**Title:** Development of a sensitive detection method using oceanic seawater samples and quantitative PCR: a case study of devil ray eDNA at seamounts

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**ABSTRACT**

Environmental DNA (eDNA) is increasingly being used in aquatic environments for monitoring species, particularly those that are of conservation concern and/or are difficult to visually observe. Quantitative PCR (qPCR) has been employed to detect low abundance species occurring in environmental water samples. However, the qPCR technique has principally been applied to freshwater habitats, with less application to pelagic marine environments. We developed a species specific eDNA assay for the Chilean devil ray, *Mobula tarapacana* to assess the capability of using eDNA to detect transient pelagic marine animals. For this pilot study, seawater samples taken at seamounts around the Azores (NE Atlantic) were tested in order to determine the suitability of this approach for detecting the target species. Samples were taken at sites where *M. tarapacana* has been previously observed, in addition to sites where its presence is not known. eDNA detection was compared to observations carried out on the same day as water sampling. The qPCR assay successfully detected *M. tarapacana* at four of five seamount sampling opportunities where the species was observed, and there is a statistically significant relationship between genetic and visual detection. Target DNA was found at one location between seamounts in the absence of visual observation. Our results highlight the importance of physical environmental factors in relation to sampling eDNA in the ocean, such as currents and eDNA dispersal ability. This method has been shown to be sensitive for detection of *M. tarapacana* DNA in seawater and therefore for the identification of important seamounts requiring conservation.

**INTRODUCTION**

Seamounts are globally important marine ecosystems where productivity, biomass and biodiversity of marine life thrives (Pitcher et al. 2007). Seamounts are common throughout the Azores archipelago (NE Atlantic Ocean), resulting from volcanic activity that characterises the region (Morato et al. 2008). The Chilean devil ray, *Mobula tarapacana*, is a large, fast, and deep-swimming animal that is known to form aggregations at some seamounts in the Azores (Sobral and Afonso 2014). *M. tarapacana* is IUCN Red Listed as Vulnerable (Pardo et al. 2016) and it is extremely susceptible to threats such as fishing, both as by-catch and as targeted fisheries (Poortvliet et al. 2014). This species is difficult to observe in the wild, and difficult to sample without restraining, and potentially injuring or killing individuals (Sobral and Afonso 2014; Thorrold et al. 2014). Furthermore, species identification in the family Mobulidae is problematic due to similar external characteristics in many species (Couturier et al. 2012). The ability to correctly and non-invasively identify *M. tarapacana* in the marine environment would provide spatial and temporal distribution data that is currently lacking for this species, and would allow the identification of seamounts of conservation importance.

Environmental DNA (eDNA) is the collective term for all DNA molecules released from organisms into the environment (Taberlet et al. 2012). eDNA techniques are non-invasive, rapid and relatively inexpensive and rely on DNA detected in the environment as a source of information. As a result, eDNA techniques have vast potential for monitoring programmes, especially in remote systems where traditional surveying methods are not feasible or cost-effective. eDNA often degrades into small fragments within hours to weeks (Dejean et al. 2011; Pilliod et al. 2014; Piaggio et al. 2014), and is therefore thought to provide approximately real-time data on species’ presence in the environment.

For targeted species detection, Polymerase Chain Reaction (PCR) is employed using primers that are designed to specifically amplify a portion of the target DNA sequence. Conventional PCR has been used for specific eDNA detection (Dejean et al. 2011; Piaggio et al. 2014; Davison et al. 2016; Simpfendorfer et al. 2016). However, quantitative (q)PCR offers a distinct advantage over traditional endpoint PCR techniques, as the addition of a florescent dye (e.g. SYBR™ Green) or a fluorescently labelled reporter probe allows the amplification of the target sequence to be monitored in real time by the qPCR instrument. Quantification is measured against a standard curve, run simultaneously with samples with a known concentration of reference DNA (Bourlat et al. 2013). Probe-based qPCR increases both specificity and sensitivity, as the use of a probe with forward and reverse primers ensures that there are three sequences to check against the target template DNA (Herder et al. 2014).

In terms of aquatic eDNA research, there has been considerable research in freshwater systems (e.g. Ficetola et al. 2008; Dejean et al. 2011; Jerde et al. 2012; Takahara et al. 2012; Laramie et al. 2014; Gustavson et al. 2015; Bylemans et al. 2016). Relatively few have been focussed on qPCR for targeted species detection in seawater samples (e.g. Foote et al. 2012; Thomsen et al. 2012; Kelly et al. 2014; Sigsgaard et al. 2016), and many of these have been carried out in controlled environments, such as aquarium tanks (Kelly et al. 2014), or inshore environments (Foote et al. 2012; Thomsen et al. 2012). These techniques have not yet been assessed for surveying biodiversity rich seamounts.

This study aims to address gaps in eDNA research by evaluating the capability of detecting a rare and threatened species, *M. tarapacana,* in oceanic seawater samples from highly biodiverse seamount environments. The ability to detect *M. tarapacana* DNA in seawater would provide a monitoring tool capable of delineating the spatial and temporal distribution of this poorly understood species and therefore be used to identify important seamounts for conservation.

**MATERIALS AND METHODS**

*eDNA sampling and extraction*

Water samples were collected above the summits of five seamounts, as well as from four far-field stations (open ocean) in the autumn of 2014 (Figure 1). Water was sampled from near to the surface (from 5-10 m depth) due to the tendency of *M. tarapacana* to be found basking in surface waters before and after foraging at greater depths of up to 2000 m (Thorrold et al. 2014). During each sampling event, samples containing 5 L of seawater were collected using Niskin bottles. The sampling bottles were single use and were rinsed with water from the sampling site several times, prior to collecting the sample. Visual observations of *M. tarapacana* were made during each sampling event, to facilitate validation of eDNA detection. Figure 1 (c) and Table 1 shows the sample details and location references for sampling events.

Three of the studied seamounts are known for attracting pelagic biodiversity, including seasonal aggregations of *M. tarapacana*: Princess Alice (location reference 5), Ambrosio (location reference 8), and Dollabarat seamounts (location reference 9). Seamount “127” and Gigante seamounts are located on the Mid-Atlantic Ridge, and little is known about their biodiversity due to their depth and location (location references 2 and 4, respectively). A total of 28 water samples were collected (Table 1), with at least one water sample taken from each location. Multiple samples were taken at some locations over an extended sampling period to coincide with the appearance of *M. tarapacana* at known aggregation sites.

Samples were frozen at -20 oC to prevent eDNA degradation. After thawing, 3 L samples were vacuum filtered through sterile Whatman 47mm diameter, 0.45µm pore size nylon filters. These were stored in 100% molecular grade ethanol and shipped to University College Dublin, Ireland for DNA extraction and genetic analysis. DNA was extracted from half filters using QIAshredder (to homogenise the DNA found on the filter) followed by the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer’s instructions and eluted in a final volume of 50µl using AE buffer (supplied with kit). Extraction blanks were included to test for contamination during this process. eDNA concentrations were determined using spectrophotometry (Biodrop; Online Resource 1). To decrease risk of contamination, sterile filtering equipment, multiple-glove changes, and separate dedicated lab spaces for eDNA extraction, pre-PCR and post-PCR processing were employed.

An additional three replicate 3 L water samples were taken from surface waters at Dublin Bay, Ireland (Figure 1(b) and Table 1) during autumn 2015 as a negative control, as there is no known record of *M. tarapacana* in Dublin Bay. A 3 L deionised water sample was taken into the field during sample collection to test for any possible contamination issues in the field or during sample processing. Samples were collected and processed following the same protocol as the Azores water samples.

*qPCR assay development*

Tissue was acquired from three *M. tarapacana* individuals from the study area. The mitochondrial *COI* gene was amplified using primers *FishF2* and *FishR2* (as described in Ward et al. 2005) and the PCR product was Sanger sequenced using a commercial facility (Macrogen, Netherlands). The resulting sequences were visualised in Geneious (R8, Biomatters Limited), along with publicly available sequences on GenBank, to generate a consensus sequence incorporating intraspecific variability in the *COI* region for primer and probe design.

Species-specific primers and a minor-groove binding (MGB) probe incorporating a 5’ FAM reporter dye and a 3’ nonfluorescent quencher were designed to amplify an 86bp region of *COI* for the target species, using Primer Express (V3.0, Life Technologies; Table 2). The specificity of the generated primers and probe were checked against the NCBI database. Although there appears to be a general lack of interspecific variability for *COI* between *M. tarapacana* and other members of the Mobulidae (the forward primer matches the related species *Mobula japanica*, as well as *M. mobular*; Figure 2), there are at least four mismatches on the reverse primer and five mismatches on the probe.

Tissue samples from individuals of *M. japanica*, as well as other co-occurring species from the Atlantic were acquired (*Dasyatis pastinaca* and *Raja undulata*), and the extracted DNA was tested with the designed forward and reverse primers using conventional end-point PCR under the following cycling conditions: initial denaturation at 95oC for 2 min followed by 35 cycles of 95oC for 30 s, annealing at 60oC for 30 s and extension at 72oC for 1 min. A final extension step was carried out at 72oC/2 min. PCR products were visualised on a 2% agarose gel stained with SYBR® Safe (Life Technologies) and a 50bp ladder (Qiagen).

To test for efficiency in the qPCR assay, SYBR™ Green (Life Technologies) was first used to detect amplification of any double stranded DNA using the primers and tissue-derived DNA from target and non-target species. SYBR™ Green PCR reactions were carried out in a 10 µl reaction volume containing 5 µl Fast SYBR™ Green Master Mix, 1 µl of each forward and reverse primer (2 µM; Applied Biosystems) and 3 µl of template DNA. The following cycling conditions were employed: 95oC for 20 s followed by 40 cycles of 95oC for 1 s and 60oC for 20 s. A melt curve was generated using 95oC for 15 s, 60oC for 60 s and 95oC for15 s.

Optimisation of the qPCR assay utilising the probe was carried out using *M. tarapacana* tissue-extracted DNA, as well as DNA extracted from other Rajiformes species (*M. japanica*, *D. pastinaca* and *R. undulata*). The qPCR consisted of a 30 µl reaction volume containing 15 µl of Bioline SensiFAST™ Probe Lo-ROX (Bioline), 3 µl of each primer (2 µM), 2.4 µl of probe (2.5 µM; Applied Biosystems) and 3 µl of template DNA. The PCR program consisted of 50oC for 2 min, 95oC for 3 min, followed by 40 cycles of 95oC for 5 s and 65oC for 20 s. All qPCR reactions were carried out using QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems).

*qPCR assay deployment using field samples*

Once assay optimisation was complete, eDNA samples were run in triplicate in the qPCR assay, along with technical blanks and a 7-point serial dilution (10:1) of muscle tissue-extracted DNA from the target species. Concentrations for the serial dilution ranged from 7.5 ng to 7.5 x 10-6 ng (or 0.0075 pg) of DNA in each reaction based on spectrophotometer quantification (Biodrop). The standard curve for *M. tarapacana* (y = -3.391x+39.161, r2 = 0.997, efficiency = 97%) was generated using 3µl of template in a total reaction volume of 30 µl. The lowest detectable concentration using the standard curve was determined to be 0.25 pg/µl at a C*q* of 37.3. A positive detection was established to be any sample that amplified in at least two out of three technical replicates, was within the dynamic range of the standard curve, and was further verified by cloning and subsequent sequencing. Where multiple samples were taken at a location, a positive genetic detection in any one sample was taken as a positive genetic detection for that sampling event.

Cloning was carried out in order to verify positive detections in the qPCR assay, using the TopoTA Cloning Kit (Life Technologies) according to the manufacturer’s instructions. PCR product from one replicate of each of the positive samples was cloned (*n* = 8) and a single clone resulting from each reaction was purified and sequenced to verify the presence of the target species. Plasmid DNA was purified using the Purelink Quick Plasmid Miniprep Kit (Life Technologies). This was followed by screening of inserts by PCR using the target primers (*Mtar-F/Mtar-R*). The construct was Sanger sequenced using M13 Forward (-20) and M13 Reverse primers at a commercial facility (Macrogen, Netherlands). The resulting sequences were aligned against a reference consensus *COI* sequence in Geneious and they were also validated against the NCBI database.

A G-test of independence was performed comparing detection results for the genetic and visual data (Table 1). Detection data were categorised into positive and negative occurrences for the 15 sampling opportunities (five cases of positive and ten cases of negative visual detection). We tested the hypothesis that the positive visual detections would be distributed equally among positive and negative genetic detections (low sensitivity) and that the negative visual detections would be similarly distributed (low specificity).

**RESULTS**

Conventional PCR methods utilising forward and reverse primers proved to be unspecific (amplifying DNA from other skates and rays) for this study. A band of the expected length was present for *M. tarapacana*, as well as all other species tested. This was not anticipated considering that there are mismatches occurring on the reverse primer (ranging from three to ten mismatches across species tested; Table 2). With the addition of the probe in the qPCR assay, it is expected that specificity would increase and only the target would be detected. The use of species-specific MGB probes has been shown to have higher fidelity than those solely relying on species-specific primers or TaqMan® probes not utilising the MGB group (Kutyavin et al. 2000). The SYBR™ Green qPCR assay to test for amplification using tissue-derived DNA from both target and non-target species was also shown not to be specific, which was in agreement with the conventional PCR analysis and showed amplification for all species. Addition of the probe to the qPCR assay resulted in greater specificity, with successful amplification of the target and no amplification for non-targets, indicating the probe-based assay was optimal for eDNA samples for the target species.

A total of eight out of the 28 Azores water samples showed a positive genetic detection for the target species. This corresponds to a positive genetic detection in five out of 15 sampling opportunities (Table 1). These were at Dollabarat and Princess Alice seamounts (location references 6 and 9, respectively) and at an ocean control site for Princess Alice (location reference 5). *M. tarapacana* had been observed at all of these sites during the sampling period, with the exception of the Princess Alice control site. However it should be noted that this detection was found at the ocean control site nearest to the seamount where *M. tarapacana* had been visually detected (Figure 4). No detectable target DNA was found at Gigante and Seamount 127. This species had not been observed in the area before (Figures 3 and 4). Ambrosio seamount samples also showed no signal for *M. tarapacana*, even though seawater samples were taken both before and after aggregations of the species had been observed during the visual census. No *M. tarapacana* DNA was found in samples taken from ocean control samples for any of these sites. Dublin Bay samples, field and extraction blanks were all negative, as were no template controls.

The concentration of target DNA found in samples was variable (Figure 3) with the concentration of target DNA detected at the Princess Alice seamount increasing over the sampling period. In all cases where PCR product was cloned, resulting sequences aligned with the *M. tarapacana* *COI* consensus sequence without any mismatches (Online Resource 2) an affirmative indication that *M. tarapacana* DNA is found in all samples with positive genetic detection. Sequences were also verified as matching 100% with *M. tarapacana* against the NCBI database. There was a statistically significant association between the visual and genetic detection (G = 9.289, p = 0.002), with four of the five positive visual detections also being detected genetically, and nine of the ten negative visual detections resulting in no genetic detection (Table 1 and Figure 4). Therefore we can reject the hypothesis that positive and negative genetic detections are random with respect to having observed or not observed *M. tarapacana* at the sampling locality.

The COI region was sequenced in three individuals of *M. tarapacana* from the Azores region showed two haplotypes. Two individuals showed a previously undocumented COI haplotype (GenBank Accession No. KY454873) that differed by one single nucleotide from the sequence data available for this species. However, this variant nucleotide was not located in either the primer or probe region.

**DISCUSSION**

eDNA is a potentially powerful, non-invasive tool for detecting the presence of low abundance and indicator species in seawater samples, as documented here for the Chilean devil ray at oceanic seamounts. This was accomplished through development of a species-specific probe-based qPCR assay for the detection of target DNA, followed by a pilot study to evaluate the capability of this method to determine the presence of the target organism in the field.

Conventional end-point PCR was first carried out on the eDNA samples to test for the presence of amplicons. It would be advantageous to use a conventional PCR method for detecting species, as there are significantly greater costs involved in the use of probe-based chemistries. While this method has been successfully used in some previous cases (Davison et al. 2016; Simpfendorfer et al. 2016) in the present case, it was insufficient to detect species-specific differences; therefore a probe-based system was adopted. Discovery of a discriminate short marker suitable for qPCR in elasmobranchs is hindered by their presumed slow mutation rates relative to other vertebrates (Dudgeon et al. 2012). Discrimination among elasmobranch species has been successful using a range of genetic markers, for example barcoding studies that utilise approximately 650bp of the *COI* region (Holmes et al. 2009), although this length of marker is unsuitable for qPCR, and will likely be unsuitable for most eDNA applications owing to the degradation of DNA into small fragments (Taberlet et al. 2012). The degradation DNA fragments might therefore preclude detection of species using end-point PCR products visualised on agarose gels. Other variable loci have been employed for species identification in elasmobranchs, such as cytochrome *b* and nicotinamide adenine dinucleotide (NADH)-ubiquinone oxidoreductase chain 2 genes (155 and 188 bp fragments, respectively; Hoelzel 2001) and 16S ribosomal DNA (280bp; Tinti et al. 2003). These markers were investigated during primer and probe design, however the only region found to be both sufficiently short and variable for *M. tarapacana* detection was *COI*. This probe based approach, in conjunction with qPCR in an optimised assay, ensured that DNA could reliably be detected in seawater samples in concentrations as low as 0.4pg/µl.

The detection of *M. tarapacana* DNA in four out of five seamount sampling opportunities with positive visual detection is a promising indication that this tool can be further developed with increased efficacy. A major challenge associated with eDNA detection techniques is the reliance on inferring species presence where little is known about the distribution of the target species. This is the case with *M. tarapacana*, as it is known to occur at some seamounts, but information is lacking regarding its ecology, wide-ranging movements or migration (Pardo et al. 2016). The presence of target DNA in open ocean control samples indicates that either the species DNA is being transported as a result of hydrographic factors into the study area, or this species was present, even if transiently, at the control site despite not being observed. Failure to detect target DNA at a location where they were observed (Ambrosio seamount) raises the possibility of inhibition in eDNA samples or heterogeneous distribution of eDNA molecules in the water, which could be mitigated by increasing the number of sample replicates (Shaw et al. 2016) and the use of an internal positive control to test for possible inhibition (Goldberg et al. 2016). Alternatively, failure to detect target DNA at this location could indicate that target DNA was transported away from the water sampling site before sampling was carried out, due to physical environmental factors such as currents and wave action.

The results of this study highlight the importance of a robust sampling regime. eDNA is subject to degradation and transport, which introduces uncertainty into the data collected (Goldberg et al. 2016). While studies focussing on elucidating the effects of both of these factors have been performed (Strickler et al. 2014; Deiner and Altermatt 2014), none has specifically investigated how degradation and transport of eDNA impacts on the use of eDNA for inferring species presence in open ocean environments. Studies focussing on these factors in the marine environment constitute an important avenue for future eDNA research. This, combined with visual observation data and presence/absence data based on eDNA, would be an essential precursor to establishing modelling approaches to improve our capabilities to identify important seamounts for conservation and our knowledge of the distribution of *M. tarapacana* both in the Azores and globally.

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**COMPLIANCE WITH ETHICAL STANDARDS**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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**TABLES AND FIGURES**

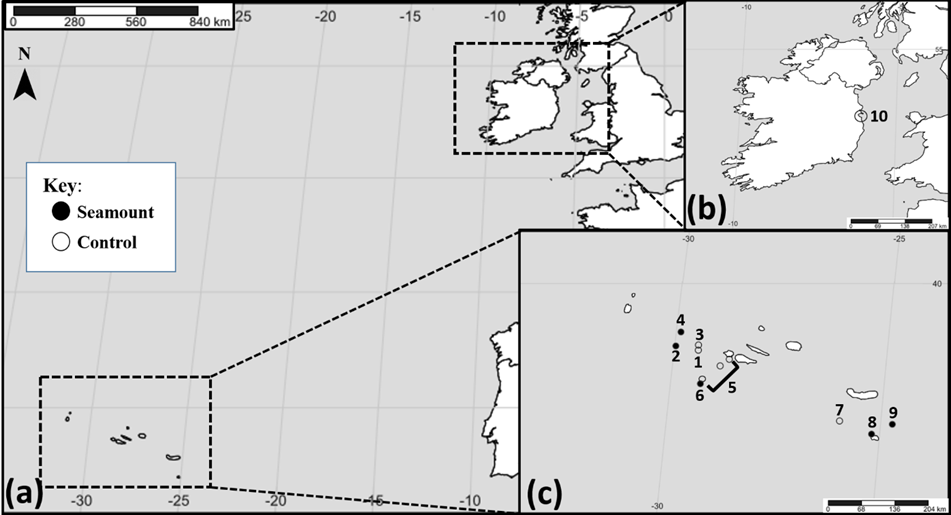


Figure 1: Map showing the wider sampling area encompassing Ireland and the Azores in the North East Atlantic (a). Also shown is Dublin Bay (b) and sampling locations in the Azores (c) with seamounts (black circles) and controls (open circles) displayed. Location numbers are shown. Further sampling details are found in Table 1.

**Mtar-F** 1 AACCACCTGCAATCTCTCAATATCA------------------------------------------------------------- 86  
**Mtar-R** 1 ---------------------------------------------------------CAACTGTCCTTCTATTATTATCTCTTCCC 86  
**Mtar-PR** 1 -------------------------------CTTGTTTGTTTGATCAATTC----------------------------------- 86  
***M.tarapacana*** 1 AACCACCTGCAATCTCTCAATATCAAACGCCCTTGTTTGTTTGATCAATTCTAATCACAACTGTCCTTCTATTATTATCTCTTCCC 86  
***M.japanica*** 1 AACCACCTGCAATCTCTCAATATCAAACACCATTATTTGTCTGATCTATCCTAATTACAACTGTCCTTCTCTWATTATCCCTCCCA 86  
***M.mobular*** 1 AACCACCTGCAATCTCTCAATATCAAACACCATTATTTGTCTGATCTATCCTAATTACAACTGTCCTTCTCTTATTATCCCTCCCA 86  
***M.birostris*** 1 AACCACCTGCAATCTCTCAGTATCAAACACCCTTATTCGTTTGATCAATTCTAATCACAACTGTCCTTCTCTTATTATCCCTCCCC 86  
***R.undulata*** 1 AACCACCAGCAATCTCTCAATACCAAACACCCCTATTCGTCTGATCAATCCTTGTTACAACTGTCTTACTTCTTATGGCCCTTCCA 86  
***D.pastinaca*** 1 AACCCCCYRCAATYTCCCAATATCARACACCTCTMTTCGTYTGATCTATTCTCATTACAACAGTYCTCCTTTTAYTATCACTCCCA 86

Figure 2: Primers (*Mtar*-F and *Mtar-R*) and probe (*Mtar-PR*) designed for targeted detection of *M. tarapacana* are shown aligned the consensus sequence, as well as consensus sequences for co-occurring species from the order Rajiformes. Mismatches are highlighted by shading.

Table 1: Comparison of visual observation and genetic detection of *Mobula tarapacana* using qPCR from each sampling location. PCR products resulting from genetic detections were sequenced for verification.



Table 2: Details of species-specific primers and the probe designed to amplifly an 86bp fragment of the COI gene of *Mobula tarapacana*.

|  |  |  |  |
| --- | --- | --- | --- |
| **Primer Name** | **Type** | **Length (bp)** | **Primer sequence 5'-3'** |
| *Mtar-F* | Forward Primer | 24 | AACCACCTGCAATCTCTCAATATC |
| *Mtar-R* | Reverse Primer | 24 | GGGAAGAGATAATAATAGGACAGT |
| *Mtar-PR* | Probe\* | 20 | CTTGTTTGTTTGATCAATTC |

\*minor-groove binding (MGB) probe incorporating a 5’ FAM reporter dye and a 3’ nonfluorescent quencher

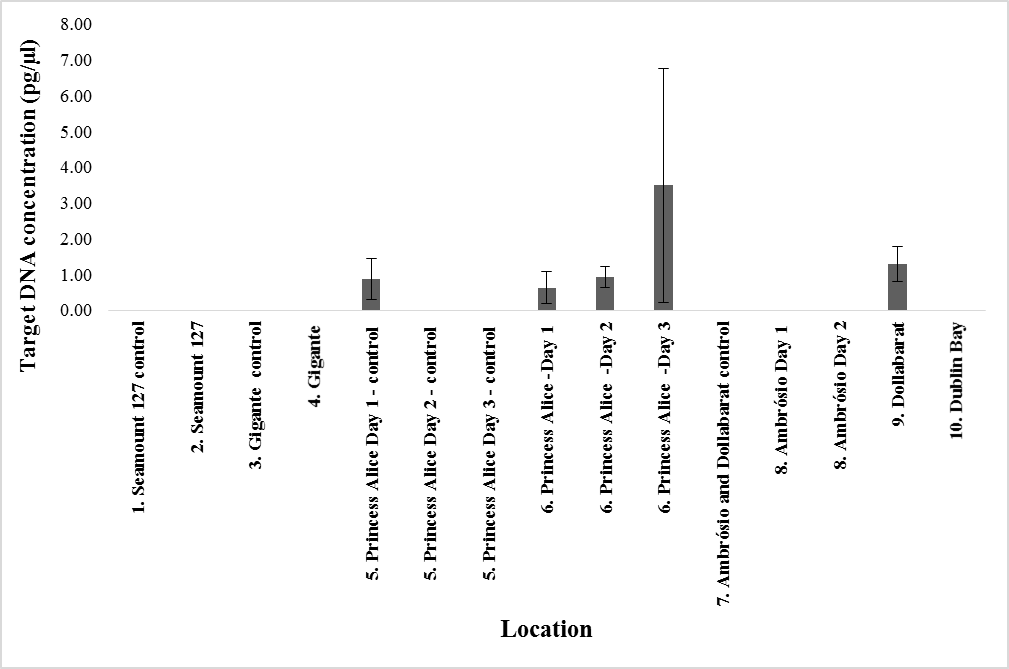


Figure 3: The average concentration of *Mobula tarapacana* DNA found at each site with 95% confidence intervals.



Figure 4: Map of the Azores, showing observational and/or genetic detection of the target species at sampling sites. Where multiple samples were taken at sites, observational and genetic data are combined. Numbers refer to the location reference seen in Table 1.