APC0264 ABL Data Report – Perley Offshore Collection

## Objectives (from Work Plan Agreement)

1. Extract and quantify 140 eDNA samples.
2. Optimize Atlantic halibut, Atlantic cod, and Atlantic wolffish qPCR assays.
3. Apply optimized qPCR assays to a subset of (114) extracted samples.
4. Develop metabarcoding libraries for COI-1 (Cytochrome oxidase I) and 12S (ribosomal RNA) for a total of 4 libraries.
5. Sequence COI and 12S libraries on MiSeq.
6. Provide raw sequencing data and corresponding sample index information.
7. Provide ABL Report upon completion of the project.

## 1. Sample Extraction & Quantification

A total of 140 samples were received. Of those, 17 were “sponge” fiber filters preserved in DNA/RNA Shield (Zymo Research) and 123 were SmithRoot filters preserved in ethanol. These were a collection of samples from 44 stations along St. Ann’s Bank (SAB) and the AZMP. There were 123 samples (each station had at least three sample replicates), and 11 Field Blanks (see Table 1).

For the extractions, all sample filters were cut in half and processed according to ABL’s eDNA extraction protocol. This particular protocol utilized the QIACube Connect (Qiagen) and a modified version of the Qiagen DNeasy Blood and Tissue Kit (240). Each set of extractions consisted of 11 samples and one Extraction Negative (ENEG). All extracted samples, Field Blanks, and ENEGs were quantified using the Qubit Fluorometer with the dsDNA High Sensitivity Assay Kit (ThermoFisher).

The highest eDNA concentrations were observed in samples from the CURDO stations (see Table 1). The lowest concentrations were observed in the samples collected from AZMP, as well as those that were preserved in DNA/RNA Shield. The latter did not incorporate well into the DNeasy buffers, despite claims from the manufacturer. It is recommended that using a DNA purification kit from Zymo Research would be more compatible with the buffer/kit chemistry.

All ENEGs had DNA concentrations that were below quantifiable limits (BQL) for the Qubit Fluorometer and considered to be 0 ng/µL. The Field Blanks for the DNA/RNA Shield-preserved collection were also BQL. However, all other Field Blanks had DNA concentrations that were detectable (Table 1). This may have implications for downstream analyses (see qPCR application section).

**Table 1:** The number of eDNA filter samples and Field Blanks processed from each station, as well the average eDNA concentration of samples by station.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Station | Collection | Preservative | No. of Replicates | No. of Field Blanks | Aver. eDNA Sample Conc. (ng/µL) | Field Blank Conc. (ng/µL) |
| BBL\_05 | AZMP | Ethanol | 2 | 0 | 2.10 | N/A |
| BBL\_07 | AZMP | Ethanol | 2 | 0 | 0.358 | N/A |
| CAM\_13C | SAB | DNA Shield | 5 | 1 | 0.789 | BQL |
| CAM\_14 | SAB | DNA Shield | 5 | 1 | 2.442 | BQL |
| CAM\_17 | SAB | DNA Shield | 4 | 1 | 0.4435 | BQL |
| CAM\_34 | SAB | Ethanol | 3 | 0 | 8.04 | N/A |
| CAM\_37 | SAB | Ethanol | 3 | 1 | 6.14 | 2.70 |
| CURDO\_1 | SAB | Ethanol | 3 | 0 | 19.90 | N/A |
| CURDO\_2 | SAB | Ethanol | 3 | 0 | 8.04 | N/A |
| CURDO\_3 | SAB | Ethanol | 3 | 0 | 16.10 | N/A |
| CURDO\_4 | SAB | Ethanol | 3 | 0 | 9.63 | N/A |
| CURDO\_5 | SAB | Ethanol | 3 | 0 | 8.10 | N/A |
| CURDO\_6 | SAB | Ethanol | 3 | 0 | 17.40 | N/A |
| CURDO\_7 | SAB | Ethanol | 3 | 0 | 8.14 | N/A |
| CURDO\_8 | SAB | Ethanol | 3 | 0 | 8.27 | N/A |
| CURDO\_9 | SAB | Ethanol | 3 | 0 | 11.80 | N/A |
| CURDO\_10 | SAB | Ethanol | 3 | 0 | 18.47 | N/A |
| GUL\_02 | AZMP | Ethanol | 2 | 0 | 0.341 | N/A |
| GUL\_03 | AZMP | Ethanol | 4 | 0 | 0.661 | N/A |
| NEC\_05 | AZMP | Ethanol | 4 | 0 | 1.72 | N/A |
| NEC\_06 | AZMP | Ethanol | 2 | 0 | 1.15 | N/A |
| SAB\_1 | SAB | Ethanol | 3 | 2 | 8.53 | 2.48, 0.56 |
| SAB\_02 | SAB | Ethanol | 3 | 0 | 9.96 | N/A |
| SAB\_03 | SAB | Ethanol | 3 | 0 | 8.19 | N/A |
| SAB\_04 | SAB | Ethanol | 3 | 1 | 8.45 | 2.12 |
| SAB\_5 | SAB | Ethanol | 3 | 0 | 5.14 | N/A |
| SAB\_6 | SAB | Ethanol | 3 | 0 | 7.20 | N/A |
| SAB\_07 | SAB | Ethanol | 3 | 0 | 5.43 | N/A |
| SAB\_08 | SAB | Ethanol | 3 | 0 | 6.36 | N/A |
| SAB\_9 | SAB | Ethanol | 3 | 0 | 4.13 | N/A |
| SAB\_10 | SAB | Ethanol | 3 | 1 | 7.30 | 0.95 |
| SAB\_13 | SAB | Ethanol | 3 | 1 | 4.04 | 2.42 |
| SAB\_14 | SAB | Ethanol | 3 | 0 | 5.64 | N/A |
| SAB\_15 | SAB | Ethanol | 3 | 1 | 6.64 | 4.76 |
| SAB\_16 | SAB | Ethanol | 3 | 0 | 3.63 | N/A |
| SAB\_19 | SAB | Ethanol | 3 | 0 | 6.12 | N/A |
| SAB\_20 | SAB | Ethanol | 3 | 1 | 6.32 | 0.28 |
| SAB\_21 | SAB | Ethanol | 3 | 0 | 3.69 | N/A |
| STAB\_01 | AZMP | Ethanol | 2 | 0 | 3.47 | N/A |
| STAB\_02 | AZMP | Ethanol | 2 | 0 | 2.77 | N/A |
| STAB\_03 | AZMP | Ethanol | 2 | 0 | 2.81 | N/A |
| STAB\_04 | AZMP | Ethanol | 2 | 0 | 1.27 | N/A |
| STAB\_05 | AZMP | Ethanol | 2 | 0 | 1.18 | N/A |
| STAB\_06 | AZMP | Ethanol | 2 | 0 | 1.17 | N/A |

## BQL = Below Quantifiable Limit.

## 2. Optimization of qPCR Assays

Assays for three species, Atlantic cod (*Gadus morhua*), Atlantic halibut (*Hippoglossus hippoglossus*), and Atlantic Wolffish (*Anarhichas lupus*), were optimized based on qPCR conditions laid out in literature sources listed in Table 2. The primer and probe sequences for each assay are outlined in Table 3.

**Table 2**. Optimized assay conditions tested by ABL.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Assay | Target Region | TA | Primer/Probe Concentration | LOD/LOQ (copies/reaction) | Reference |
| GadmorCYB\_02  (*G. morhua*) | mtDNA-*cytb* | 60°C | Gadmor\_F15076: 400nM  Gadmor\_R15155: 800nM  Gadmor\_P15102: 300nM | LOD: 1.5  LOQ: 14 | Knudsen *et al.* (2018) |
| Hal\_q  (*H. hippoglossus*) | mtDNA-*cytb* | 62°C | Hal\_qF: 750nM  Hal\_qR: 750nM  Hal\_qP: 250nM | LOD: 1.5  LOQ: 11 | Dufault *et al.* (2021) |
| Al\_CytB  (*A. lupus*) | mtDNA-*cytb* | 60°C | Al\_CytB\_F: 800nM  Al\_CytB\_R: 600nM  Al\_CytB\_P: 250nM | LOD: 3  LOQ: 11 | Chevrinais and Parent (2023) |

The qPCR cycling conditions were as follows:

1. 95°C, 10 mins
2. 50 cycles of:
   1. 95°C, 30 seconds
   2. 60°C or 62°C\*, 60 seconds (\*reading)

**Table 3.** Primer and probe sequence information for the qPCR assays. The fluorophore and quencher are included for the probes.

|  |  |  |
| --- | --- | --- |
| Primer/Probe | Sequence (5’ to 3’) | Amplicon Size |
| Gadmor\_F15076 | TTC GCA CCT AAT TTA CTC GGA G | 80 bps |
| Gadmor\_R15155 | TCG GGC TTA ACA TGA GGT GG |
| Gadmor\_P15102 | /FAM/-AGA TAA TTT CAC CCC TGC TAA CCC CAT C-/BHQ1/ |
| Hal\_qF | TTC ATC TAC TCT TTC TTC ACG | 151 bps |
| Hal\_qR | AAT AGG GCT AGG GAT GCT AA |
| Hal\_qP | /FAM /-TCA GAC TCC GAC AAA GTT CCC T-/ |
| Al\_CytB\_F | CCC TAC TGC TCA AAG AAA GGA GAT TTT AAC TC | 92 bps |
| Al\_CytB\_R | TGA ATA CCA TTG AAA TGG TTA AAA TAA ATA CAT GGT GAT A |
| Al\_CytB\_P | /6FAM/TGC GCG TAT GTA ACT AAT CAT GTA TGT ACT TAG TGC AT/ MGBNFQ/ |

The assays were optimized using TaqPath ProAmp 2X Master Mix (Applied Biosystems). However, there was observed amplification in the No Template Controls (NTC) for the Atlantic cod assay (GadmorCYB\_02). This assay underwent additional optimization to eliminate the erroneous amplification curves. By switching to PerfeCTa qPCR ToughMix (Low ROX) (QuantaBio), no further amplification was observed in the NTCs.

## 3. Application of qPCR assays and Internal Positive Control (IPC)

At this stage, only 112 samples had been provided to the ABL. Assays were applied to all 112 samples and Field Blanks available at that time, and processed in 384-well plate format (where suitable). The epMotion 5073 (Eppendorf) was used to transfer the qPCR master mix and the eDNA samples in triplicate. For application of the IPC assay, the T4 DNA primers and probe (VIC) were used. These were included in the same master mix as the *A. lupus* (FAM). The IPC was used to test for PCR inhibition.

The Standards were developed from a synthetic DNA fragment (gBlock) of the specific species assay mtDNA-cytb region (Table 4). Serial dilutions of the gBlocks were created and used for the standard curves added to the qPCR plate. The standards were plated manually, in triplicate, and ranged from 105 (100,000 copies/uL) to 100 (1 copy/uL). The NTCs were also added manually, in triplicate. The Standards and NTCs were used to assess the quality of the qPCR run. The qPCR was run on the QuantStudio 7 Flex (ThermoFisher) and analyzed using the QuantStudio Real-Time PCR Software.

**Table 4**. The gBlocks used for qPCR standard curves and LOD/LOQ dilutions.

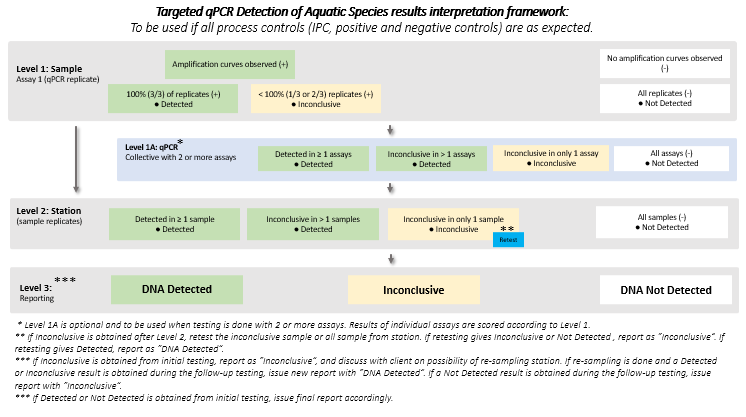
|  |  |
| --- | --- |
| gBlock | Sequence (5’ to 3’) |
| *G. morhua* | TTC GCA CCT AAT TTA CTC GGA GAT CCA GAT AAT TTC ACC CCT GCT AAC CCC ATC GTT ACC CCA CCT CAT GTT AAG CCC GAA TGA TAT TTC TTG TTT GCC TAT GCC ATC TTA CGC TCT ATT CCA AGC |
| *H. hippoglossus* | TTC ATC TAC TCT TTC TTC ACG AAA CTG GCT CAA ACA ACC CGA CCG GGC TAA ACT CAG ACT CCG ACA AAG TTC CCT TTC ACC CCT ACT TCA CTT ACA AGG ACC TCT TAG GCTT TGC AGT CCT TCT TAC TGC ATT AGC ATC CCT AGC CCT ATT |
| *A. lupus* | CCC TAC TGC TCA AAG AAA GGA GAT TTT AAC TCC TAC CCC TAA CTC CCA AAG CTA GGA TTC TGG GCT GCG CTA TGC CTT GCG CGT ATG TAA CTA TAT CAT GTA TGT ACT TAG TGC ATA TAT GTA TTA TCA CCA TGT ATT TAT TTT AAC CAT TTC AAT GGT ATT CAA GGG CGC CGC GGG CCC GG |

All CT values for the qPCRs were provided in the file “APC0264 Final qPCR Results\_20-Nov-24.xlsx”. Overall, there was no evidence of PCR inhibition detected in any of the samples. There was no detection of the target species in any of the ENEGs or NTCs, which gives confidence that there was no contamination at the extraction or processing levels. However, there were three instances of the “Inconclusive” result in two Field Blanks. The *G. morhua* cytb assay was “Inconclusive” for Field Blanks 885030 and 885095, while Field Blank 885030 was also “Inconclusive” for the *H. hippoglossus* cytb assay. The targets were “Not Detected” for all remaining Field Blanks.

Results were interpreted based on the “Targeted qPCR Detection of Aquatic Species results interpretation framework” (Gulf Lab and ZM working group; see Fig. 1). The qPCR results were first analyzed at the sample level, whereby reviewing the qPCR technical replicates per sample. A sample was “Detected” if there was amplification in all three technical replicates. If any sample was flagged as “Inconclusive” (amplification in ≤ 2 technical replicates), the assay was repeated for that sample, as well as the other samples within that station. At the sample level (after redos, not including blanks or negatives), the results were as follows:

|  |  |  |  |
| --- | --- | --- | --- |
| Target | Detected | Inconclusive | Not Detected |
| A. lupus | 51 | 23 | 27 |
| G. morhua | 52 | 38 | 11 |
| H. hippoglossus | 0 | 8 | 93 |

Next the results were analyzed at the station level, which looked at amplification in all sample replicates within that station. If the target was “Detected” in ≥ 1 sample, the result was “Detected” for that station. If the target was “Inconclusive” in > 1 sample, the result was also “Detected” for that station. “Inconclusive” on only 1 sample, that result remained “Inconclusive”. The target was considered “Not Detected” if a sample/station had no instances of amplification in any replicate. The final qPCR results interpretations were summarized in Table 5 (excludes ENEGs and Field Blanks). Note that sites identified as “Inconclusive” infer the inability to confidently determine the absence or presence of the target species.



**Figure 1:** The qPCRresults interpretation framework used to determine the presence or absence of the target species among sample replicates and station.

**Table 5:** The final qPCR results interpretation at the station level for all three target species. from applyingIPC assay and Atlantic salmon qPCR target assay on all extracted samples. The above framework (Figure 1) was used to determine presence/absence at the sample level and sample-level results were used to determine station-level results. This is generally the reporting level.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Results (based on station replicates) | | |
| Station | **A. lupus cytb** | **G. morhua cytb** | **H. hippoglossus cytb** |
| SAB\_16 | Inconclusive | Detected | Not Detected |
| SAB\_21 | Not Detected | Detected | Not Detected |
| SAB\_20 | Detected | Detected | Not Detected |
| SAB\_02 | Detected | Detected | Not Detected |
| SAB\_03 | Detected | Detected | Not Detected |
| SAB\_07 | Detected | Detected | Not Detected |
| SAB\_08 | Detected | Detected | Not Detected |
| SAB\_04 | Detected | Detected | Not Detected |
| SAB\_13 | Not Detected | Not Detected | Inconclusive |
| CURDO\_8 | Detected | Detected | Not Detected |
| CURDO\_5 | Detected | Detected | Inconclusive |
| CURDO\_2 | Detected | Detected | Inconclusive |
| CURDO\_7 | Detected | Detected | Not Detected |
| CURDO\_4 | Detected | Detected | Not Detected |
| CURDO\_1 | Detected | Detected | Not Detected |
| CURDO\_10 | Not Detected | Not Detected | Not Detected |
| CURDO\_3 | Not Detected | Detected | Not Detected |
| CURDO\_6 | Detected | Detected | Not Detected |
| CURDO\_9 | Detected | Detected | Inconclusive |
| SAB\_1 | Detected | Detected | Not Detected |
| SAB\_6 | Detected | Detected | Not Detected |
| SAB\_10 | Detected | Detected | Inconclusive |
| SAB\_5 | Detected | Detected | Not Detected |
| SAB\_9 | Detected | Detected | Not Detected |
| SAB\_14 | Detected | Detected | Not Detected |
| SAB\_19 | Detected | Detected | Not Detected |
| SAB\_15 | Detected | Detected | Not Detected |
| CAM\_34 | Detected | Detected | Inconclusive |
| CAM\_37 | Detected | Detected | Not Detected |
| CAM\_13C | Detected | Detected | Not Detected |
| CAM\_14 | Detected | Detected | Not Detected |
| CAM\_17 | Not Detected | Detected | Not Detected |

## 4. Develop metabarcoding libraries for COI and 12S

The loci used for metabarcoding were:

* COI-1:
  + mICOIintF / jgHCO2198 (Lacoursière‐Roussel et al. (2018))
    - mlCOIlintF (Leray et al. (2013))
      * Sequence: GGWACWGGWTGAACWGTWTAYCCYCC
    - jgHCO2198 (Geller et al. (2013))
      * Sequence: ACTTTCGTTCTTGATYRA
* 12S:
  + 12S\_248F\_RADS\_For / Mifish\_UR\_Miya (He et al. (2023))
    - 12S\_248F\_RADS\_For (He et al. (2022))
      * Sequence: CGTGCCAGCCACCGCGGTT
    - Mifish\_UR\_Miya (Miya et al. (2015))
      * Sequence: CATAGTGGGGTATCTAATCCCAGTTTG

The eDNA samples were randomized prior to the PCR reactions to avoid bias, and split evenly to create two libraries. PCR reactions were performed with three identical replicates per sample. The PCR products were screened on the TapeStation 4200 (Agilent Technologies) to confirm amplification in the samples and ensure there was no amplification in any associated PCR negatives (PNEG). If amplification was observed in any PNEG the sample was redone.

Once verified, PCR replicates were pooled and purified using the AMPure XP Reagent (Beckman Coulter), followed by quantification using the AccuClear Ultra High Sensitivity dsDNA Quantitation kit (Biotium). Purified PCR products were multiplexed with equal amounts per sample when possible, with a target input of 50 ng/sample for COI libraries and 25 ng/sample for 12S libraries. For samples with very low DNA (such as Field Blanks and Extraction Negatives), a maximum of 10µL was added to void diluting the final library concentration.

The multiplexed libraries were purified using AMPure XP Reagent, then quantified using the NEBNext Library Quant kit for Illumina (New England Biolabs) on the QuantStudio 7 Flex (Applied Biosystems). Two libraries, each with 77 randomly assigned samples and controls, were developed for two targets (COI-1 and 12S), for a total of four libraries.

## 5. COI and 12S Library Sequencing on the MiSeq

All libraries were sequenced using the MiSeq platform (Illumina). The MiSeq Reagent Kit v3 (600-cycle) was used for the COI-1 A and COI-1 B libraries, while the v2 kit (500-cycle) was used for the 12S A and 12S B libraries.

The COI-1 libraries were prepared to a final concentration of 15pM, with a 15% PhiX spike-in (15pM). This was empirically determined from past projects and had shown to work well. For the COI-1 A library, the run metrics looked similar to those from previous projects. However, in this instance there was evidence of some over-clustering in the flow cell. There were inconsistencies in some of the tiles (2102 and 2109 in particular). Tile 2102 produced the lowest Intensity, Density, and Clusters Passing Filter (PF). This tile also produced 0% of reads Aligned and 0% PF. The clusters passing filter should be between 80-90%, but from Table 6 one can see it is slightly lower and the confidence interval is a little high. While the clusters passing filter are expected to be lower than the raw density, this metric should be higher.

The run metrics for the COI-1 B library were very similar to library A (Table 6). There was also evidence of the cluster density being too high (over-clustering). Inconsistencies in the tiles were more evident, Tiles 1103, 1108, 2103, 2109, and 2111 in particular, with the most variability in Density and %>Q30. These tiles also had the lowest % of sequences aligned for both reads. Furthermore, in Tiles 1103 and 1108 there was evidence of some data loss. For example, the sequence read quality was 0% > Q30.

Indeed, the data were reviewed by a technician at Illumina who noticed from the raw images that the tiles with issues were over-clustered. For future runs with COI-1 libraries it was recommended aiming for a cluster density 30-40% lower than the optimal range for the kit chemistry because the library is low diversity.

**Table 6.** Summary of sequencing run parameters and data output quality metrics.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Library/Run | MiSeq Reagent Kit/Chemistry | Library Input (pM) | PhiX Spike-in (%) | % > Q30 | Cluster Density (K/mm2) | Clusters PF (%) | Data Yield (Gb) |
| COI-1 A R1 | v3 (600-cycle)  PE | 15 | 15 | 80.05 | 1118 ± 18 | 79.4 ± 13.0 | 14.0 |
| COI-1 B R1 | v3 (600-cycle)  PE | 15 | 15 | 80.93 | 1130 ± 37 | 79.30 ± 18.99 | 14.15 |
| 12S A R1 | v2 (500-cycle)  PE | 7 | 20 | 83.55 | 783 ± 33 | 90.58 ± 1.74 | 5.45 |
| 12S A R2 | v2 (500-cycle)  PE | 7 | 20 | 81.14 | 765 ± 32 | 91.23 ± 1.59 | 7.72 |
| 12S B R1 | v2 (500-cycle)  PE | 7 | 20 | 79.93 | 726 ± 39 | 89.89 ± 2.50 | 7.42 |

PE = paired end

The 12S (ribosomal RNA) libraries (12S A and 12S B) were prepared to a final concentration of 7pM with a 20% PhiX spike-in (7pM). The sequencing runs for both libraries looked good, with output quality metrics being close to or within optimal ranges for the kit chemistry (Table 6). Library 12S A was run a second time because a power outage interrupted the MiSeq run and only partial data were collected.

## 6. Provide Raw Sequencing Data

The sequence data for each of the COI-1 and 12S library sequencing runs were retrieved from the MiSeq. The sequence data (.fasta) and metadata were uploaded to the shared Teams project page “APC0264 Perley Offshore Collection”.

## Conclusions

##### Extract & quantify 140 eDNA

* 95 samples (SmithRoot filters preserved in ethanol) from SAB were initially provided to the ABL, followed by 17 samples (“sponge” filters preserved in DNA/RNA Shield).
* Another 28 samples (SmithRoot filters preserved in ethanol) from the fall AZMP were later provided.
* All samples were extracted from half-filter using Qiagen’s DNeasy Blood and Tissue kit for the QIAcube, with 14 Extraction Negatives being generated. The DNA was quantified using the Qubit dsDNA HS Assay for the Qubit v4.

##### Optimize assays and apply to a subset of 114 samples

* Assays for Atlantic cod, Atlantic halibut, and Atlantic wolffish were optimized.
* The assays were applied to 112 samples (including 11 Field Blanks) and 11 ENEGs.
  + At this point in the project only 112 samples had been provided. The AZPM samples had been provided much later and could not be processed with the assays.
* The IPC was applied to all samples with the A. lupus assay. No evidence for PCR inhibition was found.
* The presence of *A. lupus* was detected at 26 of the 32 stations screened and inconclusive at the SAB\_16 station.
* *G. morhua* was detected at 30 of the 32 stations. There were two inconclusive results for Field Blanks 885030 and 885095.
* There were no confident detections of *H. hippoglossus* at any of the stations, but there were six stations with inconclusive results (CURDO\_2, CURDO\_5, CURDO\_9, SAB\_10, SAB\_13, and CAM\_34), as well as one Field Blank (885030).

##### Develop libraries for COI-1 and 12S and sequence on the MiSeq

* Two libraries were produced (each with 77 randomized samples) for both COI-1 and 12S, for a total of 4 libraries.
* The COI-1 libraries were successfully sequenced on the MiSeq. However, there was some evidence for over-clustering on the flow cells. As a result, the output quality metrics were not as optimal as in previous projects. For future COI-1 libraries, the target cluster density should be between 800-900 K/mm2. This is roughly 30% below the optimal range for the v3 kit chemistry.
* The 12S libraries were also successfully sequenced on the MiSeq. There were no obvious issues with the runs; the sequencing output values were within optimal ranges.
* The raw sequencing data for the COI-1 and 12S libraries were uploaded to the Teams project page, “APC0264 Perley Offshore Collection”.

## References

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