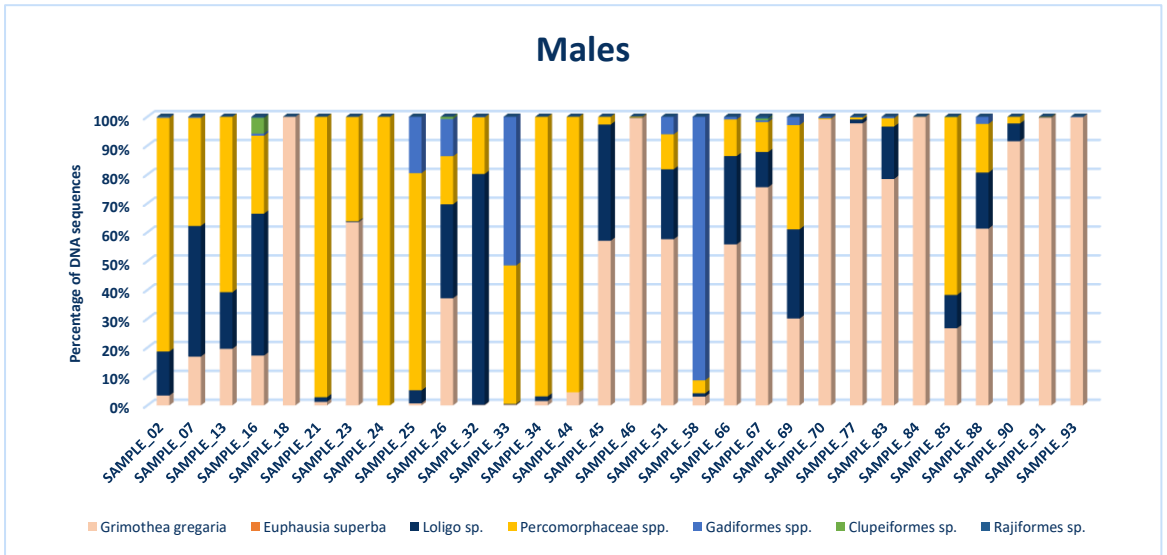


Figure 20. *Relative percentage of DNA sequences of different target prey in confirmed SAFS female and male samples.*

This predominance of Gregarious squat lobster DNA sequences in SAFS samples towards the end of the collection period was more evident went plotting average of percentage of DNA target prey taxa per collection date (Figure 21).

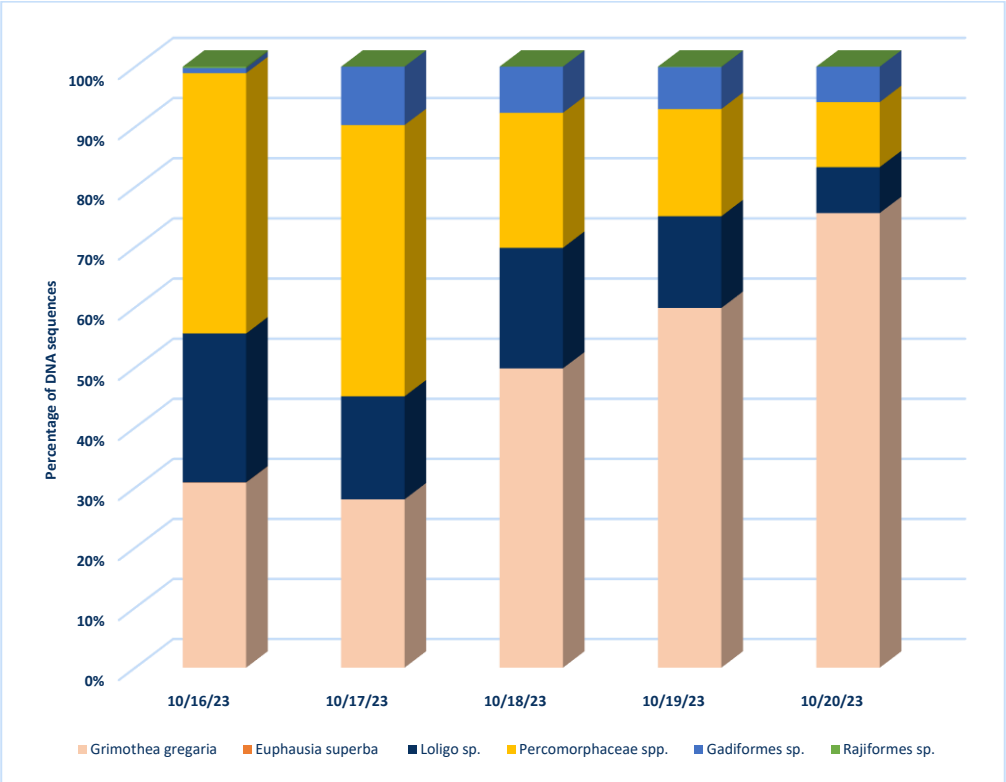


Figure 21. *Average relative percentage of DNA sequences of each targeted prey taxa per collection day.*

Comparisons of the average relative percentage of DNA sequences for targeted prey during the collection period between males and females (Figure 21) indicated an apparent predominant consumption of Gregarious squat lobster by SAFS females earlier in the collection period in comparison to males, for which the consumption of Gregarious squat lobster is more predominant during the last days of sample collection. However, as indicated earlier, the data presented here for males and females should be interpreted cautiously as the number of females (n = 12) was much smaller than males (n = 37).

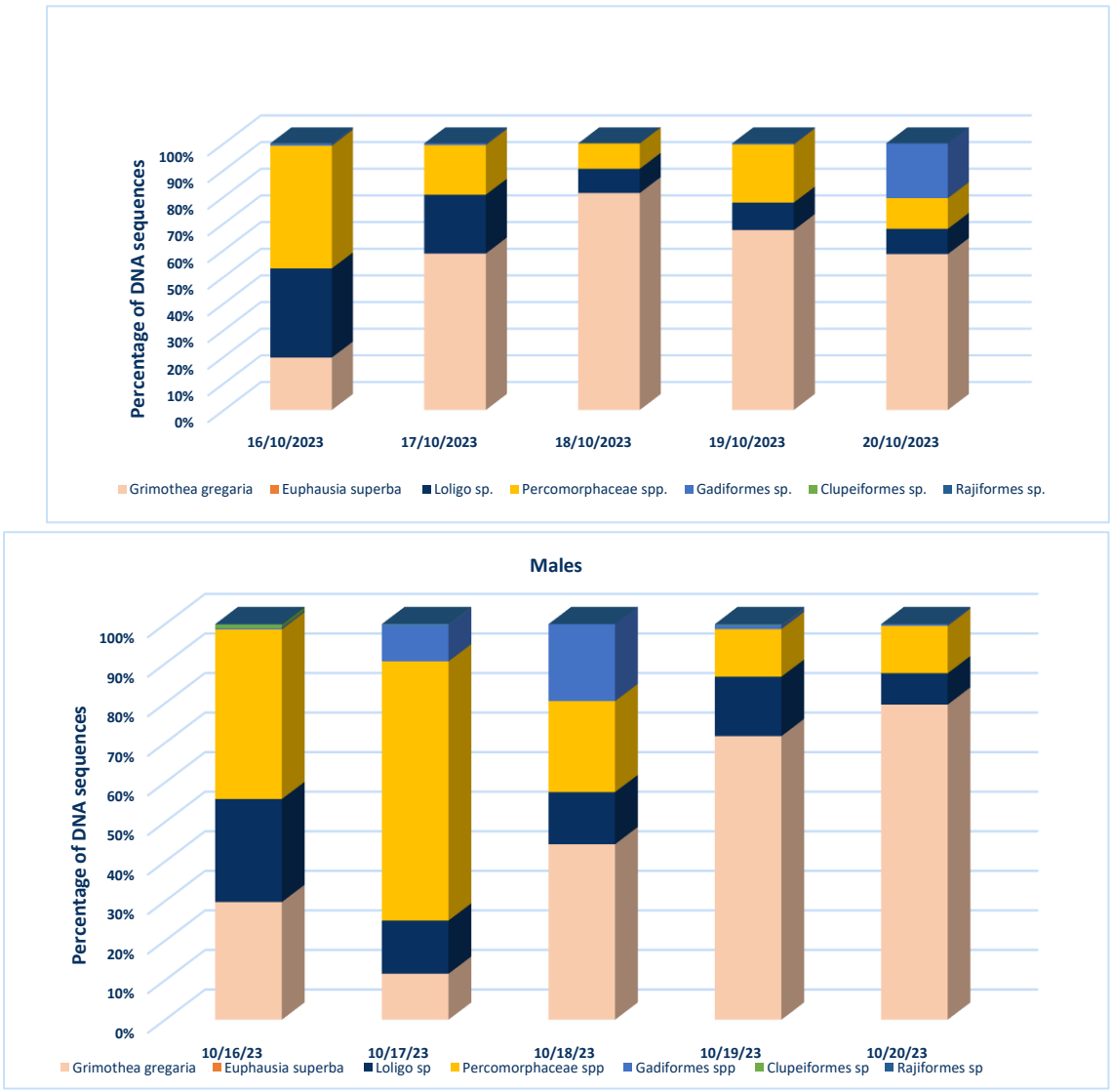


Figure 22. *Average relative percentage of DNA sequences of targeted prey in SAFS scat samples during the collection period for female (left) and male (right) confirmed samples.*

SOUTH ATLANTIC
ENVIRONMENTAL RESEARCH
INSTITUTE

www.south-atlantic-research.org

@SAERI_FI

FALKLAND ISLANDS OFFICE

Stanley Cottage North, Stanley, Falkland Islands. FIQQ 1ZZ
Tel: +500 27374 Email: info@saeri.ac.fk

UK REGISTERED OFFICE

Falkland House, 14 Broadway, Westminster, London, United Kingdom, SW1H 0BH Tel. +44 (0)203 745 1731

SAERI is a Charitable Incorporated Organisation (CIO) registered with the Charity Commission in England under Charity number 1173105. It is also entered on the register of Charities in the Falkland Islands with registration number C47.