Boskalis Cambridge Gulf Marine Sand Proposal s38 Referral WA EP Act

Referral Report No. 2 - *Proposal Setting & Existing Environment Descriptions*

ANNEX 14 - MARINE eDNA REPORT - Boskalis Cambridge Gulf.

UNIVERSITY OF CANBERRA | National eDNA Reference Centre

NRC-26-2024: ENVIRONMENTAL DNA ASSESSMENT OF NATIVE SAWFISH AND RIVERINE SHARK SPECIES IN CAMBRIDGE GULF

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EXECUTIVE SUMMARY

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The National eDNA Reference Centre (NeRC) was contracted by Boskalis Australia Pty Ltd (BKA) to undertake marine environmental DNA (eDNA) sampling, to support environmental assessment studies for a proposed marine sand sourcing operation in Cambridge Gulf (CG), in the north-east of Western Australia, in February 2024. The purpose of the eDNA sampling was to assess presence/absence and if possible indicative abundance of four sawfish species (*Anoxypristis cuspidata*, *Pristis clavata*, *Pristis zijsron* and *Pristis pristis*) and two river shark species (*Glyphis garricki* and *Glyphis glyphis*). A total of 86 environmental samples were collected, comprising 60 sediment samples and 26 water samples at 20 separate locations within CG. Sampling sites included up rivers and inlets around the coast of CG, which are the typical habitat of the target species, and the open-water areas of CG, including within BKA's proposed operational area. DNA was extracted from all samples and analysed using optimised species - specific assays for all sawfish species, and High Throughput Sequencing broad spectrum assays for both river shark species. The latter method was used as there were no pre-existing validated species-specific DNA assays for the two river-shark species.

Species-specific assays were optimised to achieve high detection sensitivity and quantitatively assess abundance of targeted sawfish species. Similarly, High Throughput Sequencing broad spectrum broad-spectrum was completed for the 86 environmental samples collected at GG, including six field negative controls, three extraction negative controls and three negative PCR testing controls to detect possible cross-contamination.

There was no detection for the four sawfish species (*Anoxypristis cuspidata*, *Pristis clavata*, *Pristis pristis or Pristis zijsron*) at any of the sampled sites using species-specific assays. There was no detection of the two river shark species *Glyphis garricki* or *Glyphis glyphis* at any of the sites using broad spectrum assays, however, a total of 55 DNA sequence reads were detected for *Anoxypristis cuspidata* at site 03 using the broad spectrum assay. This detection indicates the presence of marginally low DNA traces for this species at site 03, which could be associated to old DNA present in the environment from past occurrences of the species in the area, but not indicative of current occurrence at the time of sampling

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BACKGROUND

Australia's northern tropical rivers, estuaries and coastal waters, including Cambridge Gulf (CG) in the north-east of Western Australia (WA), provide important habitat for the following six sawfish species and two river shark species, that are listed under the Commonwealth *Environment Protection and Biodiversity Conservation Act 1999* (EPBC Act) and the WA *Biodiversity Conservation Act* (BC Act):

- Freshwater (also called Largetooth) Sawfish (*Pristis pristis)*,
- Green Sawfish (*P. zijsron)*,
- Dwarf Sawfish (*P. clavata)*,
- Narrow Sawfish (*Anoxypristis cuspidata),*
- Speartooth Shark (*Glyphis glyphis*); and
- Northern River Shark (*G. garricki*).

While CG provides suitable habitat for the six sawfish species, no published scientific papers, reports or records confirming their presence in CG have been found through comprehensive literature search. The two river shark species are reported from the Lower Ord River upstream from CG by Kyne (Charles Darwin University online news article) but no published scientific papers or reports could be found.

Boskalis Australia Pty Ltd (BKA) is proposing to develop a marine sand sourcing operation in CG and is undertaking a wide range of environmental studies to assist in assessing potential environmental impacts of the proposal. Given the potential presence of sawfish species and the reported presence of the two reiver shark species in the CG area, BKA is giving very high priority to assessing potential impacts of the proposed operation on these species. This includes undertaking surveys of their presence/absence, distrubution and abundance in the area. Conventional survey techniques for these species include setting gillnets to capture individuals. This sampling method was not adopted by BKA has it can cause injury and harm to the animals, as well as pose significant safety risks to sampling personnel, including from potential crocodile attack. The much less invasive and much safer survey technique of environmental DNA (eDNA) sampling was therefore adopted by BKA.

Sampling using eDNA technology has been used to detect Australian native sawfish species in the past (pls insert some references), demonstrating the capacity of collecting environmental samples and the suitability of high-throughput, non-destructive and sensitive to detect high priority native species in Australian waters.

The National eDNA Reference Centre (NeRC) was contracted by BKA to undertake the marine eDNA sampling and analysis program in CG in February 2024, to support the environmental assessment studies for the proposed marine sand sourcing operation. The purpose of the eDNA sampling was to assess presence/absence and if possible indicative abundance of the four sawfish species and two river shark species. This report presents the findings of the eDNA study in CG.

SAMPLE COLLECTION AND DNA EXTRACTION

METHODS

Sample Collection

Environmental samples were collected in February 2024 from 20 sites in CG, including up rivers and inlets around the coast of CG, which are the typical habitat of the target species, and the open-water areas of CG, including within BKA's proposed operational area (Figure 1).

Two replicate water samples were collected from sites 1-13 using Smith-Root 5 µM self-preserving filters attached to a Smith Root eDNA sampler. Filters were attached to a telescopic pole and 1.5 L of superficial seawater (5-20 cm depth) was filtered through each sample (Figure 2). Flow rate was adjusted to 0.3 L/min to avoid clogging of filters. All filtered samples were kept inside their individual filter casing until arrival to the main vessel at the end of each sampling day.

Water samples were not taken from sites 14 to 20 as they are located in open areas where mixing of the water column is high due to strong tidal currents, wind and waves. Seabed sediment samples were considered to be more reliable, considering that the six target species are epi-benthic, they mainly live near and on the seabed.

Three replicate seabed sediment samples were taken from benthic grabs at all 20 sites (Figure 3). Samples were taken from the superficial layers of each grab using sterile, single use plastic spoons and placing roughly 20-30 grams of soil inside 50 mL sterile falcon tubes with 35 mL of analytical grade ethanol (Figure 4).

Lastly, for quality control field negative control samples were collected at the end of each sampling event every day. 500 mL plastic bottles containing clean drinking water were opened for approximately 30 seconds over the surface of the water. Then, bottles were capped and briefly submerged at sea for approximately 10 seconds and filtered using Smith-Root 5 µM self-preserving filters, following the same sampling method used to collect water samples at each site.

Upon arrival to the main survey vessel, filter housings were removed from their packaging and the filters were extracted by carefully opening the filter housing. Sterile forceps were used to transfer the filters into 5 mL sterile tubes with 2 mL of analytical grade ethanol (Figure 5).

Photographs with a sample ID data board were taken of each step in the sampling process for each sample (Figure 2 & Figure 3).

Fixed filters and soil samples were then kept inside the walk-in freezer of the main survey vessel for the duration of the sampling program and then transported to the NeRC in Canberra for analysis.

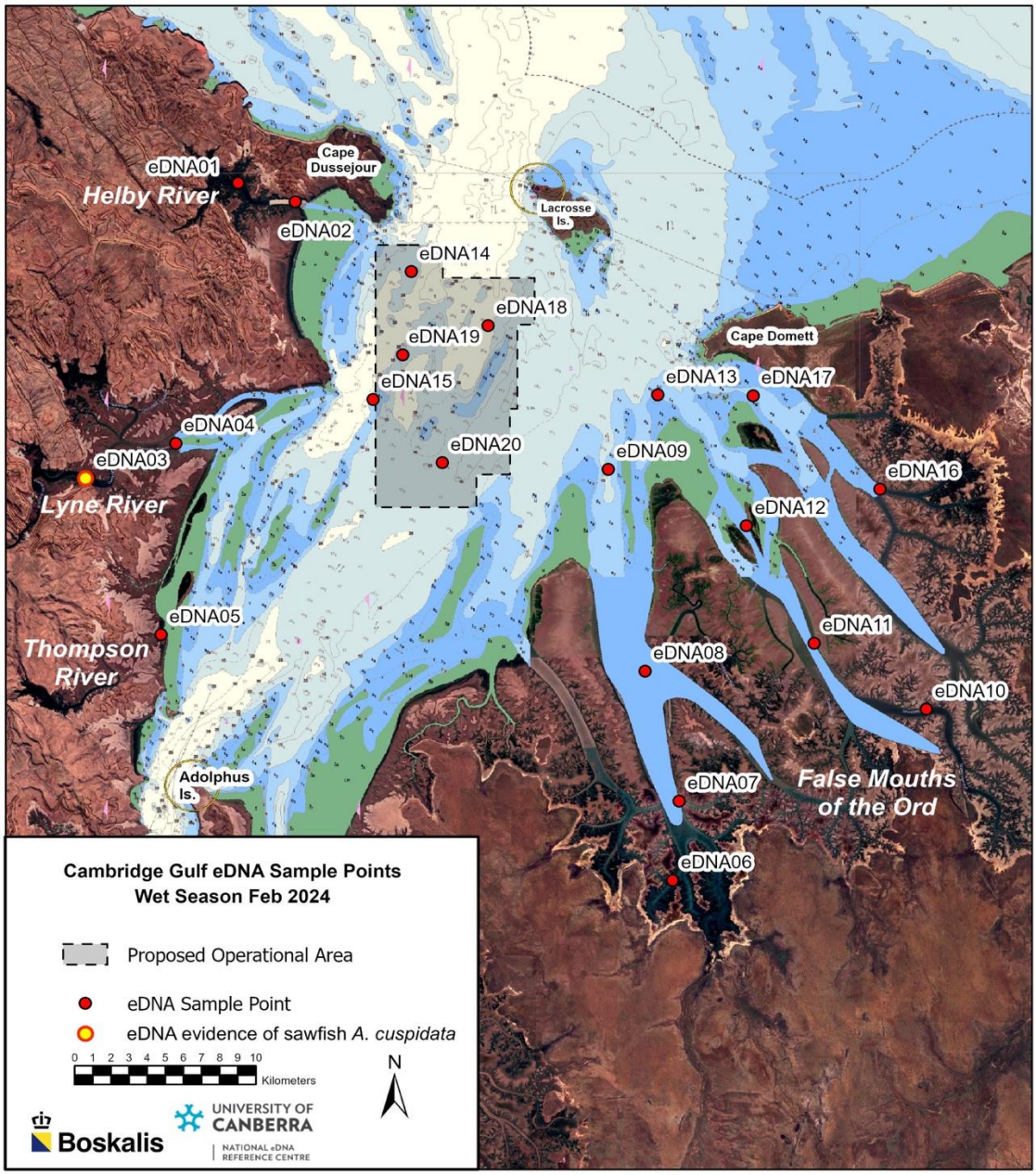

Figure 1. Sites sampled for environmental DNA analysis. Both water and seabed sediment samples were taken at Sites 1 to 13 and only seabed sediments at Sites 14 to 20. eDNA site 03 is highlighted as 55 fully curated DNA sequence reads were detected for *Anoxypristis cuspidata.*

Figure 2. Water sampling with telescopic pole.

Figure 3. Grab used for taking seabed sediment samples.

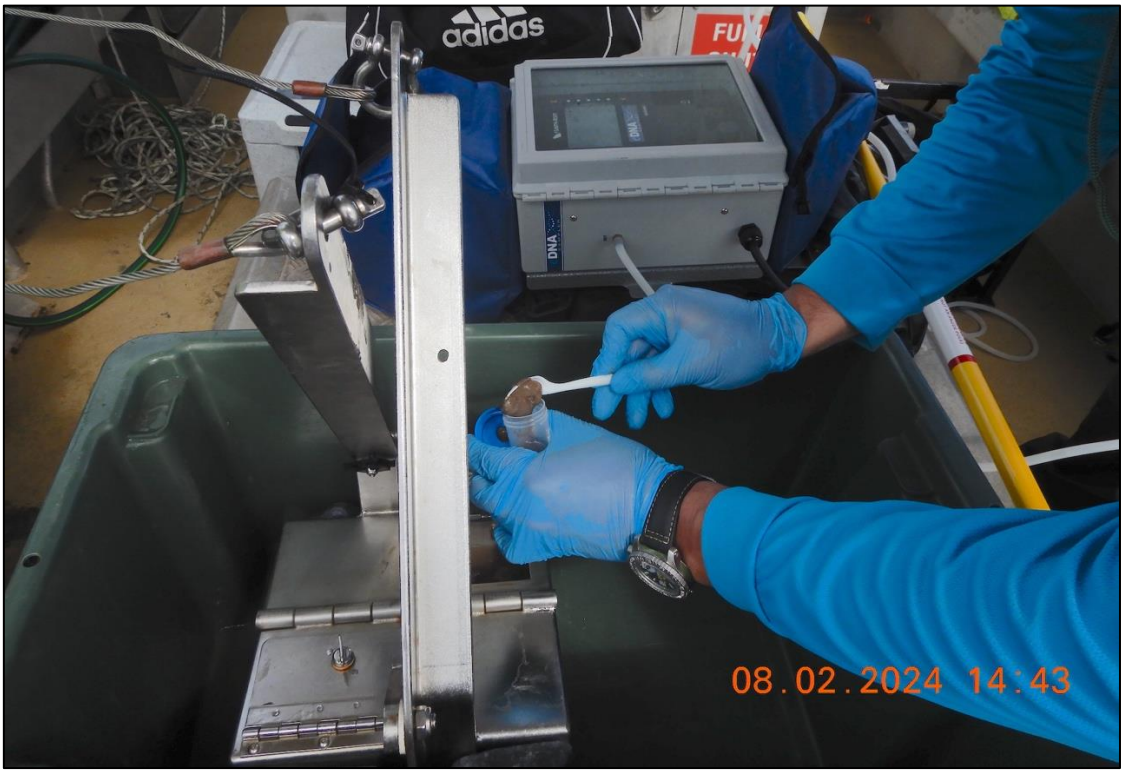

Figure 4. Taking sediment samples from the grab.

Figure 5. Processing samples on main survey vessel.

Sample Processing

eDNA extraction: Water sample filter papers

Prior to extraction, 5 mL tubes were wiped with a 10 % bleach solution to limit potentially contaminating DNA from entering the NERC Trace DNA Laboratory. On arrival of the samples at NeRC ethanol preserved filters were removed and placed into a new 5 mL tube for extraction following a modified Qiagen DNeasy Blood and Tissue Kit protocol where each filter was lysed in 560 µL of ATL buffer and 60 µL of Proteinase K before incubation at 65 °C for two and a half hours (Hinlo et al., 2017). Following incubation 630 µL of AL buffer and 630 µL of 100 % ethanol was added to the sample tubes. The Qiagen protocol was then followed as prescribed, and samples were eluted in 150 µL of buffer AE. 1:10 and 1:100 dilutions of each sample were created for downstream analyses and optimisation. This same process was completed for all field negative controls.

eDNA extraction: Sediment samples

On arrival of the samples at NeRC the Falcon tubes containing sediment samples were wiped with bleach prior to entering the NERC trace DNA laboratory. After cleaning, tubes were arranged in an extraction hood, and 1 mL syringes were used to aspirate approximately 250 – 500 mg of ethanol preserved sediment to a new Powerbead ceramic tube in preparation for bead beating. Samples were centrifuged for 5 minutes at 8000 rpm to pellet the sediment material and excess ethanol was carefully aspirated off and discarded. 60 µL of Qiagen's Proteinase K and 360 µL of Buffer ATL were then added to each tube before bead beating off tubes for 10 minutes at maximum speed. Samples were incubated overnight at 56 °C and following incubation samples were stored at 4 °C prior to commencing the secondary steps of the protocol.

Following incubation, samples were bead-beated again for 10 mins and then centrifuged for 20 mins at 8000 rpm. The supernatant was transferred to a new tube for the remainder of the extraction process where equal amounts of Buffer AL and 100 % ethanol were added to the sample tubes. 500 µL of Buffers AW1 and AW2 were added to the tubes with centrifugation in between and all samples were eluted in 150 µL of Qiagen Buffer AE. Raw extracts of all eDNA samples (filter, sediment, and control samples) were quantified using a Nanodrop One and 1:10 and 1:100 dilutions were constructed for downstream analyses. Extraction negative controls were also created for each batch of samples to control for contamination and incorrect processing of samples.

Dilution and inhibition testing

Each sample was assessed for optimal amplification (i.e., ensure detection is being achieved by primers within the operational range of the test to ensure reliable detection) by testing dilutions of sample with a broad detection fish primer set from West et al. (2020)(Fish F1-degenerate, Fish F2-degenerate and Shark COI-MINIR-degenerate primers). The effect of PCR inhibition in samples was evaluated by performing a single qPCR replicate for neat and diluted samples with melt curve analysis.

qPCR testing was performed as in West et al. (2020) using 0.1 µM of each forward primer (i.e., Fish F1 and F2), and 0.2 µM or reverse primer for each 25 µL reaction. 0. 6 µL of Sybr Green (5X) and 10 µL of Taqman Environmental Master Mix with 4 uL of template was also added to reactions with remaining volume reached using Ultra-Pure H^2O.

Thermocycling conditions were performed as in West et al. (2020) for the specified 50 cycles. The most optimal dilution (i.e., based on Cq-value and melt curve analysis) of each sample was selected for downstream targeted analyses using the sawfish species-specific Taqman assays and metabarcoding analysis. Gel electrophoresis was also used to assess and confirm the amplification of correct fragments for a subset of samples, with primers being used for downstream library preparation and metabarcoding.

Assay sensitivity and standardisation

Sawfish assays were then tested on synthetic gBlock fragments for qPCR performance (Appendix, Table 2). Synthetic fragments used in testing were sourced directly from Cooper et al. (2021) and incorporated an internal variation of the prescribed amplicon sequence to differentiate this DNA sequence from true detections and eliminate potential contamination risks. Each assay (

Table 1) was tested according to the prescribed qPCR run conditions sourced from literature.

Assays were multiplexed together to increase efficiency of testing. If multiplexing of assays was not deemed appropriate, then sensitivity testing was repeated as a single target qPCR reaction. Each 20 µL reaction contained 10 µL of Taqman Environmental Master Mix, species specific primers and probe in optimised concentrations, 1 µL of synthetic fragment and made up to 20 µL with Ultrapure H20. The optimised conditions for all species-specific assays subsequently used for eDNA testing of all eDNA samples collected from previous studies in the Kimberley Region can be found in Table 2 in the Appendix below.

Sensitivity testing was conducted by diluting the gBlock synthetic fragments to a known copy number dilution and adding 1 µL of 106 – 10-1 copy/µL template to PCR reactions across 11 replicates with eight negative control replicates added to each plate. Thermocycling conditions consisted of an initial hold cycle at 60 °C for 10 mins, followed by 55 cycles of 95 °C for 15 seconds and 60 °C for 1 min. The limit of quantification (LOQ) for each assay, wherein testing results can be reliably used to estimate DNA copy number/µL was determined as the lowest step where >85% of qPCR technical replicates amplified with less than 10% relative standard deviation from the mean cycle threshold (mean Cq).

Table 1. Primers and probe information used to detect sawfish (species-specific assays, Cooper et al. (2021)) and river sharks (metabarcoding assays, West et al. (2020)).

Primers & probes	Target gene region	Amplicon size (bp)	5'-Sequence-3'	Final Primer concentration (nM)
	Species-Specific analyses			
Anoxypristis cuspidata				
Forward	12S	155	TGCCCCAGACCCACCTAGA	500
Reverse	VIC		CCTGACGTGTTGGAGGTTAATC	500
Probe			TTCTTGCCACTAACCG	250
Pristis clavata				
Forward	12S	156	GGTGCCTTAGATCCACCTAGAG	300
Reverse	FAM		CTGACGTATTGAAGGTGGGTTCT	300
Probe			CATTTCTTGCTATCAACC	250
Pristis pristis				
Forward	12S	227	GTGCCTCAGACCCACCTAGA	300
Reverse	VIC		CATCATACTGTTCGTTTTTTCTTAGGAG	300
Probe			AAATGAACTAACCTTCAATACG	250
Pristis zijrson				
Forward	12S	160 bp	GGTGCCTTAGATCCACCTAGA	500
Reverse	FAM		CGACCTGACGTATTGAAGATAGAT	500
Probe		CCCACCACTTCTTGCTAT	250	
	Metabarcoding analysis			
Forward (FishF1-Deg)	COI	110 – 241	ACCAACCACAAAGANATNGGCAC	100
Reverse (FishF2-Deg)		TCNACNAATCATAAAGATATCGGCAC	100	
Sark COI-MINIR-Deg		GATTATTACNAAAGCNTGGGC	200	

Species-specific testing

Single species testing was conducted on all eDNA samples across each of the four sawfish target assays. qPCR reactions were conducted in 384 well plates in 20 µL volumes. Each reaction contained 3 µL of template eDNA and was tested across six replicates using the optimised qPCR setup and thermocycling conditions described in sensitivity testing. Two assays, *A. cuspidata* and *P. clavata* were multiplexed together while the remaining two species (*P. pristis* and *P. zijrson*) were performed as individual qPCR tests. This was done as

greater sensitivity was achieved for the detection of *A. cuspidata* and *P. clavata* when tested simultenously.

Positive controls were added to each plate setup which consisted of 3 µL of 10^5 copy number dilution of the respective species synthetic DNA fragments. In addition, a secondary test was performed on all eDNA samples to further assess inhibition in qPCR reactions for species specific assays. This was performed by adding Taqman Exogenous Internal Positive Control Reagents into qPCR setups (1.0 µL of 10X Exo IPC Mix and 0.20 µL of Exo IPC DNA) and performing a single replicate for all samples across target assays for the *P. pristis* assay and making up qPCR reactions to a final volume of 20 µL.

Throughout eDNA testing, a positive qPCR result was determined to be the presence of an amplification curve in any of the technical and biological replicates (i.e., 1 of 6 replicates per sample) and amplification for the gBlock synthetic fragment (positive control). For IPC testing, a positive amplification for the exogenous control suggests appropriate sample processing and lack of inhibition in samples.

Metabarcoding library preparation and sequencing

Libraries were constructed using a two-step Illumina PCR tagging approach. All samples were first amplified using the Fish F1, F2 and Shark COI MINR primers containing the Illumina adapter overhangs in triplicate qPCR reactions as described previously. Positive replicates of each eDNA sample were pooled to a total of 24 µL with 21 µL of each amplicon purified using an AMPure bead clean protocol in a 1.2X ratio of beads to sample.

Following purification, samples were added to a secondary PCR to ligate the indexing tags to amplicons following the Illumina prescribed protocol. Following tagmentation, gel electrophoresis was used to assess the correct attachment of secondary PCR tags through visualisation of increased length of the amplicon products. Amplicons were then pooled according to Cq value in pools of 10 samples per pool with 21 µL of each sample added to a pool and an AMPure clean was again conducted. Pools were quantified using a Qubit Flurometer and HS reagent and pools were normalised and visualised on a gel to confirm successful normalisation.

A final super pool was constructed from the normalised pools with a final concentration of 26.4 nM. The final run concentration for sequencing was 7 pM with 20 % Phi X added to the sequencing pool. Sequencing was conducted on the NERC Illumina MiSeq using a V3 V300 on an Illumina Miseq at the University of Canberra.

Bioinformatic pipeline

The generated Fastq.gz files were automatically demultiplexed and the primers and adapters removed by the Illumina Local Run Manager. Denoising and filtering of reads was completed in R (version 4.1.2) (R Core Team, 2019).The demultiplexed FASTQ files were quality-evaluated, denoised, and filtered using DADA2 (1.22.0) (Callahan et al., 2016).

Given that two different amplicon sizes were expected by the primer combinations used in this study, reads were trimmed at 110 bp (for FishF1-SHARKCOI amplicons) and at 140 bp (for FishF1-FISHR1 amplicons). The maximum number of expected errors for quality filtering was set at two base pairs. Filtered forward and reverse reads were merged using DADA2 (1.22.0) (Callahan et al., 2016). Chimeras were also removed using DADA2 (1.22.0) (Callahan et al., 2016).

Taxonomic information was assigned to each ASV against the curated BOLD system. Curated reference sequences for the Cytochrome oxidase 1 gene region were downloaded using the "Actinopterygii" and "Elasmobranchii" queries. Accessions were then selected only for "Australia" and filtered for accessions containing complete taxonomy assessments.

Taxonomic information was then assigned using the *assignTaxonomy* script from DADA2. ASVs without Species-Level or failing taxonomic information criteria were removed.

RESULTS

Environmental DNA yield & quality

In total, 93 eDNA samples including 60 sediment samples, 26 water samples and nine field and negative controls were processed for analysis. A total of 26 water samples passed quality control with mean DNA yield (± STD) = 22.46 ± 18.56 ng/µL. Similarly, 60 sediment samples passed quality control with mean DNA yield (± STD) = 30.99 ± 46.51 ng/µL. As expected, DNA purity (i.e., measure of pure DNA compared to the presence of contaminants in a samples, such as proteins, salts, lipids amongst others)across samples was below the accepted 1.6 ratio (Figure 6), a common condition of DNA extracted from high turbidity estuary systems such as Cambridge Gulf. Low purity indicates that molecular testing of samples could be affected by contaminants, resulting in potential false negative results.

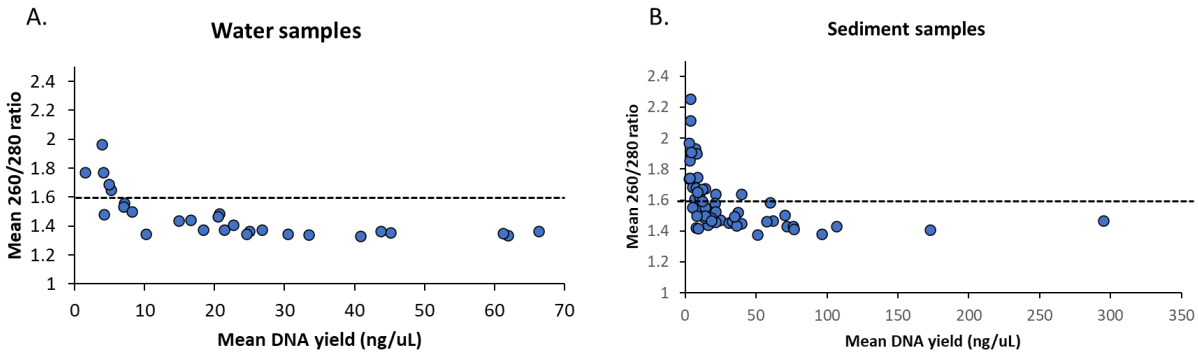

Figure 6. DNA Purity ratios for water (A) and sediment (B) samples collected during this study. Samples are considered to have high DNA purity for ratios above 1.8, acceptable DNA purity for ratios between 1.8 and 1.6, and low-quality DNA for ratios below 1.6.

Sensitivity testing

Sensitivity testing showed that two assays could be successfully multiplexed for eDNA testing purposes. The *A. cuspidata* and *P. clavata* assays reported LOQ at 10 copies/reaction and replicated efficiencies reported from the source literature of 108.6% and 110.8% respectively.

The *P. pristis* assay showed equally high efficiency at 94.5%, and reported a LOQ of 1000 copies/reaction. Efficiency of the *P. zirjson* assay was reported as 80.54 % with an assay LOQ of 100 copies/reaction (Figure 7).

To achieve greater detection sensitivity, samples were tested for *A. cuspidata* and *P. clavata in multiplexed reactions,* while testing for *P. pristis* and *P. zirjson* occurred individually.

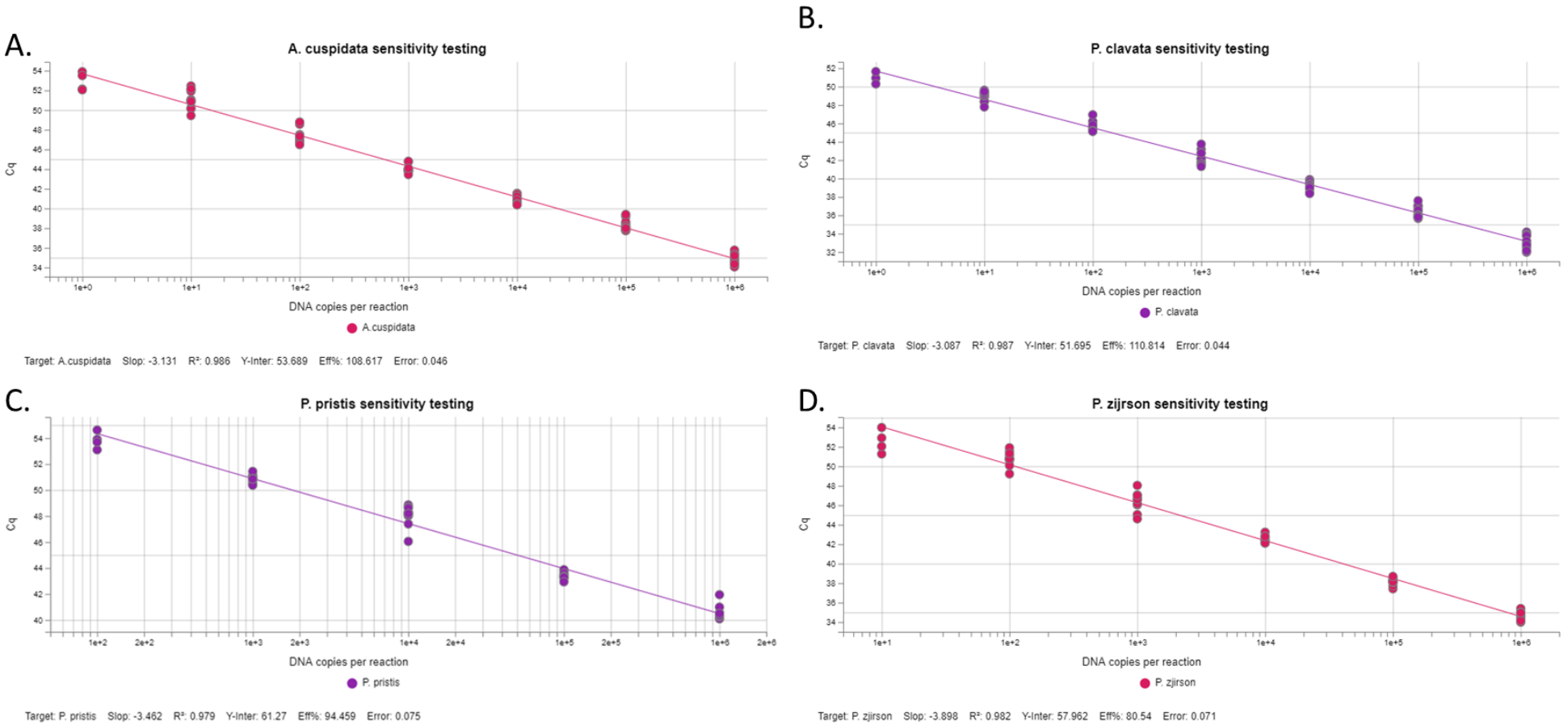

Figure 7. Sensitivity and standardisation of species-specific assays designed to estimate environmental DNA abundance for *Anoxypristis cuspidata* (A), *Pristis clavata* (B), *Pristis pristis* (C) *and Pristis zijsron* (D).

Saw fish species-specific detection

No amplification was observed for any samples across all species-specific assays in either neat or diluted sample aliquots designed to minimize inhibition, meaning that eDNA for the four sawfish species was not detected in any of the samples using this method . Positive controls showed amplification for all tests while negative and field controls (i.e. NTC) also performed as expected. Inhibition testing showed amplification for the internal positive control in each single replicate of eDNA field collected samples, suggesting no significant factors were affecting reaction performance.

Metabarcoding results

All samples showed amplification using the Fish F1, F2 and COI primer set. A 1:10 dilution was found to be most optimal for approximately 60 % of filter eDNA samples and 55 % of sediment collected samples. Gel electrophoresis suggested a range of product sizes, as expected for a broad COI primer set. A total of 13,790,261 raw reads were obtained by Illumina sequencing for 86 environmental samples, six field negative controls, three extraction negative controls and three non-template plate controls. Following read denoising and filtration for quality, a total of five environmental samples failed quality controls and were removed from the analysis (see highlighted in red, Table 3). There was a significantly higher number of curated reads in water samples (average ±SD= 71,429 ± 32,561) compared to sediment samples (40,311 ± 42,906) (Figure 8). There was amplification of DNA in extraction and field-negative controls (see highlighted in yellow, Table 3). Amplification in these controls corresponded to Sequence variances with 99.6% pairwise similarity to human DNA and bacteria, presumably associated to handling during sample collection and processing. No amplification for fish, sharks or rays was observed in any of the controls.

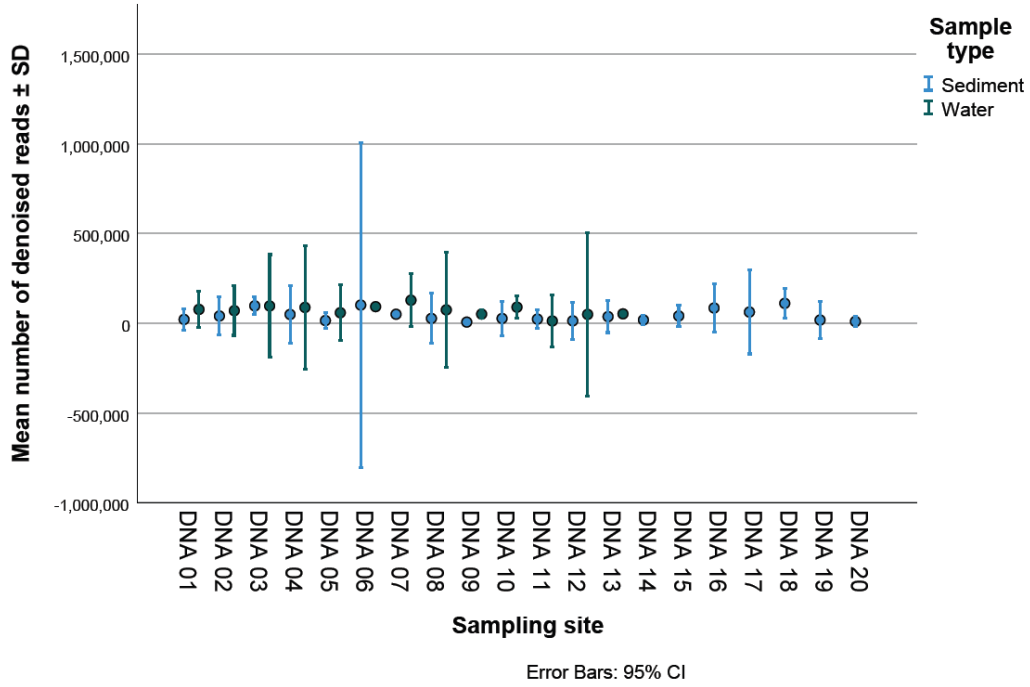

Figure 8. Mean number of curated reads obtained from sediment (blue) and water (green) samples collected across 20 different sites. Mean number of reads were significantly higher in water samples compared to sediment samples (ANOVA, $F_{1,75}$=19.659, p<0.001).

There was no detection for either *Glyphis garricki* or *Glyphis glyphis* in any of the samples of this study. Interestingly, a total of 55 fully curated reads were detected with 100% pairwise similarity to *Anoxypristis cuspidata* in sediment samples from DNA 03 (Figure 1). Although the species-specific assay optimised to detect showed no detection of DNA in the sediment sample, it is possible that the dilution and normalization steps taken as part of creating sequencing pools for high-throughput sequencing, would have boosted the low signal of *A. cuspidata* DNA. Detection for *A. cuspidata* at such a low level could indicate the presence of old DNA associated with possible historical occurrence of the species in the area, but not current occurrence, or the current presence of the species in the area in low abundance.

DELIVERABLE SUMMARY

1. A total of 60 sediment samples, and 26 water samples were collected across 20 separate locations within the Cambridge gulf. In total, 93 eDNA samples including field and negative controls were processed for analysis. A total of 26 water samples passed quality control with mean DNA yield (± STD) = 22.46 ± 18.56 ng/µL. Similarly, 60 sediment samples passed initial quality control with mean DNA yield (± STD) = 30.99 ± 46.51 ng/µL. As expected, DNA purity across samples was below the accepted 1.6 ratio, a common condition of DNA extracted from high turbidity estuary systems such as the Cambridge Gulf.
2. Species-specific assays for all sawfish species were optimised and calibrated. Sensitivity testing showed that two assays could be successfully multiplexed for eDNA testing purposes. The *A. cuspidata* and *P. clavata* assays reported LOQ at 10 copies/reaction and replicated efficiencies reported from the source literature of 108.6% and 110.8% respectively. The *P. pristis* assay showed equally high efficiency and reported a LOQ of 1000 copies/reaction. Efficiency of the *P. zirjson* assay was reported as 80.54 % with an assay LOQ of 100 copies/reaction.
3. A total of 13,790,261 raw reads were obtained by Illumina sequencing for 86 environmental samples, six field negative controls, three extraction negative controls and three non-template plate controls. A total of five environmental samples failed quality controls and were removed from the analysis (see highlighted in red, Table 3).

MAIN OUTCOMES

1. There was no detection for *Anoxypristis cuspidata*, *Pristis clavata*, *Pristis zijsron*, or *Pristis pristis* across any site when using the species-specific assays of this study.
2. There was no detection for *Glyphis garricki*, *Glyphis glyphis* across any site when using the metabarcoding assay.
4. A low number of *Anoxypristis cuspidata* sequence reads were detected in site 03 by metabarcoding. This is a very low amount of DNA being detected, which could indicate the presence of old DNA associated with possible historical occurrence of the species in the area, or the current presence of the species in the area in very low abundance.

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APPENDIX

Table 2. Synthetic gBlock oligonucleotides used to standardise all species-specific assays to estimate abundance. Internal variation of the prescribed amplicon sequence to discard control cross-contamination is highlighted in bold.

Species	Sequence 5'- 3'
Anoxypristis cuspidata	GTGCCCCAGACCCACCTAGAGGAGCCTGTTCTATAACCGATAATCCCCGTTAAACCTCACCACTTCTTGCCACTAACCG CCTATATACCGCCGTCGTCAGCTCACCCCAT**AAAACAAGGGAG**GTAAGCAAAATGGATTAACCTCCAACACGTCAG GTCGAGGTGTAGCGAATGAAGTGGA
Pristis clavata	GCTTCAAACCCAAAGGACTTGGCGGTGCCTTAGATCCACCTAGAGGAGCCTGTTCTATAACC**CCTAATAG**CCGTTAA ACCTCACCATTTCTTGCTATCAACCGCCTATATACCGCCGTCGTCAGCTCACCCCATGAGGGAACAAAAGTAAGCAAAA AGAACCCACCTTCAATACGTCAGGTCGAGGTGTAGC
Pristis pristis	TTCAAACCCAAAGGACTTGGCGGTGCCTCAGACCCACCTAGAGGAGCCTGTTCTATAACCGATAATCCCCGTTAAACC TCACCACTTCTTGCCATCAACCGCCTATATACC**TGCTGCCG**CAGCTCACCCCATGAGGGAACAAAAGTAAGCAAAAT GAACTAACCTTCAATACGTCAGGTCGAGGTGTAGCGAATGAAGTGGAAAGAAATGGGCTACATTTTCTCCTAAGAAA AAACGAACAGTATGATGAAAAACTACTT
Pristis zijsron	ACCCAAAGGACTTGGCGGTGCCTTAGATCCACCTAGAGGAGCCTGTTCT**AAATAGCCA**ATTCCCCGTTAAACCCCAC CACTTCTTGCTATCAACTGCCTATATACCGCCGTCGTCAGCTCACCCCATGAGGGGTTAAAAGTAAGCAAAATGAATCT ATCTTCAATACGTCAGGTCGAGGTGTAGCGAATGAA

Table 3. Sequenced reads and numbers remaining after each filtration step during metabarcoding analysis. Samples removed from the analysis due to quality control failure are highlighted in red. Negative control samples are highlighted in yellow.

Sample ID	Sample type	Sampling site	Raw reads	Filtered (Prhed>30 & length)	Paired reads	Chimeric sequence removal
DNA 01_1	Water	DNA 01	180445	174057	159046	84781
DNA 01_2	Water	DNA 01	165839	159443	132898	68810
DNA 01_3	Sediment	DNA 01	67406	60422	10354	10119
DNA 01_4	Sediment	DNA 01	182597	173055	50066	47965
DNA 01_5	Sediment	DNA 01	99544	92014	9565	5031
DNA 02_6	Water	DNA 02	174584	168323	152156	80788
DNA 02_7	Water	DNA 02	135829	128843	88138	59175
DNA 02_8	Sediment	DNA 02	67080	61918	5783	3332
DNA 02_9	Sediment	DNA 02	75994	71998	33254	29692
DNA 02_10	Sediment	DNA 02	174068	166710	136637	87313
DNA 03_12	Water	DNA 03	194567	186473	161122	73617

DNA 03_13	Water	DNA 03	275007	263466	207424	118703
DNA 03_14	Sediment	DNA 03	176417	169076	147755	99790
DNA 03_15	Sediment	DNA 03	178997	169077	143511	76099
DNA 03_16	Sediment	DNA 03	248422	236585	193971	114756
DNA 04_17	Water	DNA 04	228400	221166	201930	115044
DNA 04_18	Water	DNA 04	136865	132305	129285	60953
DNA 04_19	Sediment	DNA 04	176728	129170	108737	36370
DNA 04_20	Sediment	DNA 04	241150	226082	87154	61498
DNA 05_22	Water	DNA 05	155481	149610	99101	70685
DNA 05_23	Water	DNA 05	116800	110779	93874	46284
DNA 05_24	Sediment	DNA 05	67935	64497	5465	3651
DNA 05_25	Sediment	DNA 05	68343	61912	13136	5766
DNA 05_26	Sediment	DNA 05	90525	78821	47072	34905
DNA 06_26	Water	DNA 06	166466	158219	123761	92222
DNA 06_27	Sediment	DNA 06	406409	383310	284962	172421
DNA 06_28	Sediment	DNA 06	159837	152310	69734	30088
DNA 07_30	Water	DNA 07	279911	268209	238296	116452
DNA 07_31	Water	DNA 07	306264	295326	273911	139528
DNA 07_32	Sediment	DNA 07	183363	171617	87481	50001
DNA 07_33	Sediment	DNA 07	66442	61042	3	3
DNA 08_35	Water	DNA 08	147358	140292	106704	49358
DNA 08_36	Water	DNA 08	223936	215622	197692	99593
DNA 08_37	Sediment	DNA 08	101695	95276	39204	37684
DNA 08_38	Sediment	DNA 08	119419	108209	29624	15751
DNA 08_39	Sediment	DNA 08	86498	81248	0	0
DNA 09_41	Water	DNA 09	147606	137794	121072	48974
DNA 09_42	Water	DNA 09	99487	92810	66149	52534
DNA 09_43	Sediment	DNA 09	72791	66779	20708	10436
DNA 09_44	Sediment	DNA 09	95814	89251	9438	7037
DNA 09_45	Sediment	DNA 09	42506	37893	3101	1673
DNA 10_47	Water	DNA 10	280994	269288	219753	84561
DNA 10_48	Water	DNA 10	281973	268888	208402	94419
DNA 10_49	Sediment	DNA 10	130133	120623	72331	71584
DNA 10_50	Sediment	DNA 10	124535	92953	11469	5401
DNA 10_51	Sediment	DNA 10	132512	121760	2318	2318
DNA 11_52	Water	DNA 11	68547	61949	1374	1317
DNA 11_53	Water	DNA 11	106740	101475	31810	23991
DNA 11_54	Sediment	DNA 11	96225	64722	15840	7235
DNA 11_55	Sediment	DNA 11	97059	86223	19880	14068

DNA 11_56	Sediment	DNA 11	196950	188443	101659	47264
DNA 12_57	Water	DNA 12	80632	69858	28296	13521
DNA 12_58	Water	DNA 12	161066	154161	100344	84993
DNA 12_59	Sediment	DNA 12	59440	54947	10517	5258
DNA 12_60	Sediment	DNA 12	243758	228365	34089	21304
DNA 12_61	Sediment	DNA 12	72048	65791	27	27
DNA 13_63	Water	DNA 13	171687	163494	118403	52938
DNA 13_64	Water	DNA 13	173492	162925	113254	51563
DNA 13_65	Sediment	DNA 13	168682	161569	136902	78020
DNA 13_66	Sediment	DNA 13	141645	128079	36247	15194
DNA 13_67	Sediment	DNA 13	209869	196204	17131	17131
DNA 14_68	Sediment	DNA 14	178049	168419	37413	16734
DNA 14_69	Sediment	DNA 14	78640	46914	14221	9529
DNA 14_70	Sediment	DNA 14	102902	96204	40989	28630
DNA 15_71	Sediment	DNA 15	201508	190214	104348	48033
DNA 15_72	Sediment	DNA 15	193081	180524	62301	59787
DNA 15_73	Sediment	DNA 15	107206	89210	32431	14487
DNA 16_74	Sediment	DNA 16	134855	131083	114831	114831
DNA 16_75	Sediment	DNA 16	107204	100944	43081	21612
DNA 16_76	Sediment	DNA 16	131621	125545	118257	118225
DNA 17_77	Sediment	DNA 17	258084	248765	203608	170809
DNA 17_78	Sediment	DNA 17	41286	31054	9231	4842
DNA 17_79	Sediment	DNA 17	118219	108899	22847	11598
DNA 18_80	Sediment	DNA 18	246074	239555	211034	117261
DNA 18_81	Sediment	DNA 18	48051	44223	32	32

DNA 18_82	Sediment	DNA 18	235868	226467	196688	104057
DNA 19_83	Sediment	DNA 19	174598	158829	20262	9788
DNA 19_84	Sediment	DNA 19	88449	83570	0	0
DNA 19_85	Sediment	DNA 19	159570	149991	29160	26003
DNA 20_86	Sediment	DNA 20	165957	154183	45247	21693
DNA 20_87	Sediment	DNA 20	59968	54594	5008	2388
DNA 20_88	Sediment	DNA 20	112435	103245	4786	4786
EX-1-34_S91	Extraction negative	Na	93321	62558	37713	14781
EX-2-35_S92	Extraction negative	Na	65420	60035	46499	44141
EX-3-36_S93	Extraction negative	Na	37350	34757	1925	1618
Neg control (10,11,12) _62	Neg control (10,11,12) _62	Neg control_62	102504	97837	32677	32677
Neg control (13) _68	Neg control (13) _68	Neg control_68	138116	130442	34161	24047
NEG control (DNA 01 & 02) _11	Water	NEG control_11	216828	207457	204228	98621
NEG control (DNA 03,04,05) _27	Water	NEG control_27	180743	171497	148892	69768
NEG control (DNA 06,07,08) _40	Water	NEG control_40	151662	138460	57028	28816
NEG control (DNA 09) _46	Water	NEG control_46	246949	234769	202091	137626

NTCPLATE-1-30_S30	Plate negative control	Na	30427	28239	6410	2865
NTCPLATE-3-31_S88	Plate negative control	Na	186064	176924	150742	144443
NTCPLATE-4-37_S94	Plate negative control	Na	14440	11789	0	0