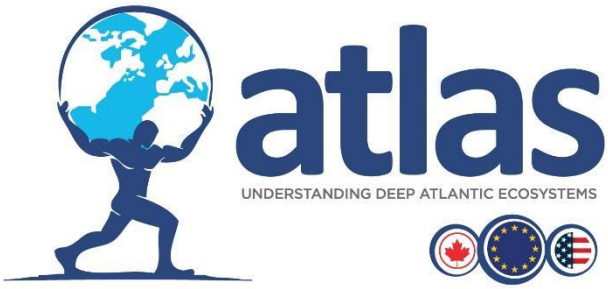

Potential and limits of metabarcoding of eDNA and qPCR

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1. Executive summary

Environmental (e)DNA methods (quantitative PCR and metabarcoding) are non-invasive, rapid and cost-efficient tools for detecting single species and monitoring biodiversity with considerable potential for informing aquatic conservation and management. Methods for implementing eDNA are constantly developing and these tools have received significant interest from industry. There have been substantial efforts to develop best practice approaches, standardisation and workflows (c.f. COST-action DNAqua-Net CA15219) that might ultimately complement or replace existing methods and develop new metrics for the implementation of the European Water Framework Directive. These eDNA based methods also have the potential to contribute to the implementation of the Marine Strategy Framework Directive. While the use of eDNA in freshwater has received by far the most attention, there is great potential for using eDNA in the marine environment to address a wide range of questions using non-invasive sampling; ranging from spatial and temporal biodiversity assessments, to assessing distribution patterns and range expansions/contractions of single species. In the ATLAS project, WP3 focused on evaluating the accuracy and sensitivity of meta-barcoding and qPCR methods with the objective of selecting a set of primers amplifying distinct DNA fragments to optimise metabarcoding across the Tree of Life, covering a maximum number of lineages, and developing species-specific probes for PCR detection of VME indicator species, fishery targets and bycatch species.

The emergence of eDNA tools to assess marine biodiversity and detect specific target marine species has generated great hopes to describe biodiversity of ecosystems that have been difficult to access (e.g. deep-sea habitats); as sampling of water and sediments is relatively simple as compared to traditional methods requiring specialised equipment (ROV, camera sledges, fishery gear, etc.). Nevertheless, few examples of such applications existed and even fewer, if any, in the deep sea. The great challenges for using eDNA techniques to assess deep-sea biodiversity are to obtain DNA from more or less blindly collected, low biomass taxa and subsequently low DNA concentration seawater or sediment samples. University College Dublin and IFREMER were tasked with evaluating the performance of next-generation genomic tools (metabarcoding of eDNA) for assessing biodiversity and quantitative qPCR (plankton samples) as a sensitive tool to detect and quantify biomass of target species. The accuracy and sensitivity of metabarcoding and qPCR will be validated on samples assessed using classical taxonomy in selected Case Studies. In respect of the development of qPCR assays, six target species were selected for assay development. Quantitative (q)PCR assays successfully detected and semi-quantified five target species showing that despite extremely low DNA concentration and the large volumes of water in which these species are found, eDNA is a very sensitive tool offering a promising method for detection of target species in the marine environment, including the deep sea. However, it was not possible to develop an assay for *Lophelia pertusa* due to low polymorphism usually encountered at mitochondrial DNA for scleractinian corals, and the lack of existing sequence data from closely related species. The metabarcoding efforts by IFREMER resulted in the development of six complementary sets of primers capable of assessing biodiversity from deep-sea sediments across the entire Tree of Life. In general, metabarcoding protocols were capable of characterising biodiversity of low biomass deep-sea sediments; even for understudied deep-sea metazoan taxa. These protocols were applied to 350 samples collected during the ATLAS-MEDWAVES cruise,

demonstrating the sensitivity of metabarcoding in deep-sea habitats. The development of eDNA species-specific assays and metabarcoding methods demonstrate the utility of eDNA-based methods for assessing and managing deep-sea biodiversity. Further, in line with the successful deployment of these tools in freshwater and in marine waters as demonstrated in this WP, these approaches could also be used to supplement or replace traditional methods such as morphology-based biodiversity used in the marine environment, including in the deep sea where specimens can be extremely small and difficult to identify (c.f. Danovaro *et al*. 2016). Similarly, future and current applications of eDNA include biodiversity assessments and baselines for Environmental Impact Studies of deep-sea industry operations such as mineral extraction (Boschen *et al*. 2016). We have demonstrated the usefulness of eDNA methods in the deep sea despite the great challenges they represent in terms of accessing samples and often low concentration of biomass.

2. Background to environmental DNA

Environmental (e)DNA is the collective term for DNA molecules that are released from living or dead organisms into the environment, which can come from sources as diverse as blood, skin, mucous, sperm, eggs and faeces. Subsequently DNA can be extracted from an environmental sample such as water, air or soil (Taberlet *et al*. 2012; Ruppert *et al*. 2019). Techniques employing eDNA can be completely non-invasive (water samples), or semi non-invasive (plankton and sediment samples), relying on DNA found in the environment as a source of information. As a result, eDNA is emerging as a valuable tool for biodiversity monitoring, especially where traditional surveying methods (e.g. transect counting, trapping, netting, trawling, electrofishing, visual observation, etc.) may not be feasible. This is especially true in marine ecosystems, where an organism's presence is concealed by water, and even more so in deep-sea environments that can be difficult to access and sample due to their vast extent and great depths. Traditional survey methods require large samples to be collected in challenging conditions, for instance in the deep sea, and may injure the target species or damage the surrounding environment. This often conflicts with the reasons for surveying in the first place. In addition, such techniques can be costly, require considerable effort and may be insensitive for cryptic or rare species, as well as requiring specialist knowledge to identify species once they have been observed or sampled (Thomsen *et al.* 2012). Besides, eDNA provides a minimalist sample and a standardisised methodology (i.e. avoiding observer effect) that can be interrogated for the presence of DNA from target species or for assessing biodiversity (Ji *et al.* 2013) not only on organisms present in the sample but for any species leaving traces of their presence (sloughed tissue, faeces, etc.).

For these reasons, eDNA is becoming increasingly used to assess biodiversity or detect the presence of specific target species from a range of different environments (Bik *et al*. 2012a; Bohman *et al*. 2012; Bush *et al*. 2019; Ruppert *et al*. 2019). There are a variety of applications in fields such as invasion biology (Jerde *et al*. 2011), for monitoring rare and endangered species (Zhu *et al*. 2011), for the detection of cryptic species (Piaggio *et al*. 2014), in the study of diet (Deagle *et al*. 2014), the inventory of communities (Ji *et al*. 2013; Cowart *et al*. 2015) and paleoecology (Willerslev *et al*. 2004). As eDNA based methods continue to be developed, they are increasingly used by scientists and managers to complement traditional survey methods.

The study of eDNA in freshwater bodies, in particular rivers, has received considerable attention both for species-specific qPCR assays and metabarcoding using multiple target gene regions. Further, the potential for using eDNA in freshwater has been the topic of several studies and concerted actions (c.f. DNAqua COST action, https://dnaqua.net/) and there have been considerable efforts to standardise eDNA approaches to supplement or replace methods in the Water Framework Directive; as for example to develop new eDNA-based scoring systems. Nevertheless, thus far there are very few examples of targeting deep-sea species and communities beyond prokaryotes and a few studies on micro-eukaryotes such as ciliates and foraminifera (Pawlowski *et al*. 2011a,b, 2016; Bik *et al*. 2012b; Creer *et al*. 2012; Lejzerowicz *et al*. 2013; Orsi *et al*. 2013; Ruff *et al*. 2014; Zhao *et al*. 2017; Schoenle *et al*. 2017). In fact, there are only a few eDNA-based studies of costal water metazoan communities (Creer *et al*. 2010; Fonseca *et al*. 2010, Cowart et al., 2015; Leray et al., 2015, 2016) and even fewer studies targeting deep-sea communities (c.f. Sinniger *et al*. 2016; Brandt *et al*. 2018; Everett & Park 2018; Guardiola et al., 2016 a,b). Further, the few existing studies are largely biased towards a reduced set of organisms, due to the lack of truly "universal" primers (Cowart *et al*. 2015; Leray *et al*. 2016).

The presence of species in deep-sea habitats is inherently difficult to survey using traditional methods as it requires either visual observations of target species or invasive sampling using fishery gear. Visual methods are limited by low visibility at depth and presence of camera sledges (Koslow *et al*. 1995), ROVs (Lorance & Trenkel 2006) or other equipment can cause disturbances making the target species (if mobile) move away from the vicinity of the survey area, potentially resulting in no detection even though the target species are present. The use of fishery gear on deep-sea species is likely to cause the mortality of sampled individuals (with the advantage that sampled individuals could be examined for multiple characters), and similar to visual observation, the presence of the gear might cause the target species to move away. ATLAS aimed to test if eDNA could be used to supplement or replace the traditional methods tool for detecting presence of target species at depth. There are advantages of using eDNA methods as compared to the visual and fisheries methods. Sampling with eDNA requires minimal logistics in comparison to the traditional methods by only requiring water samples from the survey area. Even if the target species moves away from the water sampling equipment (CTD or other water sampling methods), the DNA from the target species will remain in the vicinity. Further, visual and fisheries methods will only detect the species if it is close to the sampling equipment, while mixing of water will cause water samples represent larger volumes. It was therefore deemed desirable to develop eDNA assays for deep-sea fish that could be used to non-invasively detect target species using minimal logistics.

3. Description of work

To assess the *potential and limits of metabarcoding of eDNA and qPCR* University College Dublin and IFREMER were tasked with:

1) Agreeing on protocols for water filtration, sediment sieving and DNA extraction from multiple sources (e.g. seawater, sediment, plankton samples, VME indicator taxa, commercially valuable fish species).

2) Applying these protocols on each ATLAS cruise to collect water, sediment and plankton samples for eDNA analyses. ATLAS will evaluate the performance of next-generation genomic tools (meta-barcoding of eDNA) for assessing biodiversity and quantitative qPCR (plankton samples) as a sensitive tool to detect and quantify biomass of target species. The accuracy and sensitivity of metabarcoding and qPCR will be validated on samples assessed using classical taxonomy in selected Case Studies.

3) Finally, partners would select a set of primers amplifying distinct DNA fragments to optimise meta-barcoding across the Tree of Life covering a maximum number of lineages and develop species-specific probes for PCR detection of VME indicator species, fishery targets and bycatch species.

We developed and agreed on eDNA sampling protocols and used eDNA water samples from selected Case Studies for single species detection using qPCR for deep-sea taxa (hydrothermal vent endemic crustaceans, deep-sea fish and cold-water corals) and pelagic fish species. In addition, we assessed how well metabarcoding using a range of target loci, on sediment samples from selected Case Studies, can be used to describe deep-sea ecosystems. The methods developed and knowledge gained in ATLAS will assist in understanding the potential and limitations with emerging marine eDNA methods and how these methods can be implemented in deep-sea and open ocean environments.

3.1. Detection methods

3.1.1. qPCR

For targeted species detection, PCR is employed using primers that are designed to amplify a specific locus of the target DNA sequence. Conventional endpoint PCR has been used for targeted eDNA detection (Dejean *et al*. 2011; Piaggio *et al*. 2014; Davison *et al*. 2016). However, quantitative (q)PCR offers a distinct advantage over traditional endpoint PCR techniques, as the addition of a fluorescent dye (e.g. SYBR™ Green) or a fluorescently labelled reporter probe (e.g. MGB) 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 of 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; Figure 1). The method has been successfully applied for detecting rare (e.g. Thomsen *et al*. 2012) and invasive species (e.g. Takahara *et al*. 2013). Due to the increased sensitivity and specificity of qPCR compared to traditional PCR, as well as its ability to quantify target DNA in a sample, this represents a particularly powerful tool for eDNA studies. Wilcox *et al*. (2013) demonstrated its utility for detecting closely related, sympatric brook trout *Salvelinus fontinalis* and bull trout *S. confluentu*s at low concentrations (0.5 target copies/µl) in a freshwater environmental sample.

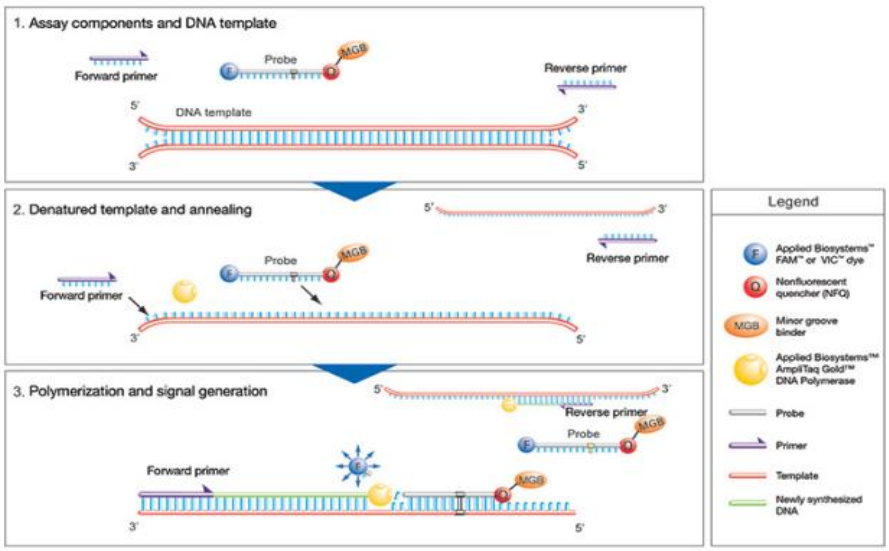

Figure 1: The TaqMan Gene Expression Assay process, which can be utilised to detect target DNA from environmental samples. (1) The temperature is raised to denature the double-stranded DNA. During this step, the signal from the fluorescent dye on the 5' end of the TaqMan probe is quenched by the NFQ on the 3' end. (2) The reaction temperature is lowered to allow the primers and probe to anneal to their specific target sequences. (3). Taq DNA polymerase synthesises new strands using the unlabeled primers and the template. When the polymerase reaches a TaqMan probe, its endogenous 5' nuclease activity cleaves the probe, separating the dye from the quencher (from http://www.thermofisher.com, with permission).

3.1.2. Metabarcoding

The extraction of DNA directly from an environmental sample was first described by Ogram *et al*. (1987) as a method to isolate microbial DNA from sediments. While once confined to the realm of microbiology, it is becoming increasingly used by ecologists. This is mainly due to the advent of molecular "barcoding", for species identification using a standardised region of DNA (Hebert *et al*. 2003, Valentini *et al*. 2009). In the case of animal DNA, the standard barcode used is a region of the mitochondrial COI (Cytochrome Oxidase Subunit I; Figure 2). Many species can be identified simultaneously, and biodiversity patterns can be analysed from eDNA (i.e. metabarcoding; Pompanon *et al*. 2011), by using semi-universal primers and PCR to amplify a short region of the COI or other gene regions and sequencing the resulting amplicons (Taberlet *et al*. 2012). Mitochondrial DNA is targeted for such studies as it is present in higher copy number than nuclear DNA, which should be reflected in the environmental sample, and also because semi-universal primers with a capacity to amplify a large number of species such as the Folmer *et al*. (1994) COI primers have been developed in conserved regions flanking otherwise variable regions, allowing species discrimination. The COI gene is thus known to be effective for biodiversity analysis of most metazoan taxa. In addition, and more importantly for massive barcoding (i.e. metabarcoding), it is a protein coding gene and indels that may distort results by causing shifts in the reading frame (Hebert *et al*. 2003) are therefore rare. Furthermore, studies have shown that barcodes as short as ~100bp from the COI region can be used to discriminate among species, where only degraded DNA is available (Meusnier *et al*. 2007; Günther *et al*. 2018). Associated initiatives for a global reference database, e.g. the Consortium for the Barcode of Life (CBOL, http://www.barcodinglife.org), indicate that the decreasing cost and advancing

technology of high throughput sequencing have no doubt facilitated the increasing use of the barcoding system (Valentini *et al*. 2009).

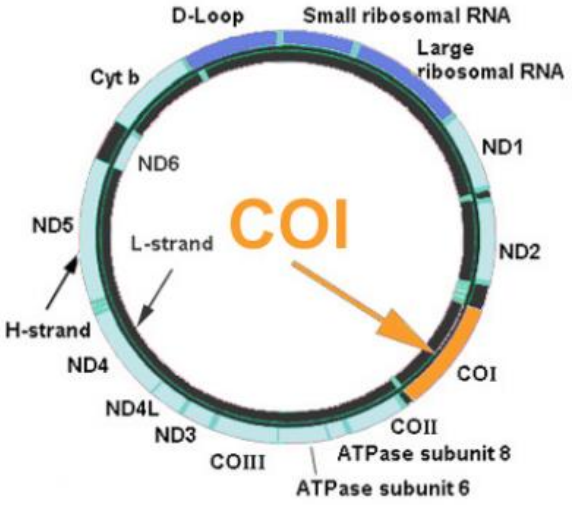

Figure 2: Diagram of the mitochondrial genome with the position of the COI gene highlighted (from Trivedi *et al*. 2016)

3.2. qPCR materials, methods and results

For the task of evaluating qPCR as a sensitive tool to detect and quantify biomass of keystone species, ATLAS analysed several scientific hypotheses listed below. ATLAS collected water samples from close to the seafloor, mid-water and surface (in accordance with sampling protocols agreed in ATLAS) in the Azores EEZ and wherever possible in the North Atlantic. In total, 196 water samples were collected for qPCR analyses (around the focal area of the Azores and Gulf of Cadiz see Figure 3, for a complete list see Appendix). These included samples from the hydrothermal vent fields Rainbow (bottom, mid and surface) and Lucky strike (bottom, mid, surface). In addition, samples from multiple seamounts around the Azores were also collected, including Gigante (bottom and surface), 127 (bottom and surface), Princesa Alice (surface), Ambrósio (surface), Formigas and Dollabarat (surface), Atlantis (bottom and surface), Irving (bottom and surface), Great Meteor (surface), Tyro (surface), and Pico Sul (surface). Samples from far-field open water stations (surface) were also collected as controls. Samples were also collected during the MEDWAVES cruise; the Formigas seamount (bottom, mid, surface), Gazul mud volcano (bottom, mid, surface), Ormond seamount (bottom, mid, surface) and from Seco de los Olivos seamount (bottom, mid, surface). Further water samples were collected by UCD from the Irish Sea and the Gulf of Cadiz. All water samples were filtered with 0.47 µm pore size nylon filters, preserved in molecular grade 96% ethanol, and sent to University College Dublin for processing. Additionally, tissue samples were collected for target species and stored in molecular grade 96% ethanol for producing species specific qPCR assays from different organisms, namely CWCs (*Leiopathes* sp., *Desmophyllum dianthus*, *Callogorgia verticillata*, *Lophelia pertusa*, *Paracalyptrophora josephinae*, *Viminella flagellum*) and fish (*Helicolenus dactylopterus*, *Beryx decadactylus*, *B. splendens*). Tissue samples for *Hoplostethus atlanticus* (sample from the Porcupine Bank) and

Galeorhinus galeus (sample from the Irish west coast) were made available from the UCD fish sample collection that is stored in molecular grade ethanol.

3.2.1. Scientific hypotheses

Can qPCR analyses help in detecting and estimating biomass (a component of Good Environmental Status) of key deep-sea fish species?
Alternative methods for estimating fish abundance and biomass are required to reduce the impact and costs of fisheries research surveys, and to increase the spatial and temporal coverage of the surveys and to help implementation of short and long term adaptive management measures.

- Species considered: the commercially important *Helicolenus dactylopterus*, the blackbelly rosefish, that has a wide distribution in the North Atlantic and has shown a recent range expansion (c.f. WP 3).
- Samples available: water samples collected close to the bottom in the Azores and in the Gulf of Cadiz.

Can qPCR analyses help identifying aggregation of vulnerable deep-sea fish species?
Some seamount associated deep-sea fish species form dense seasonal aggregations that are an easy target for industrial deep-sea trawling fishing fleets. In the Azores, bottom trawling has never occurred and was officially prohibited in the mid-2000s. Therefore, the location of fish aggregations is largely unknown preventing the identification and proper management of those seamounts.

- Species considered: Orange roughy *Hoplostethus atlanticus*. The alfonsinos, *Beryx splendens* and *B. decadactylus.*
- Samples available: Collected water samples from mid-water and close to the bottom from the Azores, Gulf of Cadiz, and samples from the Gazul mud volcano made available by the Irish Deep-Links survey.

Can qPCR analyses help identifying seamount hotspots of pelagic biodiversity?
Some seamounts are hotspots of pelagic biodiversity (mostly for large pelagic billfish, tuna, sharks, seabirds, sea-turtles and some marine mammals), however, these hotspots are difficult to identify and locate even at a regional scale. We, therefore, seek to validate if qPCR approaches can efficiently detect visiting pelagic marine animals to seamounts and therefore be used to identify important seamounts for conservation.

- Species considered: The pelagic *Thunnus obesus* and *Mobula tarapacana* (also *Galeorhinus galeus*).
- Samples available: Seamount samples collected close to the surface and samples from the Irish Sea.

Can qPCR analyses help identifying presence of hydrothermal vent species?

- Species considered: The hydrothermal vent endemic shrimp, *Mirocaris fortunata,* and mussel, *Bathymodiolus azoricus*.
- Samples available: Samples collected around the hydrothermal vent fields Rainbow (bottom), and Lucky strike hydrothermal vent field (bottom). Additionally, samples from the Atlantis and Irving seamounts were also analysed.

Can qPCR analyses help identifying hotspots of cold-water corals (CWC) in the NE Atlantic?
The location of individual CWC reefs is largely unknown in the North Atlantic and can be difficult to locate due to the significant cost of sampling large areas of the deep sea. Additionally, imagery methodologies (ROVs, drop-down cameras and AUVs) are able to sample only small portions of the seabed, making the probability of missing important communities very high. Collecting water samples and analysing it with qPCR may be an alternative technique to quickly identify the presence of CWC reefs in a certain area of the deep-sea.

- Species considered: the habitat forming coral water coral *Lophelia pertusa*.
- Samples available: samples collected close to the bottom in the Azores and the Gulf of Cadiz.

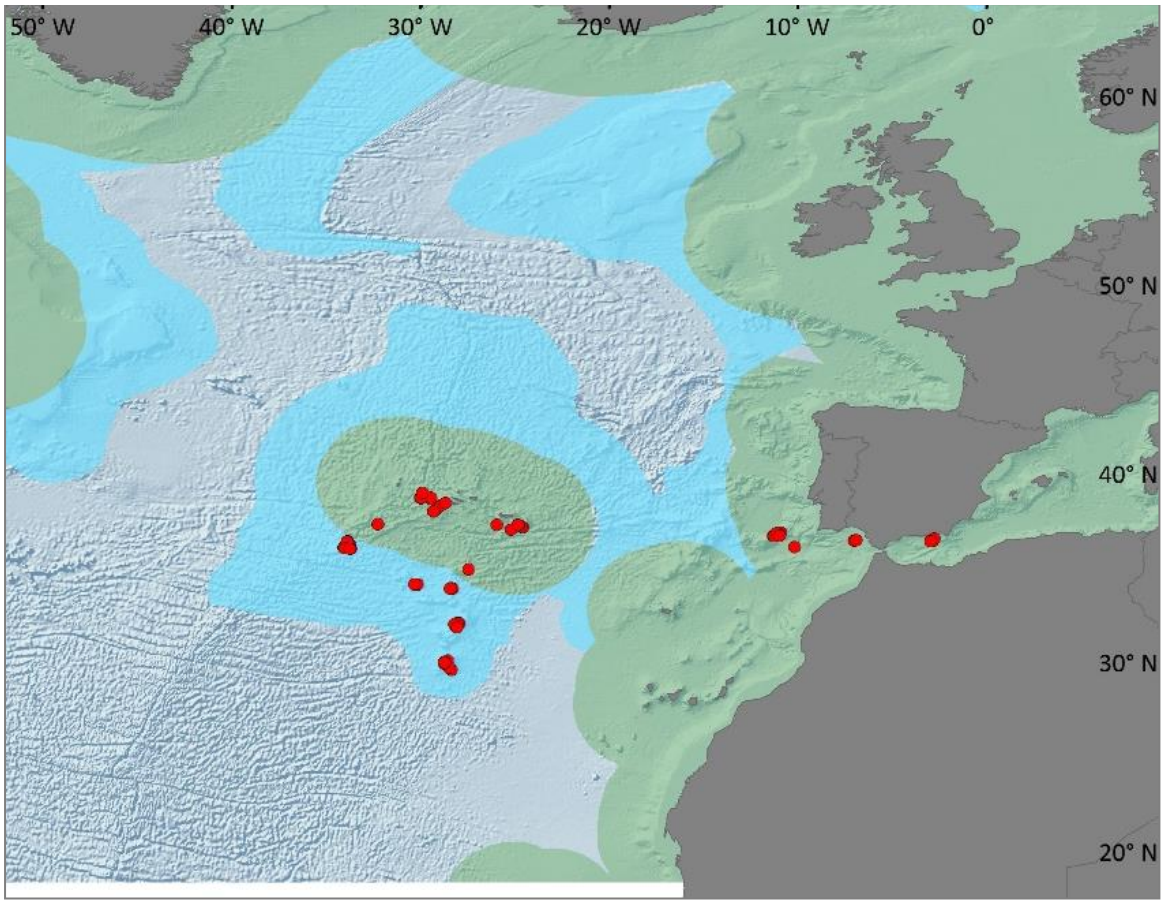

Figure 3: Location of existing water samples for eDNA analyses from the focal area of the Azores and the Gulf of Cadiz (for a complete list see Appendix).

University College Dublin (UCD) was tasked with testing the feasibility of developing species specific qPCR assays to detect the presence of representatives from marine fish, crustacean and coral taxa. This work includes development of qPCR assays for single species detection for six marine species: Fish (*Mobula tarapacana, Helicolenus dactylopterus, Hoplostethus atlanticus*, *Thunnus obesus);* the hydrothermal vent endemic shrimp, *Mirocaris fortunata*, and the deep-sea coral *Lophelia pertusa*.

The steps involved in qPCR species-specific assays (F and R primers and a labelled probe) include identifying *in-silico* suitable mitochondrial genetic regions for anchoring of primers and probes by

comparing the target species sequences to sequences from other organisms available in public repositories (e.g. Genbank and BOLD). Mitochondrial DNA is preferred as it has orders of magnitude of higher abundance in a cell as compared to nuclear DNA. Mitochondrial genetic regions should be conserved within the species but have unique sequences compared with other species. At the same time they should be relatively short (often ranging from 80-120bp to allow for detection of degraded DNA fragments). Once a region has been identified, primers that will amplify the target region are generated *in-silico* along with a probe (c.f. Figure 1). The individual components of the assay are then checked for species specificity *in-silico* by performing a BLASTn search. This search shows similarities between the primers and probes generated to sequences from other species to assess the species specificity. Several primers and probes are often needed before species-specific assays can be designed *in-silico*.

Subsequently, primers and probes are synthesised and ordered. The assays are then assessed for amplification success using several PCR amplification conditions by testing the assay on DNA from tissue samples from the focal species. If the assay fails to amplify, further primer and probes are synthesised. This procedure is repeated until amplification of target species is successful. Once amplification conditions have been established, the assay is tested on other tissue samples from non-target organisms that can be found in the same habitat as the focal species. If species specificity cannot be achieved at this stage, additional primers and probes are designed and tested until species specificity is reached. This development phase of qPCR species-specific assays relies on having access to sequence data for the focal organisms. If the amount of available sequence data are not sufficient for the target species, or closely related species, it will not be possible to develop an assay. However, if new sequence data are made available in the future it would be possible to re-interrogate the data and successfully develop assays.

Once an assay has been developed, the sensitivity of the assays is assessed by comparing the detection thresholds using known concentrations of the target species DNA. The assay is then field validated on water samples collected near or in the vicinity of a focal observation of the target species. Once, the assay has been field-validated, it can be deployed on a large scale to detect species presence. Below are the specific results from UCD's efforts for developing species specific qPCR assays (summary of the are shown in Table 1).

3.2.2. Fish qPCR assays

In an effort to test the efficacy of qPCR-based eDNA analyses across marine habitats we targeted two pelagic fish species occurring at seamounts (*Mobula tarapacana* and *Thunnus obesus*) and two deep-sea fish species (*Helicolenus dactylopterus* and *Hoplostethus atlanticus*). An assay was developed for *Mobula tarapacana* (Table 1) from tissue samples and field-validated on water samples provided by Telmo Morato at the University of the Azores where focal observations had been done. The assay was subsequently deployed on water samples where visual target species had been observed and, in addition, samples where the target species is not present were included as negative controls. The assay was able to corroborate visual observations and successfully identified the presence of the target species (Gargan *et al*. 2017). Significant *in-silico* efforts have been made to identify species specific regions that would amplify *Thunnus obesus*. However, due to the complex phylogenetics of

tuna species (c.f. Carlsson *et al*. 2004), considerable levels of morphological species and misidentification of specimens that have sequences uploaded to public databases (e.g. GenBank), it has not been possible to develop *Thunnus obesus* specific assays. Further, high levels of introgression among tunas could be another reason for the difficulties in identifying species specific regions. For instance, *c.* 5% of all *Thunnus thynnus* have a complete *Thunnus alalunga* mitochondrial genome (Carlsson *et al.* 2004). After discussions with the WP leader Telmo Morato, it was decided to change the focal species to *Galeorhinus galeus*, a species listed by the IUCN as vulnerable. A species-specific assay was developed on tissue samples from a *Galeorhinus galeus* (Table 1) caught in Irish waters and field-validated on water samples collected at a location where *Galeorhinus galeus* had been caught by fishermen within 24h. The development of species specific qPCR eDNA assays for the two pelagic species demonstrated that eDNA qPCR is very capable tool that can be used for detection of species presence at seamounts in the open ocean without the need for time consuming visual observation efforts or invasive sampling of the target species.

The University of the Azores also provided tissue samples for *Helicolenus dactylopterus*. An eDNA assay was developed using this tissue and field validated on water samples collected during the Irish marine Institute funded 2015 Deep-Links research cruise (CE15012) to the Gulf of Cadiz. Water samples were collected near the bottom in the vicinity of where visual observations (ROV video) of the target species were made. Tissue samples for *Hoplostethus atlanticus* (Table 1) were made available from previous research on the species by the UCD group (Carlsson *et al.* 2011). While analyses for *Hoplostethus atlanticus* are ongoing, primers and probes have been developed and lab validated on tissue samples from target species and non-target species and the results demonstrate that the assay is species specific. The two assays developed for deep-sea fish clearly demonstrates the capacity of eDNA to detect target species even at large depths and often low densities of target species often encountered in the deep-sea.

The successful development of eDNA assays for the target fish species found at different depths (pelagic to deep sea) demonstrated the capacity for using eDNA for assessing spatial and temporal distribution patterns of marine fish. Deployment of these assays will assist in non-invasive and low-cost survey approaches to detect target species without the need for dedicated survey equipment (ROVs, camera sledges and fisheries gear) that will ultimately complement existing monitoring techniques and aid in management and conservation efforts.

3.3. Hydrothermal vent shrimp qPCR assay

Hydrothermal vents are local discrete habitats distributed along ocean ridges and back arch basins that support many endemic species. These habitats are also being prospected for deep-sea mining of seafloor massive sulfides (c.f. http://www.nautilusminerals.com). If mining occurs, it will likely have significant effects on the species that are endemic to these habitats. As such it is vital that biodiversity and species composition is assessed prior to any mining activities to allow for monitoring effects of mining on the fauna at these habitats and to inform the development of management plans. Hydrothermal vents occur at significant depths where it is difficult to use convectional sampling methods to determine species composition and densities. To sample macro and megafauna at these habitats it is necessary to employ highly specialised sampling equipment (remotely operated vehicles

or manned submersibles) that comes with significant costs. The availability of the necessary equipment and high cost of these types of operations limits the possibility to assess which fauna are present in these habitats. However, sampling water around or near these habitats is straight forward in comparison as the only equipment needed would be a CTD rosette or other means for taking water samples. We aimed to develop an eDNA assay for a hydrothermal vent endemic species that could demonstrate the capability of eDNA as a tool to detect species presence in also in discrete deep-sea habitats. Hence, we used tissue samples of *Mirocaris fortunata*, a hydrothermal vent endemic shrimp, present at hydrothermal vents along the Mid-Atlantic Ridge, that were made available from the Irish Marine Institute funded 2011 CE11009 VENTuRE survey to the Mid-Atlantic Ridge. Primers and probes were developed (Table 1) and lab-validated on tissue samples from the target and non-target species to assess species specific. The assay is pending field-validation on water samples where the target species has been observed. This assay can demonstrate the feasibility of using eDNA as an alternative to labour intensive and high cost sampling using specialised equipment at discrete deep-sea habitats. While the analyses are ongoing the assay development is complete.

3.4. Cold-water coral qPCR assay

Deep-sea cold-water corals are found throughout the deep-sea and are keystone species by forming 3-dimensional structures supporting high biomass and considerable levels of biodiversity that may be of significant importance for fisheries species. However, coral mounds are not continuously distributed in the deep sea and, similar to hydrothermal vents, the considerable depths the corals occur at makes surveys logistically problematic and costly. Further, while there are methods of detecting the presence of hydrothermal vents (by tracking the chemical signal of the plume) and the predictability of where these habits are (ocean ridges and back-arch basins), these methods cannot be employed to detect presence of coral mounds, nor is it predictable were corals occur to same level as hydrothermal vents. The ATLAS project aimed to use the deep-water coral *Lophelia pertusa* as an indicator for the presence of cold-water coral reefs. We aimed to develop an eDNA qPCR assay for the species. However, the amount of genetic data available in public sequence repositories (GenBank and BOLD) were insufficient to locate a species specific mitochondrial region suitable for anchoring primers and probe. Further, unpublished sequence data were provided by IFREMER (Sophie Arnaud-Haond). However, these sequences did not have suitable regions developing species specific eDNA assays. In addition, sequence data from closely related organisms is currently not available and hampers efforts to assess the species specificity of target loci. After conversations with the WP leader, it was decided to not pursue further development until more sequence information is made available. The failure of developing an eDNA qPCR assay for *Lophelia pertusa* demonstrates that there are situations where there are insufficient genetic data available to allow for development of eDNA assays, even for ecologically important and abundant species. This is probably a more pronounced issue for marine species than freshwater species due to the higher biodiversity of marine ecosystems as compared to freshwater system. It is likely an even larger issue for non-charismatic or non-commercially important marine species as they are often not well-studied. While the specific issue for *Lophelia pertusa* can be resolved by sequencing whole mitochondrial genomes for cold-water coral species allowing for identification of suitable genetic regions, it is a more widespread problem across marine deep-sea benthic species as only few have been subjected to sequencing. Significant efforts to sample and sequence marine deep-sea benthic fauna are therefore required.

Table 1. eDNA qPCR assay components; forward and reverse primers and probe 5' - 3' for target species and lowest detectable DNA concentration.

Target species	Forward primer 5' - 3'	Reverse primer 5' - 3'	MGB-Probe	Lowest DNA conc. detected by standard curve pg/µl
Mobula tarapacana	AAC CAC CTG CAA TCT CTC AAT ATC	GGG AAG AGA TAA TAA TAG GAC AGT	CTT GTT TGT TTG ATC AAT TC	0.25
Helicolenus dactylopterus	AAT TAC CGC TGT TCT TCT CCT CC	GGG GTC GAA GAA GGT GGT ATT AAG	CTG CAG GCA TCA CAA TAC TCC T	0.02
Hoplostethus atlanticus	TCC TTC TAT CCC TCC CCG TC	AAT GGG GTC TCC TCC TCC TG	ACCATGCTCCTTACAG ACCG	To be assessed
Galeorhinus galeus	GTT GAA CAG TAT ATC CTC CAC TAG CAA	TTG AGG CTA GGA TTG ATG AGA TAC C	CCA TCT GTA GAT TTA GCC ATT T	0.20
Mirocaris fortunata	AGG TGT AGG TAC AGG ATG AAC TG	CGC TAG ATG TAG GGA GAA AAT TGC	CCC CAC TAG CTG CTG GAA TTG	To be assessed

3.5. Metabarcoding materials, methods and results

The objective of metabarcoding work in the framework of ATLAS was to deploy a set of probes (PCR primers) that would capture the broadest possible range of diversity across the Tree of Life, and provide inventories of biodiversity across the Mediterranean-Atlantic pathways followed by the MEDWAVES cruise, during which sediment samples were collected. Indeed, at the onset of ATLAS the main technological challenges we aimed at tackling were the identification of a comprehensive set of probes sufficiently complementary and versatile to encompass most taxa across the Tree of Life. The majority of studies were limited to one or two loci leading to detection failure of some major taxa (Cowart *et al*. 2015; Leray & Knowlton, 2016). There were also issues in assessing the contemporary biodiversity as deep-sea sediments can also contain older archived DNA that does not represent the contemporary diversity (Dell'Anno *et al*. 2004; Corinaldesi *et al*. 2011).

The development of metabarcoding protocols (sampling, loci, DNA extraction methods and bioinformatic pipelines) were performed in the framework of ATLAS and in collaboration with the Abyss/eDNAbyss projects (IFREMER-Génoscope, CEA). As such, it relied on protocols for sediment sampling developed in the Abyss project. A first assay, aiming at developing protocols suitable for a diversity of deep-sea ecosystems based on sediment, was performed on a set of samples from mesopelagic areas, mud volcano and Atlantic seamounts visited during the MEDWAVES cruise (Orejas, 2016), and other samples collected from IFREMER cruises in the Mediterranean (Justiniano, 2016) and external partners on inactive hydrothermal vents from the North Atlantic (Lodvigsen et al. 2016).

To address the first challenge (loci combinations generating representative biodiversity), a set of six primers were selected to reveal eukaryotic and prokaryotic diversity across these different ecosystems. To address the second challenge (excluding non-contemporary DNA in samples), a series of five combinations of preservation and extraction methods (Figure 4) were compared in order to focus on contemporary DNA while avoiding older DNA not representing current assemblages. Significant efforts were made to take advantage of improvements by using the recently developed modified COI primers (Leary *et al*. 2013) to assess the diversity of nematodes that represent the

dominant component of meiofaunal diversity (Boucher & Lambshead 1995), but are not well captured by "universal" primers used thus far. Although 18S primers are better to capture nematodes than COI, 18S shows insufficient taxonomic resolution (Derycke *et al*. 2010; Bhadury *et al*. 2016; Avó *et al*. 2017). However, it appears that the nematodes in our inventories are still underrepresented and that further efforts using other gene regions (loci) should could improve the representation of nematodes in metabarcoding studies.

In summary, the work gathered a comprehensive set of markers (loci) encompassing most taxa on the Tree of Life, though there is still scope for improving the detection and representation of nematode and fungal sequences. The protocols developed for sample preservation and nucleic acid extraction were chosen to minimise the presence of archived nucleic acid not representing contemporary fauna assemblies. A publication is being drafted presenting the molecular protocols developed and the primers selected, as well as the preservation method (Brandt *et al*. in preparation). Protocols will be made available after acceptance.

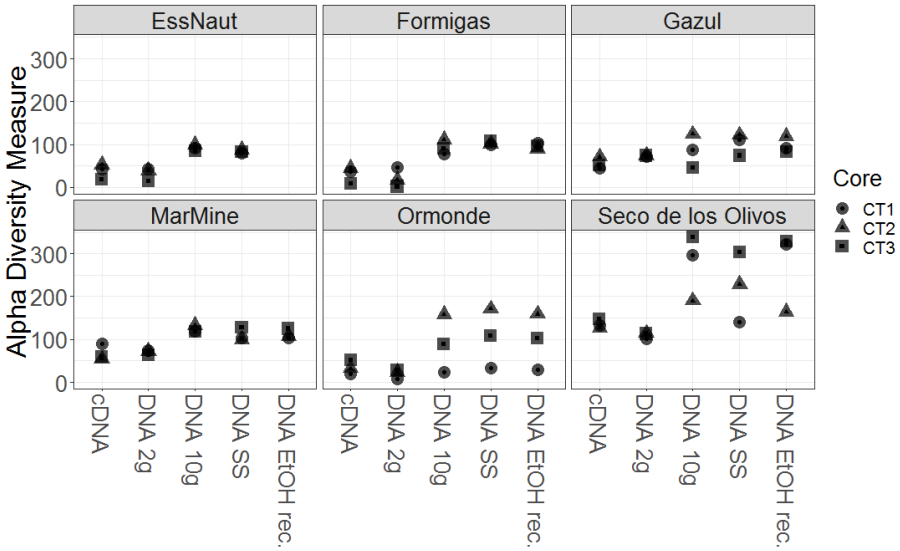

Figure 4: Alpha-diversity characterised through metabarcoding in three sediment cores of each of the 6 sites analysed, including 4 MEDWAVES sites (the four right ones), with a comparison of diversity obtained by using as starting material RNA (cDNA), DNA extracted from 2g sediment, or DNA extracted from 10g sediments, modified or not for size selection (SS) or ethanol reprecipitation (EtOH).

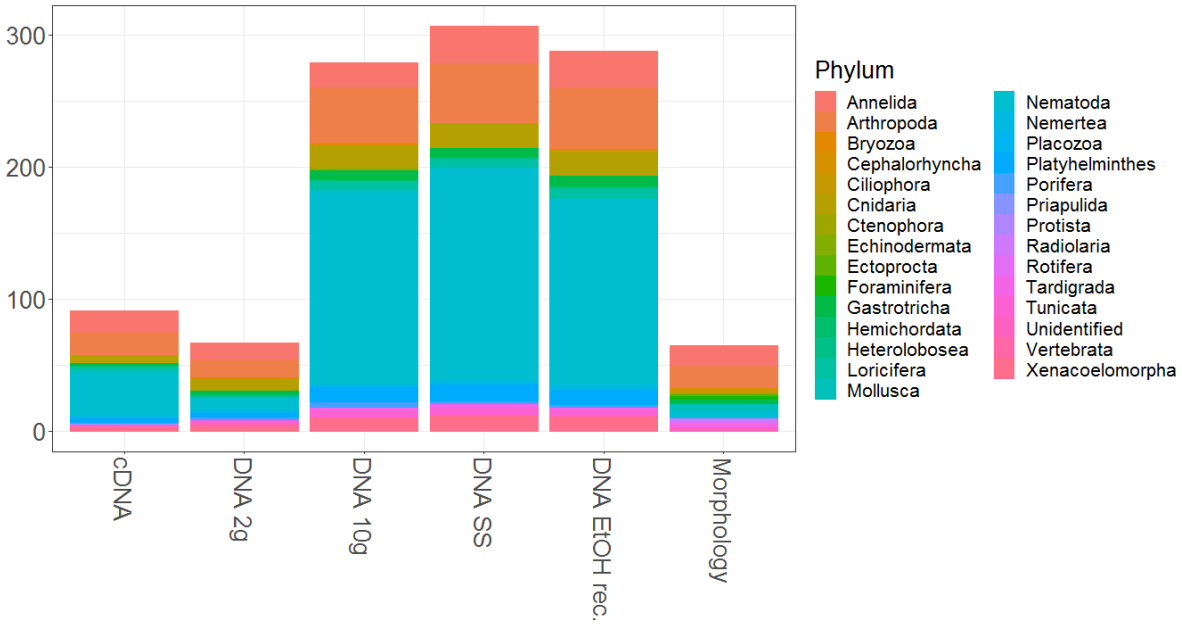

Figure 5: Diversity and proportions of the main metazoan taxa retrieved with metabarcoding, depending on the treatment applied to nucleic acids (cDNA= RNA extraction followed by cDNA synthesis before metabarcoding libraries; DNA 2g = extraction from 2 grams sediment, i.e. the same amount allowed by kits for RNA; DNA 10g = extraction from 10 grams sediment; DNA SS= extraction from 10g sediment with size selection to expel fragments smaller than 1kb; DNA EtOH rec.= extraction from 10g sediment followed by a reconcentration of DNA by precipitation with Ethanol; morphology = morphological inventory of the sister core, sampled on the same coordinates with the same multicore gear). This example comes from the analysis of cores from Formigas seamount, and, in overall, shows the higher diversity obtained with a larger amount of material and the better results obtained with DNA from 10g sediment.

The markers chosen also allowed us to perform subsequent community analysis showing clear patterns of biogeographic segregation (Figure 6).

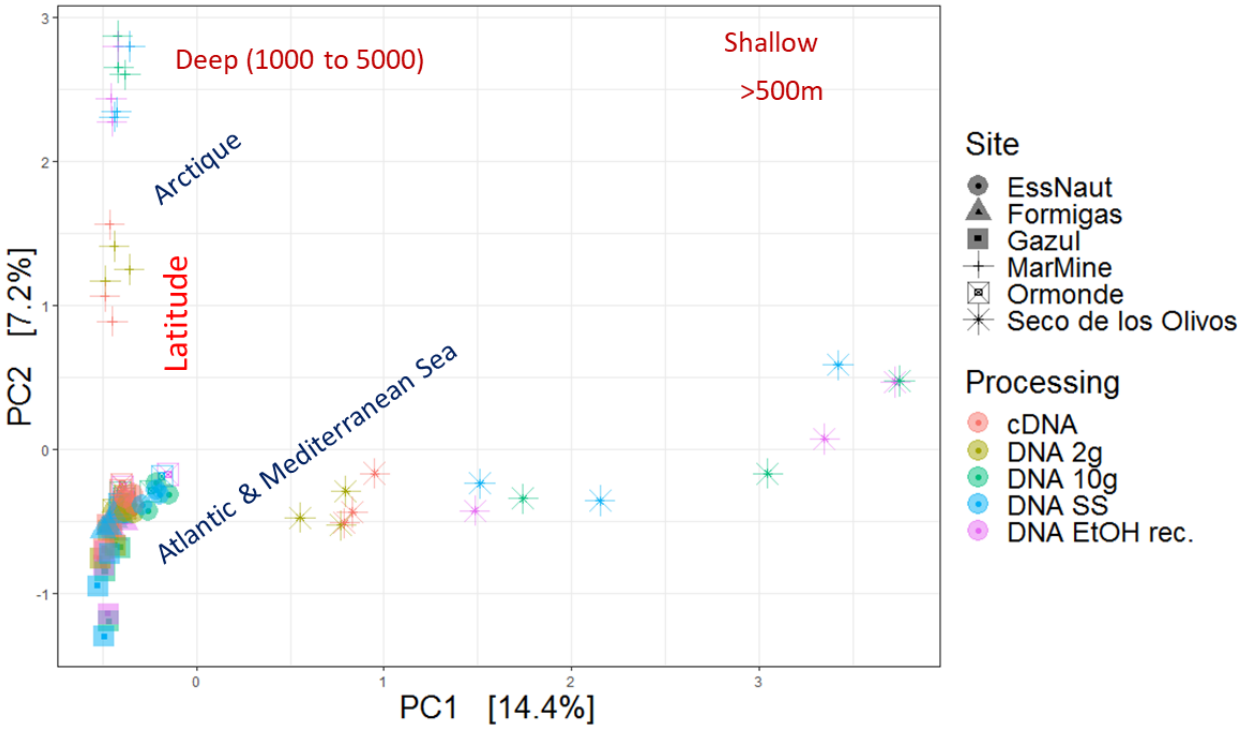

Figure 6: Illustration of the segregation of eukaryotic communities as revealed using Cytochrome Oxidase I, analysed for beta diversity with Jaccard distance (based on presence-absence data), for all treatments tested for nucleic acid extraction (RNA, DNA from 2 grams sediment, DNA from 10 grams sediment, and DNA from 10 grams sediment treated to exclude small fragment either through size selection –SS- or through Ethanol re-precipitation), and for six sites spanning from the Mediterranean to the Arctic. It appears clearly that the treatment effect is secondary compared to the site effect, when comparing samples from different depth and biogeographic regions: the first axis clearly separates Seco de los Olivos, the shallowest site sampled, being located above 500 meters while all others are below 1000, while the second axis shows the large community difference between samples from the Mediterranean and the Azores compared to those located near the Arctic.

Based on protocols developed in the ATLAS project, all samples from the MEDWAVES cruise were analysed together with other samples collected in the framework of Abyss project in the Mediterranean and the Atlantic. Eukaryotes, and more specifically metazoans were the main target for ATLAS. A total of about 350 samples were analysed (Figure 7). Data analyses are still ongoing, but have already delivered useful information as to identifying the drivers of community structure and segregation.
For metazoans, the OTU community composition is most often affected by the depth of site rather than the depth in the sediment, while the opposite tends to be observed for microbial communities.

Box cores and extra tube cores have been sampled and analysed by IEO and IFREMER to also determine morphological biodiversity inventories at the same sites. Comparison of both biodiversity inventories (metabarcoding vs morphological) is ongoing, or will be performed by both institutions. Preliminary results obtained at IFREMER support previous studies (e.g. Cowart et al., 2015) showing rather different lists. Among other reasons, this is due to the large spectra of organisms captured with metabarcoding, for example, when considering meiofauna, while morphological inventories are more limited due to the extensive time and expertise needed to complete them.

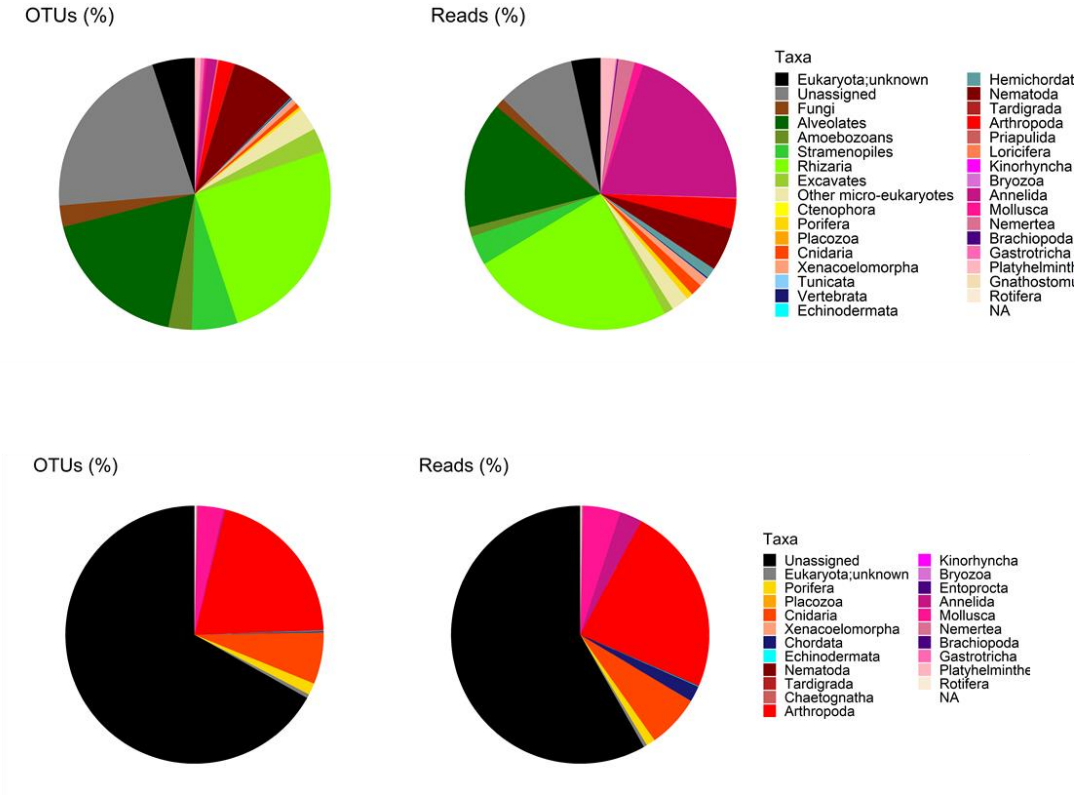

Figure 7: Biodiversity inventoried using a 18S-V1 marker (upper panel) and a COI marker (lower panel) on samples from the Mediterranean and the Atlantic, including all MEDWAVES samples (a total of approximately 300 sediment samples). On the left the % of Operational Taxonomic Unts (OTU) on a presence/absence dataset, while on the right is detailed the amount of reads (sequence) identified for each of the same large taxonomic groups. The figure shows the complementarity of both markers in identifying taxa from micro-eucaryotes and metazoans representative of the meiofauna (preferably 18S-V1) versus metazoan, principally representative of the macrofauna (COI).

4. Discussion

The development of qPCR assays has been successful for five of the seven target species. Three of the assays (*Helicolenus dactylopterus, Mobula tarapacana* and *Galeorhinus galeus*) have been validated on water samples from locations where the target species is known to occur and one of these assays has been published (*Mobula tarapacana*, Gargan *et al*. 2017). However, water samples from locations where *Hoplostethus atlanticus* and *Mirocaris fortunata* are known to occur are needed for field-validation of these two assays. We have only failed to develop an assay for *Lophelia pertusa* due to lack of available genetic resources to perform *in silico* primer and probe development. Additional sequence information from multiple cold-water coral species are needed before a species-specific qPCR eDNA assay can be developed. The detection limitation of these assays varied among species from 0.02 pg/µl (*Helicolenus dactylopterus*) to 0.25 pg/µl (*Mobula tarapacana*). While our results clearly indicate the potential for eDNA qPCR assays for marine species it is also evident that there are limitations to the approach; namely lack of genetic data, misidentified specimens and species introgression. Further mitochondrial genetic resources are needed to enable qPCR eDNA assay development for *Lophelia pertusa*, and this is likely the case for many marine species as the public genetic repositories lack sequence information for many marine species due to the vast biodiversity in this environment. Furthermore, sequences present in public repositories for some species groups might be based on morphological misidentified specimens (e.g. tuna-like species). In addition, previous historic introgression among species can make the mitochondrial genome less suitable for species identification (some 5% of Mediterranean *Thunnus thynnus* have *Thunnus alalunga* mitochondria). While it is difficult to mitigate for introgression the lack of sequence information could be resolved by establishing well curated sequence repositories by whole mitochondrial genome sequencing of marine species.

Within ATLAS we have improved our understanding of the potential of eDNA based approaches by developing five novel species-specific qPCR assays for different marine taxa. We have demonstrated that species-specific eDNA methods are very sensitive across different marine ecosystems (pelagic to deep-sea). eDNA may be a powerful non-invasive tool for the detection of target species and to assess species distributions over space and time. It is clear that the method could in the future complement existing monitoring methods, some of them which are invasive, costly and time consuming. Preliminary results of this work were presented at a WP4 workshop in Edinburgh in January 2019 and helped inform the choice of species used for connectivity modelling in WP4 (deliverables D4.4 and D4.5).

The protocols developed in ATLAS for metabarcoding of environmental sediment samples from the deep-sea have been successfully developed at IFREMER. These chains of protocols, from sampling to preservation of sediment, choice of nucleic acid and extraction protocol, and finally amplification of a set of loci, allow grasping a much broader range of lineages than loci previously used for assessing biodiversity. The protocols developed also limit the “contamination” by old archived DNA (Corinaldesi *et al*. 2011, 2018; Dell'Anno *et al*. 2015). The approaches developed and used in ATLAS demonstrate that metabarcoding is a powerful tool capable of assessing deep-sea biodiversity and that it can be deployed as a method for management of deep-sea habitats (e.g. hydrothermal vents, cold seeps,

seamounts, abyssal plains and coral/sponge gardens) to a taxonomic level previously requiring significant morphological expertise and time.

A limitation of these protocols, however, is that despite the use of multiple markers to recover the broadest possible range of biodiversity, the protocols do not completely solve the problem of nematodes that represent a very important component in the deep sea and are still poorly captured by available primers. Also, we acknowledge that fungi are an important component of the Tree of Life that are well represented in the marine realm and suitable loci for detection of fungal DNA should be developed for inclusion in future versions of the protocol. Community inventories will thus still have a large gap for these taxa, a problem similar to those for single species assays that will need to be solved in future years by establishing well-curated sequence repositories.

In summary, ATLAS concludes that qPCR is a promising method for the detection of target species in the marine environment, including the deep-sea. Additionally, the metabarcoding protocols were capable of assessing biodiversity from low biomass deep-sea sediments across the entire Tree of Life; even for the under-studied deep-sea metazoan taxa. Therefore, these eDNA methods were shown to be of potential use for assessing and managing deep-sea biodiversity, both in terms of assessing commercially important fish stocks (e.g. qPCR) and of assessing hidden biodiversity (metabarcoding) in the context of deep-sea mining and other environmental impact assessments. However, we have also shown that great challenges remain to maximise the potential of eDNA approaches in the deep sea and highlight that significant efforts to sample and sequence deep-sea benthic fauna are required to enable well-curated sequence repositories.

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6. Appendix

Environmental DNA samples made available to UCD

Sample	Lat	Long	Site location[1]	Collection date	Depth	DNA status	Species interrogated	*eDNA Detection*
1PAL	38.0049	-29.2988	Princesa Alice (PAL) seamount	27/08/2014	Surface	Extracted	*Mobula tarapacana*	*yes*
2PAL	38.0049	-29.2988	Princesa Alice (PAL) seamount	27/08/2014	Surface	Extracted	*Mobula tarapacana*	*yes*
3PAL	38.3428	-28.8815	Far field from PAL - no visual	27/08/2014	Surface	Extracted	*Mobula tarapacana*	*no*
4PAL	38.1212	-29.1659	Far field from PAL - VOLTA	27/08/2014	Surface	Extracted		
1SMA	37.0523	-25.1890	Ambrósio seamount no visual	24/08/2014	Surface	Extracted	*Mobula tarapacana*	*no*
2SMA	37.2374	-24.7253	Dollabarat seamount	25/08/2014	Surface	Extracted	*Mobula tarapacana*	*yes*
3SMA	37.2374	-24.7253	Dollabarat seamount	26/08/2014	Surface	Extracted		
4SMA	37.0523	-25.1890	Ambrósio seamount	27/08/2014	Surface	Extracted	*Mobula tarapacana*	*yes*
5SMA	37.0523	-25.1890	Ambrósio seamount	27/08/2014	Surface	Extracted		
6SMA	37.0523	-25.1890	Ambrósio seamount	28/08/2014	Surface	Extracted		
7SMA	37.3007	-25.9620	Far field from SMA no visual	30/08/2014	Surface	Extracted	*Mobula tarapacana*	*no*
8SMA	37.3007	-25.9620	Far field from SMA	30/08/2014	Surface	Extracted		
5PAL	38.0040	-29.3019	Princesa Alice (PAL) seamount	19/09/2014	Surface	Extracted		
6PAL	37.9991	-29.3006	Princesa Alice (PAL) seamount	19/09/2014	Surface	Extracted	*Mobula tarapacana*	*yes*
7PAL	38.0134	-29.3071	Far field from PAL - no visual	19/09/2014	Surface	Extracted	*Mobula tarapacana*	*yes*
8PAL	38.4684	-28.6843	Far field from PAL - no visual	19/09/2014	Surface	Extracted	*Mobula tarapacana*	*no*

9PAL	38.0049	-29.2988	Princesa Alice (PAL) seamount	26/09/2014	Surface	Extracted	*Mobula tarapacana*	*yes*
10PAL	38.0049	-29.2988	Princesa Alice (PAL) seamount	26/09/2014	Surface	Extracted		
11PAL	38.0134	-29.3071	Far field from PAL - no visual	26/09/2014	Surface	Extracted	*Mobula tarapacana*	*yes*
12PAL	38.4684	-28.6843	Far field from PAL - no visual	26/09/2014	Surface	Extracted	*Mobula tarapacana*	*no*
1GIG	38.6420	-29.4339	Far field from GIG - no visual	25/09/2014	Surface	Extracted	*Mobula tarapacana*	*no*
2GIG	38.7222	-29.9728	Seamount 127 no visual	25/09/2014	Surface	Extracted	*Mobula tarapacana*	*no*
3GIG	38.7260	-30.0158	Seamount 127	25/09/2014	Surface	Extracted		
4GIG	38.7255	-29.9681	Seamount 127	25/09/2014	Surface	Extracted		
5GIG	38.9891	-29.8908	Gigante seamount no visual	26/09/2014	Surface	Extracted	*Mobula tarapacana*	*no*
6GIG	38.7402	-29.4442	Far field from GIG - no visual	26/09/2014	Surface	Extracted	*Mobula tarapacana*	*no*
7GIG	38.9899	-29.8905	Gigante seamount	26/09/2014	Surface	Extracted		
8GIG	38.9881	-29.8815	Gigante seamount	26/09/2014	Surface	Extracted		
64PE388-02-10-1	37.3333	-32.2667	Lucky strike	12/05/2014	off bottom	On filter		
64PE388-02-10-2	37.3333	-32.2667	Lucky strike	12/05/2014	mid	On filter		
64PE388-02-10-3	37.3333	-32.2667	Lucky strike	12/05/2014	sub surface	On filter		
64PE388-29-01-4	36.2365	-33.9105	Rainbow	15/05/2014	bottom	On filter		
64PE388-29-01-5	36.2365	-33.9105	Rainbow	15/05/2014	mid	On filter		
64PE388-29-01-6	36.2365	-33.9105	Rainbow	15/05/2014	surface	On filter		
64PE388-30-01-7	36.2478	-33.9245	Rainbow	15/05/2014	bottom	On filter		
64PE388-30-01-8	36.2478	-33.9245	Rainbow	15/05/2014	mid	On filter		
64PE388-30-01-9	36.2478	-33.9245	Rainbow	15/05/2014	surface	On filter		
64PE388-41-01-10	36.1614	-33.9020	Rainbow	16/05/2014	bottom	On filter		
64PE388-41-01-11	36.1614	-33.9020	Rainbow	16/05/2014	mid	On filter		
64PE388-41-01-12	36.1614	-33.9020	Rainbow	16/05/2014	surface	On filter		

64PE398-0301A-1	36.2297	-33.9339	Rainbow	08/04/2015	off bottom	On filter		
64PE398-0301A-2	36.2297	-33.9339	Rainbow	08/04/2015	mid	On filter		
64PE398-0301A-3	36.2297	-33.9339	Rainbow	08/04/2015	sub surface	On filter		
64PE398-0610-4	36.2367	-33.8394	Rainbow	09/04/2015	bottom	Extracted		
64PE398-0610-5	36.2367	-33.8394	Rainbow	09/04/2015	mid	On filter		
64PE398-0610-6	36.2367	-33.8394	Rainbow	09/04/2015	surface	On filter		
64PE398-1210-7	36.2192	-33.8603	Rainbow	11/04/2015	bottom	Extracted		
64PE398-1210-8	36.2192	-33.8603	Rainbow	11/04/2015	mid	On filter		
64PE398-1210-9	36.2192	-33.8603	Rainbow	11/04/2015	surface	On filter		
64PE398-1501-10	36.1014	-34.0605	Rainbow	11/04/2015	bottom	Extracted		
64PE398-1501-11	36.1014	-34.0605	Rainbow	11/04/2015	mid	On filter		
64PE398-1501-12	36.1014	-34.0605	Rainbow	11/04/2015	surface	On filter		
64PE398-1609-13	36.2362	-33.8937	Rainbow	12/04/2015	bottom	Extracted		
64PE398-1609-14	36.2362	-33.8937	Rainbow	12/04/2015	mid	On filter		
64PE398-1609-15	36.2362	-33.8937	Rainbow	12/04/2015	surface	On filter		
64PE398-1609-16	36.2362	-33.8937	Rainbow	12/04/2015	at the plume	Extracted	*Helicolenus dactylopterus, Mirocris fortunata, Hoplostethus atlanticus*	*no, no, no*
64PE398-4221-17	36.2626	-33.8662	Rainbow	22/04/2015	bottom	Extracted		
64PE398-4221-18	36.2626	-33.8662	Rainbow	22/04/2015	at the plume	Extracted	*Helicolenus dactylopterus, Mirocris fortunata, Hoplostethus atlanticus*	*no, no, no*
64PE398-4221-19	36.2626	-33.8662	Rainbow	22/04/2015	mid	On filter		
64PE398-4221-20	36.2626	-33.8662	Rainbow	22/04/2015	surface	On filter		
64PE398-4501-21	36.2295	-33.7738	Rainbow	23/04/2015	bottom	Extracted		

64PE398-4501-22	36.2295	-33.7738	Rainbow	23/04/2015	at the plume	Extracted		
64PE398-4501-23	36.2295	-33.7738	Rainbow	23/04/2015	mid	On filter		
64PE398-4501-24	36.2295	-33.7738	Rainbow	23/04/2015	surface	On filter		
64PE398-4601-25	36.2301	-33.7330	Rainbow	23/04/2015	bottom	Extracted		
64PE398-4601-26	36.2301	-33.7330	Rainbow	23/04/2015	at the plume	Extracted		
64PE398-4601-27	36.2301	-33.7330	Rainbow	23/04/2015	mid	On filter		
64PE398-4601-28	36.2301	-33.7330	Rainbow	23/04/2015	surface	On filter		
64PE398-4701-29	36.3184	-33.7929	Rainbow	23/04/2015	bottom	Extracted		
64PE398-4701-30	36.3184	-33.7929	Rainbow	23/04/2015	plume	On filter		
64PE398-4701-31	36.3184	-33.7929	Rainbow	23/04/2015	mid	On filter		
64PE398-4701-32	36.3184	-33.7929	Rainbow	23/04/2015	surface	On filter		
64PE398-4801-33	36.4244	-33.8719	Rainbow	23/04/2015	bottom	Extracted		
64PE398-4801-34	36.4244	-33.8719	Rainbow	23/04/2015	surface	On filter		
64PE398-4908-35	36.3760	-33.8572	Rainbow	24/04/2015	bottom-plume	Extracted	*Helicolenus dactylopterus, Mirocris fortunata, Hoplostethus atlanticus*	*no, no, no*
64PE398-4908-36	36.3760	-33.8572	Rainbow	24/04/2015	mid	On filter		
64PE398-4908-37	36.3760	-33.8572	Rainbow	24/04/2015	surface	On filter		
64PE412-11-C1-1	36.2333	-33.8688		30/06/2016	Bottom	Extracted		
64PE412-11-C1-2	36.2333	-33.8688		30/06/2016	Plume	On filter		
64PE412-11-C1-3	36.2333	-33.8688		30/06/2016	Mid-water	On filter		
64PE412-11-C1-4	36.2333	-33.8688		30/06/2016	Surface	On filter		
64PE412-19-C1-5	36.3950	-33.8775		02/07/2016	Bottom	Extracted	*Helicolenus dactylopterus, Mirocris fortunata, Hoplostethus atlanticus*	*no, no, no*

64PE412-19-C1-6	36.3950	-33.8775		02/07/2016	Mid-water	On filter		
64PE412-19-C1-7	36.3950	-33.8775		02/07/2016	Surface	On filter		
64PE412-21-C1-8	36.3900	-33.8900		03/07/2016	Bottom	Extracted		
64PE412-21-C1-9	36.3900	-33.8900		03/07/2016	Mid-water	On filter		
64PE412-21-C1-10	36.3900	-33.8900		03/07/2016	Surface	On filter		
64PE412-30-C1-11	36.4014	-33.8925		04/07/2016	Bottom	Extracted		
64PE412-30-C1-12	36.4014	-33.8925		04/07/2016	Mid-water	On filter		
64PE412-30-C1-13	36.4014	-33.8925		04/07/2016	Surface	On filter		
64PE412-37-C1-14	36.0685	-33.9819		06/07/2016	Bottom	Extracted	*Helicolenus dactylopterus, Mirocris fortunata, Hoplostethus atlanticus*	*no, no, no*
64PE412-37-C1-15	36.0685	-33.9819		06/07/2016	Mid-water	On filter		
64PE412-37-C1-16	36.0685	-33.9819		06/07/2016	Surface	On filter		
64PE412-40-C1-17	36.0291	-33.6968		06/07/2016	Bottom	Extracted		
64PE412-40-C1-18	36.0291	-33.6968		06/07/2016	Mid-water	On filter		
64PE412-40-C1-19	36.0291	-33.6968		06/07/2016	Surface	On filter		
64PE412-48-C1-20	36.0350	-33.7407		09/07/2016	Bottom	Extracted		
64PE412-48-C1-21	36.0350	-33.7407		09/07/2016	Mid-water	On filter		
64PE412-48-C1-22	36.0350	-33.7407		09/07/2016	Surface	On filter		
43-V15-1	34.9336	-27.4455	Pico Sul seamount	07/07/2015	Surface	Extracted	*Mobula tarapacana*	*no*
43-V15-2	33.9363	-28.2969	Tyro seamount	08/07/2015	Surface	On filter		
43-V15-3	31.9403	-28.1304	Irving seamount	09/07/2015	Surface	On filter		
43-V15-4	30.1299	-28.5464	Great Meteor seamount	10/07/2015	Surface	Extracted	*Mobula tarapacana*	*no*
43-V15-5	29.6078	-28.3431	Great Meteor seamount	11/07/2015	Surface	Extracted	*Mobula tarapacana*	*no*
43-V15-6	29.7445	-28.4901	Great Meteor seamount	11/07/2015	Surface	On filter		

43-V15-7	29.9463	-28.7370	Great Meteor seamount	12/07/2015	Surface	On filter		
43-V15-8	30.0457	-28.7202	Great Meteor seamount	12/07/2015	Surface	On filter		
43-V15-9	30.0488	-28.7254	Great Meteor seamount	13/07/2015	Surface	On filter		
43-V15-10	30.0209	-28.7451	Great Meteor seamount	13/07/2015	Surface	On filter		
43-V15-11	34.1269	-30.2494	Atlantis seamount	22/07/2015	Surface	On filter		
43-V15-12	34.1291	-30.1743	Atlantis seamount	23/07/2015	Surface	Extracted	*Mobula tarapacana*	*no*
43-V15-13	34.1476	-30.1562	Atlantis seamount	24/07/2015	Surface	Extracted	*Mobula tarapacana*	*no*
43-V15-14	33.9684	-28.3576	Tyro seamount	25/07/2015	Surface	Extracted	*Mobula tarapacana*	*no*
43-V15-15	33.9146	-28.4404	Tyro seamount	26/07/2015	Surface	Extracted	*Mobula tarapacana*	*no*
43-V15-16	32.1019	-27.9261	Irving seamount	27/07/2015	Surface	Extracted	*Mobula tarapacana*	*no*
43-V15-17	32.0472	-27.9690	Irving seamount	28/07/2015	Surface	Extracted	*Mobula tarapacana*	*no*
43-V15-18	32.1077	-28.0501	Irving seamount	29/07/2015	Surface	On filter		
DOP15-D03-W1	34.1528	-30.3057	Atlantis seamount	22/09/2015	Bottom	Extracted		
DOP15-D04-W1	32.0298	-28.2027	Irving seamount	24/09/2015	Bottom	Extracted		
DOP15-D05-W1	31.8732	-28.0610	Irving seamount	25/09/2015	Bottom	Extracted		
DOP15-D05-W2	31.8788	-28.0575	Irving seamount	25/09/2015	Bottom	Extracted		
MEDW-ST13-1	36.4505	-7.0023	Gazul	22/09/2016	Bottom	Extracted		
MEDW-ST13-2	36.4505	-7.0023	Gazul	22/09/2016	Mid-Water	Extracted	*Mobula tarapacana*	*no*
MEDW-ST13-3	36.4505	-7.0023	Gazul	22/09/2016	Surface	Extracted	*Mobula tarapacana*	*no*
MEDW-ST16-4	36.5008	-6.8857	Gazul	23/09/2016	Bottom	Extracted		
MEDW-ST16-5	36.5008	-6.8857	Gazul	23/09/2016	Mid-Water	Extracted	*Mobula tarapacana*	*no*
MEDW-ST16-6	36.5008	-6.8857	Gazul	23/09/2016	Surface	Extracted	*Mobula tarapacana*	*no*
MEDW-ST39-7	36.8827	-10.9060	Ormonde	25/09/2016	Bottom	Extracted		

MEDW-ST39-8	36.8827	-10.9060	Ormonde	25/09/2016	Mid-Water	Extracted	*Mobula tarapacana*	*no*
MEDW-ST39-9	36.8827	-10.9060	Ormonde	25/09/2016	Surface	Extracted	*Mobula tarapacana*	*no*
MEDW-ST41-10	36.7970	-10.9848	Ormonde	26/09/2016	Bottom	Extracted		
MEDW-ST41-11	36.1299	-10.1520	Ormonde	26/09/2016	Mid-Water	Extracted	*Mobula tarapacana*	*no*
MEDW-ST41-12	36.1299	-10.1520	Ormonde	26/09/2016	Surface	Extracted	*Mobula tarapacana*	*no*
MEDW-ST43-13	36.7095	-11.1428	Ormonde	26/09/2016	Bottom	Extracted		
MEDW-ST43-14	36.7095	-11.1428	Ormonde	26/09/2016	Mid-Water	On filter		
MEDW-ST43-15	36.7095	-11.1428	Ormonde	26/09/2016	Surface	On filter		
MEDW-ST50-16	37.1588	-24.5595	Formigas	30/09/2016	Bottom	Extracted		
MEDW-ST50-17	37.1588	-24.5595	Formigas	30/09/2016	Mid-Water	On filter		
MEDW-ST50-18	37.1588	-24.5595	Formigas	30/09/2016	Surface	On filter		
MEDW-ST52-19	37.2163	-24.6655	Formigas	30/09/2016	Bottom	Extracted		
MEDW-ST52-20	37.2163	-24.6655	Formigas	30/09/2016	Mid-Water	On filter		
MEDW-ST52-21	37.2163	-24.6655	Formigas	30/09/2016	Surface	On filter		
MEDW-ST69-22	37.2837	-24.7873	Formigas	01/10/2016	Bottom	Extracted		
MEDW-ST69-23	37.2837	-24.7873	Formigas	01/10/2016	Mid-Water	On filter		
MEDW-ST69-24	37.2837	-24.7873	Formigas	01/10/2016	Surface	On filter		
MEDW-ST71-25	37.3000	-24.8200	Formigas	01/10/2016	Bottom	Extracted		
MEDW-ST71-26	37.3000	-24.8200	Formigas	01/10/2016	Mid-Water	On filter		
MEDW-ST71-27	37.3000	-24.8200	Formigas	01/10/2016	Surface	On filter		
MEDW-ST150-28	36.7197	-11.2981	Ormonde	20/10/2016	Bottom	Extracted		
MEDW-ST150-29	36.7197	-11.2981	Ormonde	20/10/2016	Mid-Water	On filter		
MEDW-ST150-30	36.7197	-11.2981	Ormonde	20/10/2016	Surface	On filter		
MEDW-ST151-31	36.7164	-11.2464	Ormonde	20/10/2016	Bottom	On filter		
MEDW-ST151-32	36.7164	-11.2464	Ormonde	20/10/2016	Mid-Water	On filter		
MEDW-ST151-33	36.7164	-11.2464	Ormonde	20/10/2016	Surface	On filter		

MEDW-ST159-34	36.8462	-11.1048	Ormonde	20/10/2016	Bottom	Extracted		
MEDW-ST159-35	36.8462	-11.1048	Ormonde	20/10/2016	Mid-Water	On filter		
MEDW-ST159-36	36.8462	-11.1048	Ormonde	20/10/2016	Surface	On filter		
MEDW-ST164-37	36.7001	-10.9856	Ormonde	21/10/2016	Bottom	Extracted		
MEDW-ST164-38	36.7001	-10.9856	Ormonde	21/10/2016	Mid-Water	On filter		
MEDW-ST164-39	36.7001	-10.9856	Ormonde	21/10/2016	Surface	On filter		
MEDW-ST172-40	36.5167	-2.8494	Seco Olivos	23/10/2016	Bottom	Extracted	*Helicolenus dactylopterus, Mirocris fortunata, Hoplostethus atlanticus*	*no, no, no*
MEDW-ST172-41	36.5167	-2.8494	Seco Olivos	23/10/2016	Mid-Water	On filter		
MEDW-ST172-42	36.5167	-2.8494	Seco Olivos	23/10/2016	Surface	On filter		
MEDW-ST188-43	36.4227	-2.8350	Seco Olivos	24/10/2016	Bottom	Extracted	*Helicolenus dactylopterus, Mirocris fortunata, Hoplostethus atlanticus*	*no, no, no*
MEDW-ST188-44	36.4227	-2.8350	Seco Olivos	24/10/2016	Mid-Water	On filter		
MEDW-ST188-45	36.4227	-2.8350	Seco Olivos	24/10/2016	Surface	On filter		
MEDW-ST191-46	36.5637	-2.7600	Seco Olivos	24/10/2016	Bottom	Extracted		
MEDW-ST191-47	36.5637	-2.7600	Seco Olivos	24/10/2016	Mid-Water	On filter		
MEDW-ST191-48	36.5637	-2.7600	Seco Olivos	24/10/2016	Surface	On filter		
MEDW-ST204-49	36.4227	-2.9633	Seco Olivos	24/10/2016	Bottom	On filter		
MEDW-ST204-50	36.4227	-2.9633	Seco Olivos	24/10/2016	Mid-Water	On filter		
MEDW-ST204-51	36.4227	-2.9633	Seco Olivos	24/10/2016	Surface	On filter		
UCD IS R1	52.9111	-6.0306	Irish Sea Wicklow	07/08/2018	Bottom	Extracted	*Galeorhinus galeus*	*yes*
UCD IS R2	52.9111	-6.0306	Irish Sea Wicklow	07/08/2018	Bottom	Extracted	*Galeorhinus galeus*	*yes*

UCD IS R3	52.9111	-6.0306	Irish Sea Wicklow	07/08/2018	Bottom	Extracted	*Galeorhinus galeus*	*yes*
UCD IS R1	53.2977	-6.1476	Irish Sea Dublin Bay no visual	08/11/2015	Surface	Extracted	*Mobula tarapacana*	*no*
UCD IS R2	53.2977	-6.1476	Irish Sea Dublin Bay no visual	08/11/2015	Surface	Extracted	*Mobula tarapacana*	*no*
UCD IS R3	53.2977	-6.1476	Irish Sea Dublin Bay no visual	08/11/2015	Surface	Extracted	*Mobula tarapacana*	*no*
Deep-Links 3.1 R1	36.9291	-7.1545	Anastasya mud volcano	01/11/2015	Bottom	Extracted	*Helicolenus dactylopterus*	*yes*
Deep-Links 3.1 R2	36.9291	-7.1545	Anastasya mud volcano	01/11/2015	Bottom	Extracted	*Helicolenus dactylopterus*	*yes*
Deep-Links 3.1 R3	36.9291	-7.1545	Anastasya mud volcano	01/11/2015	Bottom	Extracted	*Helicolenus dactylopterus*	*yes*
BAz 2018 ST03 W01	38.2943	-28.1927	PN6	03/06/2018	819m depth	On filter		
BAz 2018 ROV3 ST07 W01	38.2954	-28.1135	PN5	04/06/2018	522m depth	On filter		
BAz 2018 D5 ST17	38.2059	-28.1601	PS	07/06/2018	919m depth	On filter		
BAz 2018 D6 ST20 W01	38.4308	30.0051	G127	08/06/2018	382m depth	On filter		
BAz 2018 D7 ST27 W01	38.5905	-29.4947	EG	14/06/2018	682m depth	On filter		
BAz 2018 D8 ST36 W01	38.5905	-29.5004	EG	16/06/2018	571m depth	On filter		
BAz 2018 D10 ST43 W01	38.4430	-30.0246	G127 NW	18/06/2018	526m depth	On filter		
BAz 2018 D10 ST43 W02	38.4423	-30.0248	G127 NW	18/06/2018	427m depth	On filter		
BAz 2018 D11 ST45 W01	38.4313	-29.5749	G127ENE	19/06/2018	400m depth	On filter		
BAz 2018 D12 ST50 W01	38.5816	-29.5102	GS	21/06/2018	682m depth	On filter		
BAz 2018 D12 ST50 W02	38.5838	-29.5105	GS	21/06/2018	367m depth	On filter		

BAz 2018 D14 ST54 W01	39.0016	-29.5516	GNW	22/06/2018	512m depth	On filter
BAz 2018 D14 ST54 W02	38.5959	-29.5516	GNW	22/06/2018	402m depth	On filter
BAz 2018 D15 ST57 W01	38.4229	-30.1320	GAS	23/06/2018	557m depth	On filter
BAz 2018 D15 ST57 W02	38.4158	-30.1348	GAS	23/06/2018	440m depth	On filter

1no visual indicates that no visual observations were done at the time of sampling.

7. Document information

EU Project N°	678760	Acronym	ATLAS
Full Title	A trans-Atlantic assessment and deep-water ecosystem-based spatial management plan for Europe		
Project website	www.eu-atlas.org		

Deliverable	N°	D3.5	Title	Potential and limits of metabarcoding of eDNA and qPCR
Work Package	N°	WP3	Title	Biodiversity and Biogeography

Date of delivery	Contractual	31st October 2019	Actual	6th November 2019
Dissemination level	x	PU Public, fully open, e.g. web		
		CO Confidential restricted under conditions set out in Model Grant Agreement		
		CI Classified, information as referred to in Commission Decision 2001/844/EC		

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Version log			
Issue Date	Revision N°	Author	Change