TWInning Laboratory for an Innovative Global Hub To Explore the Deep

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TWILIGHTED Scientific Training Handbook on imaging, stable isotope and eDNA analysis

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1. Overview

This scientific training handbook introduces scientific methods that have proven powerful to improve understanding of deep-sea biodiversity, biological interactions and food web structure and functioning, and that are core methods in the EU TWILIGHTED project. Specifically, this includes (1) imaging, (2) stable isotope analysis, and (3) environmental DNA analysis. For each of these methods, the handbook provides an introduction to the theoretical foundations and applications in deep-sea research, method strengths and limitations, and sampling and laboratory analysis protocols. Also provided are suggestions for further reading and links to software packages for statistical analysis, synthesis, visualization and interpretation of actual data sets. The handbook contains all methods and protocols taught during the 2025 EU TWILIGHTED Summer Training Workshop at GEOMAR Helmholtz Centre for Ocean Research Kiel, Germany, June 10-20, 2025 (see Appendix 2), and is intended as introductory material to aid deep-sea knowledge and skills transfer within the TWILIGHTED consortium and the wider scientific community.

1.1. Project introduction

TWILIGHTED is a Horizon Europe Twinning project that seeks to revolutionize deep-sea research, development and innovation (RD&I) in Portugal, ultimately benefiting all of Europe. Collaborating closely with GEOMAR (Germany) and NTNU (Norway), TWILIGHTED aims to transform ARDITI (Madeira, Portugal) into a global hub for deep-sea RD&I. Focusing on the Mesophotic Zone (40-200 m) and the Twilight Zone (200-1,000 m), and capitalizing on Madeira's unique proximity to deep waters, TWILIGHTED will help accelerate our global understanding of the ocean and its essential role in sustaining life on earth.

TWILIGHTED's key objectives are to: (1) Collaborate across research institutes in Europe, (2) Elevate the research profile of Portugal and especially the European Outermost Region of Madeira, (3) Innovate low-cost alternatives to state-of-the-art deep-sea research technologies, (4) Democratize deep-sea research, (5) Globalize deep-sea RD&I and (6) Share ocean science across stakeholders.

To achieve its objectives, TWILIGHTED adopts state-of-the-art approaches to training and networking. Development activities include staff exchanges, expert visits, training schools, joint research missions, the International Twilighted Conference and novel cross-sector workshops stimulating creativity in solving the ocean's greatest challenges (the Impossible Things Workshops). Such capacity-building will not only catalyze deep-sea RD&I in Madeira but also ensure a lasting impact on the deep-sea scientific landscape, sustainable ocean policy, and Madeira's socioeconomic development.

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2. Handbook contributors

Theme 1: Imaging

Julian Stauffer
GEOMAR

Julian is a PhD student in Henk-Jan Hoving's deep-sea biology working group at GEOMAR. His main study focus lies in mapping benthic habitats of oceanic island slopes with hydroacoustic and optical methods. This includes imagery data from ROV and towed camera systems as well as bathymetry data from multibeam echosounders.

Nis Hansen, GEOMAR

Nis is a PhD student in the deep-sea biology working group at GEOMAR. He is working with imaging sensors, including PELAGIOS, stereo camera, baited camera landers and ZooScan, to study gelatinous zooplankton communities in the North Atlantic and Mediterranean Sea.

Theme 2: Stable isotope analysis

Robert Priester,
OKEANOS, Azores

Robert is a shark ecologist based on Faial Island in the Azores, Portugal, currently pursuing a PhD on the role of oceanic island shark nurseries in supporting broader Atlantic populations. He uses a multidisciplinary approach—combining biotelemetry, biologging, aerial and underwater video surveys, and stable isotope analysis—to investigate how juvenile sharks use island-associated habitats. He aims to understand the biotic and abiotic drivers of their behavior and explore how these insights can inform management and conservation strategies.

Jan Dierking, GEOMAR

Jan is a marine ecologist at GEOMAR, with a particular interest in the structure and functioning of marine food webs and the trophic ecology of species and groups within these webs in our rapidly changing seas. His work has focused on diverse systems, from coral reefs to temperate seas and more recently, the deep-sea. Stable isotope analysis has been a tremendously useful tool along the way, often in combination with other methods like stomach content analysis and via the integration with biological and physical long-term data sets.

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Theme 3: environmental DNA analysis

Véronique Merten, GEOMAR

Véronique's research focuses on deep-sea organisms, with a particular interest in cephalopods and cetaceans. By combining eDNA analysis (5+ years of experience) with techniques like biologging, net trawls, and video observations, she studies predator-prey interactions and establishes biodiversity baselines to trace ecosystem changes and identify biogeographic patterns.

Ina Vornsand, GEOMAR

Ina started working with eDNA during the course of her master studies and wrote my thesis centered around the analysis of predator-prey dynamics between cephalopods and cetaceans using eDNA metabarcoding. As part of her project, she has conducted every step of the eDNA workflow, from sampling and filtration to lab processing and final bioinformatic analysis. Since then, she has been involved in several other eDNA projects, initially as a student assistant and now as a research assistant. These projects were primarily focused on cephalopods and cetaceans around the Azores, but also include work conducted off Cabo Verde and Madeira.

Deep-sea ecology

Henk-Jan Hoving, GEOMAR

Henk-Jan is marine biologist, leading a research program on biodiversity, adaptation and food webs of deep-sea fauna with a focus on pelagic invertebrates. Via the application of state-of-the-art deep-sea technology during oceanic research expeditions, he uses in-situ observations, molecular genetics including eDNA analysis and physical sampling to investigate baseline biodiversity data, behavioral and food web interactions and organismal contributions to the biological carbon pump in a rapidly changing ocean.

All GEOMAR contributors are members of the Research Unit "Marine Evolutionary Ecology" within Research Division 3 "Marine Ecology".

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3. Motivation and concept of this handbook

Deep-sea organisms and biological communities are notoriously hard to study. Due to the challenging deep-sea environment, which is characterized by darkness and high pressure, specialized gear is needed to collect specimens, samples and observations. At the same time, deep-sea technology is often expensive as is the ship time on large research vessels that is needed to deploy instruments. Additionally, each deep-sea instrument is subject to a sampling bias. For example, trawls and plankton nets are able to collect organisms, but they sample only a selection of the community. E.g., many delicate gelatinous taxa cannot withstand the sheer of the net and are damaged, and fast moving and larger taxa may be able to avoid the nets.

In light of these difficulties, deep-sea biological research has traditionally focused on description and exploration more strongly than shallow water ecology. However, in an era of rapid anthropogenic change, there is now an urgent need to understand not only biodiversity, but move towards functional understanding of deep-sea ecosystems (Sander et al. 2025, Howell et al. 2020). The methods covered in the TWILIGHTED Scientific Training School have proven powerful in this regard, and are core tools in marine ecology and deep-sea biology research at GEOMAR. All of them are currently also benefiting from technological and methodological advances that increase the potential for low(er) cost applications, one of the core goals of the TWILIGHTED project.

Specifically, in-situ observations using still or video camera systems (Theme 1: Imaging) can allow us to document fragile taxa like gelatinous zooplankton, but also provides information on animal behavior, posture and interactions. Secondly, the analysis of environmental DNA (Theme 3: eDNA) is a relatively novel tool for biodiversity censuses, which has proven to be efficient and relatively cost effective. This technique allows the detection of taxa based on genetic traces they leave behind in the environment (mucus, feces, skin cells). Using general or specific primers, the presence but also the spatial distribution of deep-sea organisms and the composition of biological communities can be reconstructed from water volumes of just a few liters. Thirdly, the analysis of the stable isotope composition of animal tissues (Theme 2: stable isotope analysis) provides a time-integrated view of the feeding of organisms over time. This powerful application has led to the emergence of the research field stable isotope ecology, which addresses a wide range of scientific topics including the trophic ecology of organisms and the structure of food webs, including the sources of organic material and organic matter flows.

This handbook provides a brief background on theoretical foundations, an introduction to the sampling and analysis protocols, and information on laboratory pipelines for the respective methods in the molecular and biochemical laboratories at GEOMAR, and the subsequent work (statistical analysis, synthesis, visualization and interpretation) with resulting data sets. The goal of this handbook is to serve as a first primer for readers, but will not replace reading the background literature and gaining practical hands-on experience with these methods, e.g., in the context of EU TWILIGHTED training activities.

References:

Sander et al. (2025) Deep-sea Research and Management Needs. Future Science Brief N°. 12 of the European Marine Board, Ostend, Belgium. DOI: 10.5281/zenodo.14928917

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This project has received funding from the European Union's Horizon Europe program under Grant Agreement 101158714

Howell et al. (2020). A Blueprint for an Inclusive, Global Deep-Sea Ocean Decade Field Program. Frontiers in Marine Science 7:584861. doi: 10.3389/fmars.2020.584861

4. Theme 1: Imaging

Contributors: Julian Stauffer and Nis Hansen

4.1. What will readers take away from this section?

This handbook section elaborates on imaging, with a particular focus on food fall experiments with camera landers to investigate benthic scavenging communities.

4.2. Background: Relevance of imaging for deep-sea research

In-situ imaging has emerged as an advantageous and non-invasive approach to study deep-sea organisms in their natural habitats. Optical instruments provide valuable information about distribution, diversity, abundance, behavior, intra- and interspecific interactions, and the role of marine taxa in ocean dynamics. Furthermore, in situ observation methods can be used to describe and map vulnerable marine ecosystems (VME) as baseline for conservation measures. A great variety of technologies and platforms, including cameras mounted on remotely operated vehicles (ROVs), automated underwater vehicles (AUVs), towed camera systems, profiling camera systems, and baited camera landers, are used for various purposes. For example, towed in situ camera systems, including PELAGIOS (Hoving et al., 2019) but also ROVs contributed significantly to the study of fragile gelatinous zooplankton, which are challenging to sample with conventional gear like nets. Imaging systems deployed with an ROV were used to study the locomotion of deep-sea octopus (Katija 2025) and camera platforms were used to attract the elusive giant squid *Architeuthis dux* (Robinson 2021). Optical methods are also used for ground-truthing habitat mapping approaches (Misiuk 2024). The increased use of optical methods to study marine life led to the development of new software for annotation (i.e. identify and label organisms in images and video). Among these, BIIGLE (Langenkämper et al., 2017) has emerged as a powerful, open source and web-based software with the possibility of sharing datasets with colleagues worldwide.

This handbook focuses on the application of imaging techniques to study food falls. Food falls are dead pelagic animals that sink through the water column and end up on the seafloor where they act as input of concentrated organic material to an otherwise food depleted habitat (Stockton & DeLaca 1982, Smith et al. 1985). Food falls can fuel the deep seafloor and are an important food source for scavenging organisms. They can act as express pathways of carbon from the surface layers of the oceans to the deep seafloor where carbon can be stored over long timescales. This makes them a potentially important pathway of the biological carbon pump (Higgs et al. 2014). Food falls, especially small- to medium-sized food falls, are challenging to study since they are scavenged relatively fast and are thus hard to detect (Smith 1985, Soltwedel et al. 2003, Sweetman et al. 2014). This makes it difficult to assess the contribution of food falls to the global carbon cycle. Optical methods can be used to either study the distribution of natural food falls (Stauffer et al. 2022) or camera landers with experimental food falls can be deployed to

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measure scavenging rates and study scavenging communities and their succession (Scheer et al. 2022).

4.3 Short technical background on the method

Baited camera landers are used to study scavenging communities using a time-lapsed camera in combination with bait (Scheer et al. 2022). They can be used to study scavenging communities without the necessity of finding natural food falls. Landers are platforms equipped with various sensors that are lowered to the seafloor. They can be deployed as moorings (e.g. connected to the water surface via buoys) or with trigger-released weights. Specifically, baited camera landers are lander platforms equipped with camera sensors, lights, bait and additional sensors. The bait is often placed on a bait plate with a chessboard pattern of known size to allow sizing of the scavenging organisms. This can alternatively be achieved with sizing lasers. Time-lapse imaging systems like the Ocean Imaging System (OIS) can be used to take images in fixed time intervals (oceanimagingsystems.com). A more cost-effective camera system is the PlasPI camera which can be deployed in shallow waters of < 150 m and has an integrated flash and batteries (Purser et al. 2020). Its successor, the PLasPI TDM, is rated to 200 m and has integrated multispectral, temperature and pressure sensors (Zinzindohoué et al. 2023).

Lander platforms can be big steel constructions but can also be designed as small, easy-to-deploy units. The baby-lander of GEOMAR (Figure 4.1) is a small lander which can also be deployed by hand in shallow depths. It was constructed to fit a PlasPI camera and can be equipped with additional sensors like a pressure and temperature logger. This setup was proven to be useful to study experimental food falls in the Baltic Sea from research vessels like RV ALKOR but also hand-deployed at the local Pier.

Although sampling of image and video data can be challenging, especially in the deep sea, the true challenge of in situ imaging lies in the analysis of the huge amount of acquired data. Robust training sets and functional AI tools are often still missing for deep-sea imagery data, meaning that a lot of projects still rely on manual annotations. BIIGLE contains a range of tools that help streamline the annotation process and facilitate data sharing and exporting of annotation data. It is an open-source web-based tool which can be accessed via web browsers. The internal data structure of BIIGLE is partitioned into projects, volumes, annotations and labels. A project can contain several volumes of video and image data. A project can for example be a research cruise and the volumes can then be the different stations that were sampled during the cruise. Annotations can be created as different shapes including point and rectangle annotations as well as whole frame annotations for video data or whole image annotations for still images. These annotations mark the pixel coordinates of objects on an image or a specific or several frames of a video file. Labels are then attached to these annotations, describing the object (e.g. "Crustacean", "Fish"). Labels are organized in hierarchical "label trees" which can be created by any user and can be shared between different projects. BIIGLE contains a permission system that controls the permission of users to read or edit different projects and label trees. There are four different permission levels a user can have in a project: admin, expert, editor and guest. The annotations can be exported in different reporting formats. Pixel coordinates of the annotations can be exported and later used to train neural networks such as YOLO for automated object detection and segmentation. If sizing lasers were used, they can be annotated in BIIGLE with the

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help of the DELPHI tool which can automatically detect laser points on images (Schoening et al. 2015). BIIGLE will then transform pixel size to true size, enabling measuring of organisms or calculating observed area directly in the software.

4.4 Approach from sampling to data analysis

Baited camera landers are prepared with cameras, sensors, weight, and bait before being deployed. It is advised to test the whole system before deployment. The camera is programmed to the desired settings and has to be synchronized with additional sensors (e.g. via time). The bait is weighed and put on the lander platform. Usually, when deployed from a research vessel (Figure 4.1) the lander is lowered to the seafloor by a winch. The lander is connected to floating buoys and will be retrieved after hours or days of sampling. It is important to keep a protocol and to note settings, time, positions and problems. After retrieving the lander, the bait is weighed again so scavenging rates can later be calculated. The image and sensor data are retrieved, stored and immediately backed-up on several storage media.

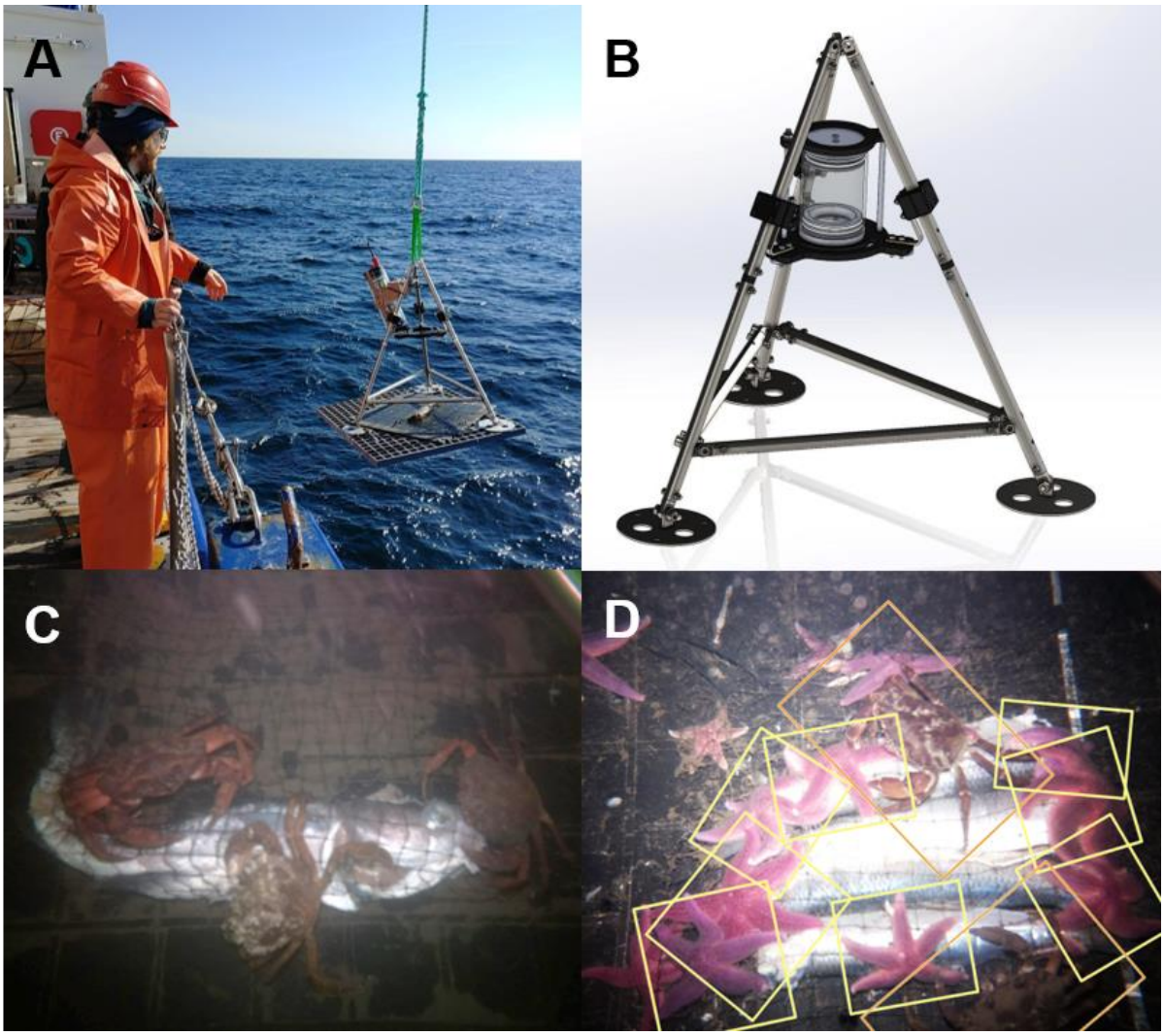

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Figure 4.1. Baited camera lander and analysis. A) The baited lander is deployed by winch over the side of research vessel RV ALKOR. B) Design of the used lander frame with a downward-looking PLasPI camera. This design is modular, compact and light-weight and can also be deployed by hand to shallow depths. C) Image obtained from the lander with Carcinus maenas *feeding on the bait Atlantic cod* Gadus morhua*. D) Annotated image with* Asterias rubens *and Carcinus maenas scavenging on* Clupea harengus *bait.*

Back at GEOMAR the image data is converted to the iFDO (image FAIR Digital Object) format and stored on the ELEMENTS server (Schoening et al. 2022). This can also already happen on the research vessel if the infrastructure is present. From the server, we upload image data to BIIGLE. Now the annotation process can start. Here it is advised to follow a detailed protocol containing rules after which organisms are annotated and identified. Image data often only allows for identifying organisms as morphotypes or Operational Taxonomic Units (OTUs). This will either require the construction of a classification tree and morphotype catalogue for the specific dataset or general classification schemes like CATAMI or SMarTaR-ID can be used (Althaus et al. 2015, Howell et al. 2019). Annotations should be done in a format that allows the later training of object detection models. This means that bounding boxes should be created tightly around the annotated object and where applicable, polygons should be used.

After the annotation process, the produced data will be analyzed to answer research questions and test hypotheses. With this setup we can for example test if different bait types (e.g. jellyfish and fish) attract different scavenging communities and express different scavenging rates. Moreover, diurnal cycles of scavenging communities can be investigated. Software needed along the whole process are BIIGLE, analysis tools like RStudio or Python as well as software to program and retrieve data from the sensors (Positeam 2025). Other useful software tools to handle image and video data are FFmpeg and ExifTool, which can be used to extract still frames from videos and manipulate image and video metadata (ffmpeg.org, exiftool.org).

4.5 References

Althaus, F., Hill, N., Ferrari, R., Edwards, L., Przeslawski, R., Schönberg, C. H. L., Stuart-Smith, R., Barrett, N., Edgar, G., Colquhoun, J., Tran, M., Jordan, A., Rees, T., & Gowlett-Holmes, K. (2015). A Standardised Vocabulary for Identifying Benthic Biota and Substrata from Underwater Imagery: The CATAMI Classification Scheme. PLOS ONE, 10(10), e0141039-. https://doi.org/10.1371/journal.pone.0141039

Higgs, N. D., Gates, A. R., & Jones, D. O. B. (2014). Fish Food in the Deep Sea: Revisiting the Role of Large Food-Falls. PLOS ONE, 9(5), 1–9. https://doi.org/10.1371/journal.pone.0096016

Hoving, H., Christiansen, S., Fabrizius, E., Hauss, H., Kiko, R., Linke, P., & Albrecht, C. (2019). The Pelagic In situ Observation System (PELAGIOS) to reveal biodiversity , behavior , and ecology of elusive oceanic fauna. Ocean Science, 1327–1340.

Howell, K. L., Davies, J. S., Allcock, A. L., Braga-Henriques, A., Buhl-Mortensen, P., Carreiro-Silva, M., Dominguez-Carrió, C., Durden, J. M., Foster, N. L., Game, C. A., Hitchin, B., Horton, T., Hosking, B., Jones, D. O. B., Mah, C., Laguionie Marchais, C., Menot, L., Morato, T., Pearman, T. R. R., … Wagner, D. (2020). A framework for the development of a global

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This project has received funding from the European Union's Horizon Europe program under Grant Agreement 101158714

standardised marine taxon reference image database (SMarTaR-ID) to support image-based analyses. PLOS ONE, 14(12), e0218904-. https://doi.org/10.1371/journal.pone.0218904

Katija, K., Huffard, C. L., Roberts, P. L. D., Daniels, J., Erickson, J., Klimov, D., Ruhl, H. A., & Sherman, A. D. (2025). In situ light-field imaging of octopus locomotion reveals simplified control. Nature, 646(8086), 865–871. https://doi.org/10.1038/s41586-025-09379-z

Langenkämper, D., Zurowietz, M., Schoening, T., & Nattkemper, T. W. (2017). BIIGLE 2.0 - Browsing and Annotating Large Marine Image Collections. Frontiers in Marine Science, Volume 4-2017. https://doi.org/10.3389/fmars.2017.00083

Misiuk, B., & Brown, C. J. (2024). Benthic habitat mapping: A review of three decades of mapping biological patterns on the seafloor. Estuarine, Coastal and Shelf Science, 296, 108599. https://doi.org/https://doi.org/10.1016/j.ecss.2023.108599

Posit team (2025). RStudio: Integrated Development Environment for R. Posit Software, PBC, Boston, MA. URL http://www.posit.co/.

Purser, A., Hoge, U., Lemburg, J., Bodur, Y., Schiller, E., Ludszuweit, J., Greinert, J., Dreutter, S., Dorschel, B., & Wenzhöfer, F. (2020). PlasPI marine cameras: Open-source, affordable camera systems for time series marine studies. HardwareX, 7, e00102. https://doi.org/https://doi.org/10.1016/j.ohx.2020.e00102

Scheer, S. L., Sweetman, A. K., Piatkowski, U., Rohlfer, E. K., & Hoving, H. J. T. (2022). Food fall-specific scavenging response to experimental medium-sized carcasses in the deep sea. Marine Ecology Progress Series, 685, 31–48. https://www.int-res.com/abstracts/meps/v685/p31-48/

Schoening, T., Kuhn, T., Bergmann, M., & Nattkemper, T. W. (2015). DELPHI—fast and adaptive computational laser point detection and visual footprint quantification for arbitrary underwater image collections. Frontiers in Marine Science, Volume 2-2015. https://doi.org/10.3389/fmars.2015.00020

Schoening, T., Durden, J.M., Faber, C. et al. Making marine image data FAIR. Sci Data 9, 414 (2022). https://doi.org/10.1038/s41597-022-01491-3

Smith, C. R. (1985). Food for the deep sea: utilization, dispersal, and flux of nekton falls at the Santa catalina basin floor. Deep Sea Research Part A. Oceanographic Research Papers, 32(4), 417–442. https://doi.org/https://doi.org/10.1016/0198-0149(85)90089-5

Soltwedel, T., von Juterzenka, K., Premke, K., & Klages, M. (2003). What a lucky shot! Photographic evidence for a medium-sized natural food-fall at the deep seafloor. Oceanologica Acta, 26(5), 623–628. https://doi.org/https://doi.org/10.1016/S0399-1784(03)00060-4

Stauffer, J. B., Purser, A., Griffiths, H. J., Smith, C. R., & Hoving, H.-J. T. (2022). Food falls in the deep northwestern Weddell Sea. Frontiers in Marine Science, Volume 9-2022. https://doi.org/10.3389/fmars.2022.1055318

Stockton, W. L., & DeLaca, T. E. (1982). Food falls in the deep sea: occurrence, quality, and significance. Deep Sea Research Part A. Oceanographic Research Papers, 29(2), 157–169. https://doi.org/https://doi.org/10.1016/0198-0149(82)90106-6

Sweetman, A. K., Smith, C. R., Dale, T., & Jones, D. O. B. (2014). Rapid scavenging of jellyfish carcasses reveals the importance of gelatinous material to deep-sea food webs.

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This project has received funding from the European Union's Horizon Europe program under Grant Agreement 101158714

Proceedings of the Royal Society B: Biological Sciences, 281(1796), 20142210. https://doi.org/10.1098/rspb.2014.2210

Zinzindohoué, C. G. F., Schoening, T., Lima, E. B., & Fiedler, B. (2023). PlasPi TDM: Augmentation of a low-cost camera platform for advanced underwater physical-ecological observations. HardwareX, 15, e00470. https://doi.org/https://doi.org/10.1016/j.ohx.2023.e00470

5. Theme 2: Stable isotope analysis in deep-sea research

Contributors: Jan Dierking and Robert Priester

5.1 What will readers take away from this section?

This section is intended as a first primer on the use of stable isotope analysis in deep-sea research and provides background on basic concepts of stable isotopes as ecological tracers, on sampling procedures and sample preparation, and on software packages to process, analyze and interpret stable isotope datasets.

5.2 Background: Relevance of stable isotope analysis for deep-sea research and examples of applications in the field

The deep sea is Earth's largest ecosystem, yet it remains one of the least understood due to its inaccessibility and the logistical challenges of sampling and in-situ observations (Danovaro et al., 2014). In this context, stable isotope analysis (SIA) has emerged as a key tool in deep-sea ecology, offering a means to infer ecological relationships, energy pathways and habitat use with a relatively limited sampling effort (Boecklen et al., 2011, Shipley et al., 2017).

SIA measures the natural ratios of stable isotopes—typically of carbon ($^{13}C/^{12}C$, expressed as $\delta^{13}C$) and nitrogen ($^{15}N/^{14}N$, expressed as $\delta^{15}N$), increasingly also sulfur ($^{34}S/^{32}S$, expressed as $\delta^{34}S$) —in animal tissues or other organic material. These ratios reflect integrated information over time, capturing dietary assimilation and trophic interactions (Peterson and Fry, 1987). These useful properties have led to the emergence of an entire research field, "stable isotope ecology", covering a range of topics from the trophic ecology of individual species, to the assessment of resource partitioning among co-occurring species, to the reconstruction of food webs including the identification of sources of organic matter at their base (Eglite et al., 2023).

Applications of SIA in the deep-sea have been equally diverse. It has been used to investigate the trophic structure of hydrothermal vent communities, revealing reliance on chemosynthetic carbon fixation rather than surface-derived input (Bergquist et al., 2007, Chang et al., 2018). In submarine canyon systems and abyssal plains, SIA has helped delineate vertical and lateral transfer of organic matter from continental margins, shedding light on benthic-pelagic coupling (Demopoulos et al., 2017). Studies on deep-sea fishes and invertebrates have used SIA to identify ontogenetic dietary shifts (Priester et al., 2024a), niche breadth and trophic positions (Loutrage et al., 2025), and foraging strategies over depth gradients (Ñacari et al., 2023). Multiple studies have focused on the characterization of the structure of oceanic (deep-sea) food webs, including dedicated studies focusing on the important role of gelatinous taxa (the "jelly web") (Chi

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et al. 2021). More recently, compound-specific isotope analysis (CSIA) of amino acids has allowed finer-scale resolution of trophic positions, even in taxa with complex or mixed diets.

Overall, the high integrative power, limited sample requirements, and applicability across taxa make stable isotope analysis a highly promising approach for deep-sea ecological research. When paired with other methods such as stomach content analysis, fatty acid analysis or telemetry, SIA provides critical insight into the performance of species and the functioning of deep-sea ecosystems in a changing ocean.

5.3 Short technical background on the method

How does it work?

Stable isotope analysis (SIA) is based on measuring the relative abundance of naturally occurring stable isotopes—most commonly of carbon and nitrogen—in biological or environmental samples. These ratios are expressed in delta (δ) notation as parts per thousand (‰) deviations from international standards (e.g., Vienna Pee Dee Belemnite for carbon; atmospheric N_2 for nitrogen).

Approach from sampling to lab work to data

Sampling: The process begins with the collection of biological tissues (e.g., muscle, liver, skin, whole organism) or environmental material (e.g., particulate organic matter, sediments). Tissue choice is essential as it affects the temporal resolution of isotopic information: metabolically active tissues reflect recent diet, while inert tissues (e.g., chitinous parts, collagen) integrate longer-term signals (Vander Zanden et al., 2015).

To ensure comparability, samples must be handled carefully. This often involves rinsing, freezing or drying, and homogenizing. Lipids and urea (especially in elasmobranchs (Carlisle et al., 2017) and deep-sea species (Hoffman and Sutton, 2010)) can bias $\delta^{13}C$ and $\delta^{15}N$ values, so chemical extraction or mathematical correction may be necessary depending on the target taxa and tissue (Post et al., 2007, Sweeting et al., 2006).

Laboratory Processing: In the lab, prepared samples are combusted in an elemental analyzer, converting organic matter into simple gases (CO_2 and N_2). These are introduced into an isotope ratio mass spectrometer (IRMS), which separates isotopes based on their mass and quantifies their ratios relative to reference gases.

The resulting isotope ratios provide information on ecological axes: $\delta^{13}C$ and $\delta^{34}S$ reflect the source of primary production (e.g., pelagic vs benthic, coastal vs. offshore photosynthetic vs chemosynthetic), while $\delta^{15}N$ increases with each trophic transfer (~3–4‰), enabling estimates of trophic position.

Data Interpretation: Raw isotope data are corrected against standards and often normalized using laboratory reference materials. Analysts then interpret values in ecological context, either qualitatively (e.g., niche shifts, trophic overlap) or quantitatively using mixing models (e.g., MixSIAR, SIAR) or niche metrics (e.g., SIBER). Increasingly, compound-specific isotope analysis (CSIA) of amino acids is used to disentangle baseline variability from true trophic effects, especially useful in spatially heterogeneous systems like the deep-sea.

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Together, this workflow—from careful sampling to robust interpretation—makes SIA a reliable, cost-effective, and highly scalable method for ecological inference in deep-sea environments.

5.4 Recommended sources for further reading on stable isotope analysis

- General overview of current state of the art of SIA - Shipley and Matich (2020)
- Interesting review of animal tracking using SIA - Trueman and Glew (2019)
- The foundation reference - Peterson and Fry (1987)
- Shark stable isotope ecology - Priester et al. (2024b)
- Characterization of a pelagic (deep-sea) food web – Chi et al (2021)

5.5 Software packages for the analysis of stable isotope data sets

- R and R Studio
- Packages:
    - dplyr
    - tibble
    - tidyr
    - ggplot
    - Plotly
    - nicheROVER
    - MixSIAR

5.6 Protocol for stable isotope sample collection and processing

Field sampling

Excise a clean piece of white muscle without skin (size of a pea is sufficient) and place into labelled Eppendorf tube. Freeze samples as soon as possible (if possible -20 °C), and do not add ethanol or other conservatives! Wipe sampling equipment clean between individuals. Depending on the accessibility of the freezer, samples can be kept on ice during sampling of longer stations. If appropriate, collect samples from the same station and store them together in labelled boxes/Ziploc bags.

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Sample conservation

Freeze-dry samples 24 hours. Afterwards, store vacuum sealed or in an exsiccator, alternatively in the freezer until further processing.

Sample processing

1. Homogenization
   Homogenize the samples using a ball mill or mortar and pestle.
2. Lipid extraction
   Put samples into glass scintillation vials with foil lined caps. Add 10 mL of 100% Petroleum Ether, sonicate samples for 15 min., and decant used solution. Repeat this previous step for 2nd rinse.

3. Urea extraction (shark and some deep-sea fishes only)
   Rinse samples in 10mL DIW, sonicate samples for 15 minutes, and decant. Repeat this previous step for 2nd and 3rd rinse. Freezedry samples again over night.

4. Weighing in
   Transfer the pre-determined mass (muscle at ZLCA, Geomar = 50 ± 10 µg) into tin cups using a micro-scale. Then fold the tin cups and place them into dedicated 96-well plates. Only for sulfur analysis: add vanadium pentoxide (V2O5) to the tin cup prior to folding (400 ± 100 µg), which will catalyse the complete oxidation of sulfur during analysis.

5. Conservation
   Store weighed in samples vacuum sealed or in an exsiccator until analysis.

Literature

KIM, S. L. & KOCH, P. L. 2012. Methods to collect, preserve, and prepare elasmobranch tissues for stable isotope analysis. Environmental Biology of Fishes, 95, 53-63.

5.7 References

BERGQUIST, D. C., ECKNER, J. T., URCUYO, I. A., CORDES, E. E., HOURDEZ, S., MACKO, S. A. & FISHER, C. R. 2007. Using stable isotopes and quantitative community characteristics to determine a local hydrothermal vent food web. *Marine Ecology Progress Series,* 330**,** 49-65.

BOECKLEN, W. J., YARNES, C. T., COOK, B. A. & JAMES, A. C. 2011. On the use of stable isotopes in trophic ecology. *Annual review of ecology, evolution, and systematics,* 42**,** 411-440.

CARLISLE, A. B., LITVIN, S. Y., MADIGAN, D. J., LYONS, K., BIGMAN, J. S., IBARRA, M. & BIZZARRO, J. J. 2017. Interactive effects of urea and lipid content confound stable

Funded by the European Union

This project has received funding from the European Union's Horizon Europe program under Grant Agreement 101158714

isotope analysis in elasmobranch fishes. *Canadian Journal of Fisheries Aquatic Sciences,* 74**,** 419-428.

CHANG, N.-N., LIN, L.-H., TU, T.-H., JENG, M.-S., CHIKARAISHI, Y. & WANG, P.-L. 2018. Trophic structure and energy flow in a shallow-water hydrothermal vent: Insights from a stable isotope approach. *PLoS One,* 13**,** e0204753.

CHI, X., DIERKInG, J, HOVING, H.-J., LÜSKOW, F., DENDA, A., CHRISTIANSEN, B., SOMMER, U., HANSEN, T., JAVIDPOUR, J.. 2021. Tackling the jelly web: trophic ecology of gelatinous zooplankton in oceanic food webs of the eastern tropical Atlantic assessed by stable isotope analysis. Limnology and Oceanography 66:289-305.

DANOVARO, R., SNELGROVE, P. V. & TYLER, P. 2014. Challenging the paradigms of deep-sea ecology. *Trends in ecology and evolution,* 29**,** 465-475.

DEMOPOULOS, A. W., MCCLAIN-COUNTS, J., ROSS, S. W., BROOKE, S. & MIENIS, F. 2017. Food-web dynamics and isotopic niches in deep-sea communities residing in a submarine canyon and on the adjacent open slopes. *Marine Ecology Progress Series,* 578**,** 19-33.

EGLITE, E., MOHM, C., DIERKING, J.. 2023. Stable isotope analysis in food web research: Systematic review and a vision for the future for the Baltic Sea macro-region. AMBIO 52:319-338.

HOFFMAN, J. C. & SUTTON, T. T. 2010. Lipid correction for carbon stable isotope analysis of deep-sea fishes. *Deep Sea research part I: Oceanographic research papers,* 57**,** 956-964.

LOUTRAGE, L., SPITZ, J., BRIND'AMOUR, A. & CHOUVELON, T. 2025. Carbon and Nitrogen Stable Isotopes Evidence High Trophic Segregation Within a Meso-to Bathypelagic Micronektonic Invertebrate Community From Canyons in the North-East Atlantic. *Marine Ecology,* 46**,** e70005.

ÑACARI, L. A., ESCRIBANO, R., HARROD, C. & OLIVA, M. E. 2023. Combined use of carbon, nitrogen and sulfur stable isotopes reveal trophic structure and connections in deep-sea mesopelagic and demersal fish communities from the Southeastern Pacific Ocean. *Deep Sea Research Part I: Oceanographic Research Papers,* 197**,** 104069.

PETERSON, B. J. & FRY, B. 1987. Stable isotopes in ecosystem studies. *Annual review of ecology and systematics,* 18**,** 293-320.

POST, D. M., LAYMAN, C. A., ARRINGTON, D. A., TAKIMOTO, G., QUATTROCHI, J. & MONTANA, C. G. 2007. Getting to the fat of the matter: models, methods and assumptions for dealing with lipids in stable isotope analyses. *Oecologia,* 152**,** 179-189.

PRIESTER, C. R., AFONSO, P., TRUEMAN, C. N., MENEZES, G., GRAÇA, G. & FONTES, J. 2024a. Contrasting ontogenetic shifts in habitat and metabolism of three sympatric key deep-sea fishes. *Marine Ecology Progress Series,* 729**,** 185-199.

PRIESTER, C. R., DIERKING, J., HANSEN, T., ABECASIS, D., FONTES, J. M. & AFONSO, P. 2024b. Trophic ecology and coastal niche partitioning of two sympatric shark species in the Azores (mid-Atlantic). *Marine Ecology Progress Series,* 726**,** 113-130.

SHIPLEY, O. N., BROOKS, E. J., MADIGAN, D. J., SWEETING, C. J. & DEAN GRUBBS, R. 2017. Stable isotope analysis in deep-sea chondrichthyans: recent challenges, ecological insights, and future directions. *Reviews in Fish Biology and Fisheries,* 27**,** 481-497.

SHIPLEY, O. N. & MATICH, P. 2020. Studying animal niches using bulk stable isotope ratios: an updated synthesis. *Oecologia***,** 1-25.

Funded by the European Union

This project has received funding from the European Union's Horizon Europe program under Grant Agreement 101158714

SWEETING, C., POLUNIN, N. & JENNINGS, S. 2006. Effects of chemical lipid extraction and arithmetic lipid correction on stable isotope ratios of fish tissues. *Rapid Communications in Mass Spectrometry: An International Journal Devoted to the Rapid Dissemination of Up-to-the-Minute Research in Mass Spectrometry,* 20**,** 595-601.

TRUEMAN, C. N. & GLEW, K. S. J. 2019. Isotopic tracking of marine animal movement. *Tracking animal migration with stable isotopes.* Elsevier.

VANDER ZANDEN, M. J., CLAYTON, M. K., MOODY, E. K., SOLOMON, C. T. & WEIDEL, B. C. 2015. Stable Isotope Turnover and Half-Life in Animal Tissues: A Literature Synthesis. *PLOS ONE,* 10**,** e0116182.

6. Theme 3: Environmental DNA analysis in deep-sea research

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6.1 What will readers take away from this section?

This section is intended as a first primer on the use of eDNA analysis in deep-sea research, including basic concepts of the use of eDNA to characterize biodiversity, technical background on the method, as well as links to further reading and suggested software packages.

6.2 Background: Relevance of environmental DNA for deep-sea research, including examples of applications

Environmental DNA (eDNA) has emerged as a powerful tool for deep-sea research, enabling the detection of biodiversity in remote and hard-to-access environments without the need for direct observation or specimen collection (Merten et al., 2021; Visser et al., 2021). Environmental DNA is defined as genetic material that organisms shed into their environment, such as skin cells, feces, and saliva. (Andruszkiewicz Allan et al., 2021) This DNA can be extracted from environmental samples such as water or sediment without first isolating the organisms it is coming from. By using appropriate marker genes, the DNA can be extracted and identified to family, genus or species level. Environmental DNA analysis in the marine environment has already been applied successfully to study the biodiversity and distribution of fishes or invertebrates (Beng and Corlett, 2020; Merten et al., 2023; Rourke et al., 2022; Stat et al., 2017; Thomsen et al., 2016; Thomsen and Willerslev, 2015; Valentini et al., 2016; Visser et al., 2021), to detect invasive or rare species (Deagle et al., 2003; Jerde et al., 2011), and to investigate population genetics (Andres et al., 2023; Parsons et al., 2025, 2018; Sigsgaard et al., 2017; Tsuji et al., 2020).

Most eDNA studies on pelagic marine diversity have focused on surface waters, with relatively few targeting the deep sea. In the mesopelagic zone, eDNA analysis was found to be more effective than net trawls for detecting marine metazoan communities, based on the volume of seawater filtered (Govindarajan et al., 2021). In the Labrador Sea (500–3000 m), eDNA sampling yielded fish diversity estimates comparable to conventional trawls, though DNA concentrations decreased significantly below 1400 m (McClenaghan et al., 2020). Similarly, in Southwest Greenland, eDNA reads from 188–918 m showed strong correlations with biomass and abundance data from trawling, with dominant taxa consistent across both methods (Thomsen

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et al., 2016). Marine sediments are another important eDNA source; deep-sea sediment metabarcoding between 100–2250 m revealed 1,629 molecular operational taxonomic units (MOTUs), with metazoans as one of the most dominant groups (Guardiola et al., 2015). However, a global analysis of sediment from bathyal and abyssal zones highlighted major gaps in reference databases, with many MOTUs remaining unassigned (Sinniger et al., 2016). Beyond biodiversity assessments, eDNA also holds promise for population genetics by enabling the detection of intraspecific genetic variation and population structure, even in low-abundance or elusive species (Andres et al., 2023). Yet, while eDNA enhances traditional biodiversity assessments, especially in remote and understudied environments like the deep sea, it must be integrated with conventional methods to overcome limitations related to taxonomic resolution, reference data availability and absolute abundance estimates (Merten et al., 2021).

6.3 Short technical background on the method

With the development of next-generation sequencing technologies, which are capable of quickly sequencing hundreds of samples at relatively low costs, eDNA metabarcoding has become a valuable approach to investigate the marine environment (Deiner et al., 2017; Pawlowski et al., 2020; Ruppert et al., 2019). DNA metabarcoding enables the identification of species groups within a given sample by analyzing barcode sequences amplified from eDNA (Figure 6.1). To achieve this, DNA templates are first amplified using Polymerase Chain Reaction (PCR) and the resulting DNA concentrations are quantified, for example using a Qubit fluorometer or gel electrophoresis. Following quantification, individual samples are pooled in equimolar concentrations to ensure balanced representation, allowing multiple samples to be sequenced simultaneously in a single sequencing run.

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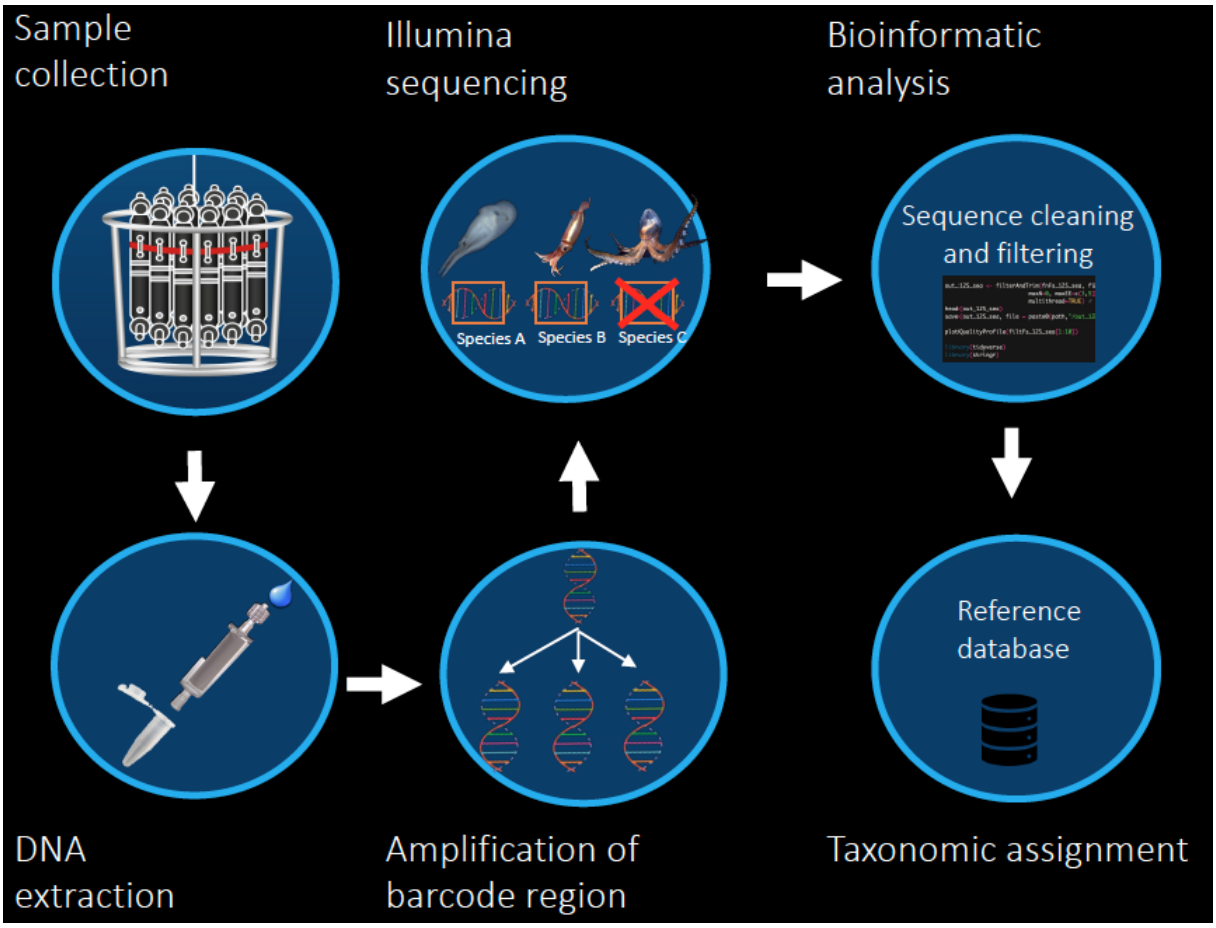

Figure 6.1: Schematic representation of the eDNA metabarcoding workflow, from sample collection to sequence analysis.

The choice of the barcode sequence for eDNA studies is of fundamental importance as it greatly impacts the results. DNA barcodes (or primers) are short DNA molecules that need to be conserved in the target group, but that amplify a highly variable region that should be unique to a single species and not shared with others. Some barcodes are able to distinguish specimens to the family level, others to genus or even species level. In eDNA studies, the DNA fragment that the barcode amplifies (amplicon) is usually rather short (< 300 bp), since eDNA is mostly degraded (Taberlet et al., 2018). However, eDNA stemming from recently shed skin cells or saliva might contain complete cells or long DNA fragments and could be used for eDNA analysis as well (Deiner et al., 2017).

A combination of short and long barcodes promises a higher coverage of DNA present in the sample than just using one meta-barcode, since an environmental sample could contain single cells and free-floating DNA (Bista et al., 2017). DNA barcodes used for eDNA studies need to be represented in a reference database in which every DNA sequence was retrieved from barcoding of a voucher specimen that has been identified morphologically by a taxonomist. Since there is still a massive gap in sequences for a variety of organisms, it is vital to establish a study-specific local database for the organism group of interest, covering the chosen marker genes (Miya, 2021).

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Primer selection is a critical step in eDNA studies, as it determines which taxonomic groups will be effectively amplified and detected. Primers need to target conserved regions flanking variable sequences to ensure broad coverage across the desired taxa while allowing discrimination at the family, genus, or species level. The choice of primers depends on the study objectives—for example, whether the focus is on a broad biodiversity assessment or on specific taxonomic groups like fish or marine mammals. Additionally, primers should be evaluated for specificity, amplification efficiency, and minimal bias to avoid preferential amplification of certain taxa over others. In silico testing against reference databases, followed by empirical validation using mock communities or known samples, is often recommended to ensure the chosen primers perform reliably in environmental samples, which typically contain degraded and low-concentration DNA (Zhang et al., 2020).

Despite its potential to revolutionize biodiversity assessments and other fields in biology, the precision and accuracy of eDNA metabarcoding has limitations like any other method (Thomsen and Willerslev, 2015). In the field and laboratory, several controls are needed to detect contaminations, false-positives or false-negatives (Ficetola et al., 2015). Equipment needs to be cleaned rigorously between all steps. When analyzing sequencing data, one of the most important challenges is to recognize artefactual sequences that can be mistaken for rare species (Kunin et al., 2010). Sequence errors can occur at various steps by replication errors during PCR and during sequencing. A variety of programs have been developed to identify and remove both PCR and sequencing errors (Coissac et al., 2012). The appropriate parameters of the bioinformatic steps can highly influence the results (Baker et al., 2018).

6.4 Recommended sources for further reading on eDNA analysis

Deiner, K., Bik, H. M., Mächler, E., Seymour, M., Lacoursière-Roussel, A., Altermatt, F., Creer, S., Bista, I., Lodge, D. M., de Vere, N., Pfrender, M. E., & Bernatchez, L. (2017). Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. Molecular Ecology, 26(21), 5872–5895. https://doi.org/https://doi.org/10.1111/mec.14350

Ruppert, K. M., Kline, R. J., & Rahman, M. S. (2019). Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global eDNA. Global Ecology and Conservation, 17, e00547. https://doi.org/https://doi.org/10.1016/j.gecco.2019.e00547

Barnes, M.A., Turner, C.R. The ecology of environmental DNA and implications for conservation genetics. Conservation Genetics 17, 1–17 (2016). https://doi.org/10.1007/s10592-015-0775-4

Visser, F., Merten, V. J., Bayer, T., Oudejans, M. G., W de Jonge, D. S., Puebla, O., H Reusch, T. B., Fuss, J., & T Hoving, H. J. (2021). Deep-sea predator niche segregation revealed by combined cetacean biologging and eDNA analysis of cephalopod prey. In Sci. Adv (Vol. 7). https://doi.org/10.1126/sciadv.abf5908

Merten, V., Bayer, T., Reusch, T. B. H., Puebla, O., Fuss, J., Stefanschitz, J., Lischka, A., Hauss, H., Neitzel, P., Piatkowski, U., Czudaj, S., Christiansen, B., Denda, A., & Hoving, H.-J. T. (2021). An Integrative Assessment Combining Deep-Sea Net Sampling, in situ Observations and Environmental DNA Analysis Identifies Cabo Verde as a Cephalopod

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This project has received funding from the European Union's Horizon Europe program under Grant Agreement 101158714

Biodiversity Hotspot in the Atlantic Ocean. Frontiers in Marine Science, 8. https://doi.org/10.3389/fmars.2021.760108

6.5 Environmental DNA analysis protocol

Once environmental DNA has been collected, sample analysis generally comprises two main components: laboratory processing and subsequent bioinformatic analysis of the sequencing data.

Appendix 1 provides an example of an eDNA laboratory workflow optimized for DNA extraction from Sterivex filters (Merck Millipore) and the use of a primer pair targeting the 18S rRNA gene of cephalopods (de Jonge et al., 2021). When using different filter types and/or different primers, respective steps of the protocol should be adjusted accordingly.

6.6 Useful programs and packages for bioinformatic analysis

Programs:

- Python
- Cutadapt (Martin, 2011)
- RStudio

Useful packages in R:

- DADA2 (Callahan et al., 2016)
- Vegan (Oksanen et al., 2019)
- ggplot2
- Rcpp
- stringr
- tidyverse
- tidyr
- dplyr
- phylotools
- DECIPHER
- RSQLite
- openxlsx
- readxl
- pairwise.adonis (Martinez, 2020)

6.7 References

Andres, K.J., Lodge, D.M., Andrés, J., 2023. Environmental DNA reveals the genetic diversity and population structure of an invasive species in the Laurentian Great Lakes. Proc Natl Acad Sci U S A 120. https://doi.org/10.1073/pnas.2307345120

Funded by the European Union

This project has received funding from the European Union's Horizon Europe program under Grant Agreement 101158714

Andres, K.J., Lodge, D.M., Sethi, S.A., Andrés, J., 2023. Detecting and analysing intraspecific genetic variation with eDNA: From population genetics to species abundance. Molecular Ecology 32, 4118–4132. https://doi.org/10.1111/mec.17031

Andruszkiewicz Allan, E., Zhang, W.G., C. Lavery, A., F. Govindarajan, A., 2021. Environmental DNA shedding and decay rates from diverse animal forms and thermal regimes. Environmental DNA 3, 492–514. https://doi.org/https://doi.org/10.1002/edn3.141

Baker, C.S., Steel, D., Nieukirk, S., Klinck, H., 2018. Environmental DNA (eDNA) From the Wake of the Whales: Droplet Digital PCR for Detection and Species Identification. Frontiers in Marine Science 5, 133. https://doi.org/10.3389/fmars.2018.00133

Beng, K.C., Corlett, R.T., 2020. Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects. Biodiversity and Conservation 29, 2089–2121. https://doi.org/10.1007/s10531-020-01980-0

Bista, I., Carvalho, G.R., Walsh, K., Seymour, M., Hajibabaei, M., Lallias, D., Christmas, M., Creer, S., 2017. Annual time-series analysis of aqueous eDNA reveals ecologically relevant dynamics of lake ecosystem biodiversity. Nat Commun 8. https://doi.org/10.1038/ncomms14087

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nature Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869

Coissac, E., Riaz, T., Puillandre, N., 2012. Bioinformatic challenges for DNA metabarcoding of plants and animals. Molecular Ecology 21, 1834–1847. https://doi.org/10.1111/j.1365-294X.2012.05550.x

de Jonge, D., Merten, V., Bayer, T., Puebla, O., Reusch, T.B.H., Hoving, H.-J.T., 2021. A novel metabarcoding primer pair for environmental DNA analysis of Cephalopoda (Mollusca) targeting the nuclear 18S rRNA region. Royal Society Open Science. https://doi.org/10.1098/rsos.201388

Deagle, B.E., Bax, N., Hewitt, C.L., Patil, J.G., 2003. Development and evaluation of a PCR-based test for detection of *Asterias* (Echinodermata: Asteroidea) larvae in Australian plankton samples from ballast water. Mar. Freshwater Res. 54, 709–719.

Deiner, K., Bik, H.M., Mächler, E., Seymour, M., Lacoursière-Roussel, A., Altermatt, F., Creer, S., Bista, I., Lodge, D.M., de Vere, N., Pfrender, M.E., Bernatchez, L., 2017. Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. Mol Ecol 26, 5872–5895. https://doi.org/https://doi.org/10.1111/mec.14350

Deiner, K., Renshaw, M.A., Li, Y., Olds, B.P., Lodge, D.M., Pfrender, M.E., 2017. Long-range PCR allows sequencing of mitochondrial genomes from environmental DNA. Methods in Ecology and Evolution 8, 1888–1898. https://doi.org/10.1111/2041-210X.12836

Ficetola, G.F., Pansu, J., Bonin, A., Coissac, E., Giguet-Covex, C., De Barba, M., Gielly, L., Lopes, C.M., Boyer, F., Pompanon, F., Rayé, G., Taberlet, P., 2015. Replication levels, false presences and the estimation of the presence/absence from eDNA metabarcoding data. Molecular Ecology Resources 15, 543–556. https://doi.org/10.1111/1755-0998.12338

Govindarajan, A.F., Francolini, R.D., Jech, J.M., Lavery, A.C., Llopiz, J.K., Wiebe, P.H., Zhang, W. (Gordon), 2021. Exploring the Use of Environmental DNA (eDNA) to Detect Animal Taxa in the Mesopelagic Zone. Frontiers in Ecology and Evolution 9, 146. https://doi.org/10.3389/fevo.2021.574877

Funded by the European Union

This project has received funding from the European Union's Horizon Europe program under Grant Agreement 101158714

Guardiola, M., Uriz, M.J., Taberlet, P., Coissac, E., Wangensteen, O.S., Turon, X., 2015. Deep-Sea, Deep-Sequencing: Metabarcoding Extracellular DNA from Sediments of Marine Canyons. PLOS ONE 10, e0139633. https://doi.org/10.1371/journal.pone.0139633

Jerde, C.L., Mahon, A.R., Chadderton, W.L., Lodge, D.M., 2011. "Sight-unseen" detection of rare aquatic species using environmental DNA. Conservation Letters 4, 150–157. https://doi.org/10.1111/j.1755-263X.2010.00158.x

Kunin, V., Engelbrektson, A., Ochman, H., Hugenholtz, P., 2010. Wrinkles in the rare biosphere: pyrosequencing errors can lead to artificial inflation of diversity estimates. Environmental Microbiology 12, 118–123. https://doi.org/10.1111/j.1462-2920.2009.02051.x

Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal; Vol 17, No 1: Next Generation Sequencing Data Analysis. https://doi.org/10.14806/ej.17.1.200

Martinez, A., 2020. pairwiseAdonis: Pairwise multilevel comparison using adonis. R package version 0.4.

McClenaghan, B., Fahner, N., Cote, D., Chawarski, J., McCarthy, A., Rajabi, H., Singer, G., Hajibabaei, M., 2020. Harnessing the power of eDNA metabarcoding for the detection of deep-sea fishes. PLOS ONE 15, e0236540. https://doi.org/10.1371/journal.pone.0236540

Merten, V., Bayer, T., Reusch, T.B.H., Puebla, O., Fuss, J., Stefanschitz, J., Lischka, A., Hauss, H., Neitzel, P., Piatkowski, U., Czudaj, S., Christiansen, B., Denda, A., Hoving, H.-J.T., 2021. An Integrative Assessment Combining Deep-Sea Net Sampling, in situ Observations and Environmental DNA Analysis Identifies Cabo Verde as a Cephalopod Biodiversity Hotspot in the Atlantic Ocean. Front Mar Sci 8. https://doi.org/10.3389/fmars.2021.760108

Merten, V., Puebla, O., Bayer, T., Reusch, T.B.H., Fuss, J., Stefanschitz, J., Metfies, K., Stauffer, J.B., Hoving, H.J.T., 2023. Arctic nekton uncovered by eDNA metabarcoding: diversity, potential range expansions and benthopelagic coupling. Environmental DNA 5, 503–518. https://doi.org/10.1002/edn3.403

Miya, M., 2021. Environmental DNA Metabarcoding: A Novel Method for Biodiversity Monitoring of Marine Fish Communities. Ann Rev Mar Sci 14, 161–185. https://doi.org/https://doi.org/10.1146/annurev-marine-041421-082251

Oksanen, J.F., Blanchet, G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2019. vegan: Community Ecology Package. R package version 2.5-6 https://CRAN.R-project.org/package=vegan.

Parsons, K.M., Everett, M., Dahlheim, M., Park, L., 2018. Water, water everywhere: environmental DNA can unlock population structure in elusive marine species. Royal Society Open Science 5, 180537. https://doi.org/10.1098/rsos.180537

Parsons, K.M., May, S.A., Gold, Z., Dahlheim, M., Gabriele, C., Straley, J.M., Moran, J.R., Goetz, K., Zerbini, A.N., Park, L., Morin, P.A., 2025. Using eDNA to Supplement Population Genetic Analyses for Cryptic Marine Species: Identifying Population Boundaries for Alaska Harbour Porpoises. Molecular Ecology 34, e17563. https://doi.org/10.1111/mec.17563

Pawlowski, J., Apothéloz-Perret-Gentil, L., Altermatt, F., 2020. Environmental DNA: What's behind the term? Clarifying the terminology and recommendations for its future use in biomonitoring. Mol Ecol 29, 4258–4264. https://doi.org/https://doi.org/10.1111/mec.15643

Rourke, M.L., Fowler, A.M., Hughes, J.M., Broadhurst, M.K., DiBattista, J.D., Fielder, S., Wilkes

Funded by the European Union

This project has received funding from the European Union's Horizon Europe program under Grant Agreement 101158714

Walburn, J., Furlan, E.M., 2022. Environmental DNA (eDNA) as a tool for assessing fish biomass: A review of approaches and future considerations for resource surveys. Environmental DNA 4, 9–33. https://doi.org/10.1002/edn3.185

Ruppert, K.M., Kline, R.J., Rahman, M.S., 2019. Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global eDNA. Glob Ecol Conserv 17, e00547. https://doi.org/https://doi.org/10.1016/j.gecco.2019.e00547

Sigsgaard, E.E., Nielsen, I.B., Bach, S.S., Lorenzen, E.D., Robinson, D.P., Knudsen, S.W., Pedersen, M.W., Jaidah, M.A., Orlando, L., Willerslev, E., Møller, P.R., Thomsen, P.F., 2017. Population characteristics of a large whale shark aggregation inferred from seawater environmental DNA. Nature Ecology & Evolution 1, 0004. https://doi.org/10.1038/s41559-016-0004

Sinniger, F., Pawlowski, J., Harii, S., Gooday, A.J., Yamamoto, H., Chevaldonné, P., Cedhagen, T., Carvalho, G., Creer, S., 2016. Worldwide Analysis of Sedimentary DNA Reveals Major Gaps in Taxonomic Knowledge of Deep-Sea Benthos. Frontiers in Marine Science 3, 92. https://doi.org/10.3389/fmars.2016.00092

Stat, M., Huggett, M.J., Bernasconi, R., DiBattista, J.D., Berry, T.E., Newman, S.J., Harvey, E.S., Bunce, M., 2017. Ecosystem biomonitoring with eDNA: metabarcoding across the tree of life in a tropical marine environment. Scientific Reports 7, 12240. https://doi.org/10.1038/s41598-017-12501-5

Taberlet, P., Bonin, A., Zinger, L., Coissac, E., 2018. Environmental DNA: For biodiversity research and monitoring. Oxford University Press, Oxford, UK.

Thomsen, P.F., Møller, P.R., Sigsgaard, E.E., Knudsen, S.W., Jørgensen, O.A., Willerslev, E., 2016. Environmental DNA from seawater samples correlate with trawl catches of subarctic, deepwater fishes. PLoS ONE 11, e0165252. https://doi.org/10.1371/journal.pone.0165252

Thomsen, P.F., Willerslev, E., 2015. Environmental DNA – An emerging tool in conservation for monitoring past and present biodiversity. Biological Conservation 183, 4–18. https://doi.org/10.1016/j.biocon.2014.11.019

Tsuji, S., Maruyama, A., Miya, M., Ushio, M., Sato, H., Minamoto, T., Yamanaka, H., 2020. Environmental DNA analysis shows high potential as a tool for estimating intraspecific genetic diversity in a wild fish population. Molecular Ecology Resources 20, 1248–1258. https://doi.org/10.1111/1755-0998.13165

Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P.F., Bellemain, E., Besnard, A., Coissac, E., Boyer, F., Gaboriaud, C., Jean, P., Poulet, N., Roset, N., Copp, G.H., Geniez, P., Pont, D., Argillier, C., Baudoin, J.-M., Peroux, T., Crivelli, A.J., Olivier, A., Acqueberge, M., Le Brun, M., Møller, P.R., Willerslev, E., Dejean, T., 2016. Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. Molecular Ecology 25, 929–942. https://doi.org/10.1111/mec.13428

Visser, F., Merten, V.J., Bayer, T., Oudejans, M.G., de Jonge, D.S.W., Puebla, O., Reusch, T.B.H., Fuss, J., Hoving, H.J.T., 2021. Deep-sea predator niche segregation revealed by combined cetacean biologging and eDNA analysis of cephalopod prey. Sci Adv 7, eabf5908. https://doi.org/10.1126/sciadv.abf5908

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Zhang, S., Zhao, J., Yao, M., 2020. A comprehensive and comparative evaluation of primers for metabarcoding eDNA from fish. Methods Ecol Evol 11, 1609–1625. https://doi.org/10.1111/2041-210X.13485

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Appendix 1 – Protocol for eDNA metabarcoding of cephalopods

Protocol for environmental DNA extraction and library preparation with application of the *Ceph18S* Primer (DeJonge et al, 2021). Prepared by Julia Stefanschitz, *Deep-Sea Biology Working Group*, GEOMAR, Kiel, January 2024. Revised by Ina Vornsand, *Deep-Sea Biology Working Group*, GEOMAR, Kiel, December 2025.

1 eDNA extraction

1.0 Important points before starting

- Extractions of eDNA from Sterivex-Filters are performed with the Blood and Tissue Kit (QIAGEN)
- Work under clean bench
- During extraction and after eluation only open tube under clean bench and one tube at a time to avoid cross contamination
- Prepare one blank extraction as an extraction control (=Lab Blank) per round
- use filter tips, nitrile gloves and mask

1.1 Lysis and digestion

Day 1

1. Move Sterivex filters from -80°C to 4°C in the morning for gentle thawing
2. Start extraction in the afternoon around 15:00-16:00
3. Clean **Extraction clean bench** with 70% EtOH & RNase/DNase Away and let it sit for 5-10 min, wipe consumables/reagents with RNase Away before transferring them under the clean bench.
4. Wipe outside of each filter with RNase/DNase Away.
5. Make sure that filters are empty (no water inside). If not, empty with Luer-Lock syringe (5 ml). Use adapter (CT67.1) and remove water from the outlet side of the filter.
6. Add 720 µl (pre-warmed) ATL-buffer and 80 µl Proteinase K into the inlet side of the filter. It works best if you tilt the tip slightly so air can escape while adding reagent.
7. Incubate filters for **minimum 4h or overnight at 56 °C** with agitation (in hybridisation-oven or shaking incubator).

1.2 Extraction

Day 2

1. Back at **extraction clean bench** (the next morning): Add 600 µl AL-Buffer and 600 µl ≥96 % Ethanol to sterile 2 ml Eppendorf tubes.
2. Transfer buffer mix from Sterivex filters to new sterile 2 ml Eppendorf tubes using 5 ml disposable Luer-Lock syringes.

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3. Pipette 600 µl of each sample to AL-Ethanol mix to obtain equal volumes for all samples (total of 1800 µl per tube) and vortex thoroughly.
4. Pipette 600 µl of the mixture (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube.
5. Centrifuge at ≥6000 x g (8000 rpm) for 1 min. Discard flow-through and collection tube.
6. Place the DNeasy Mini spin column in a new 2 ml collection tube
7. Repeat steps 4.-6. until all of the mixture has passed the column (usually takes 2-3 times).
8. Place the DNeasy Mini spin column in a new 2 ml collection tube
9. Add 500 µl Buffer AW1, centrifuge for 1 min at ≥6000 x g (8000 rpm). Discard flow-through and collection tube.
10. Place the DNeasy Mini spin column in a new 2 ml collection tube.
11. Add 500 µl Buffer AW2, centrifuge for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.
12. Use a new collection tube and centrifuge again for 1 min at 14,000 rpm to remove all residual ethanol.
13. Place the DNeasy Mini spin column in a clean 1.5 ml microcentrifuge tube, and pipet 65 µl of pre-heated (60°C) Buffer AE directly onto the DNeasy membrane.
14. Incubate at room temperature for 10 min, and then centrifuge for 1 min at ≥ 9,000 x g (10,000 rpm) to elute.

1.3 Quantification (optional)

- Measure DNA concentration (ng/µl) with Nanodrop or Qubit

1.4 Storage

- Store DNA extracts at -20°C until further use, or keep in fridge (4°C) if processed within a week.

2 Library Preparation

2.0 Important points before starting

- When library prep is started, register run at Sequencing center.
- Prepare primer aliquots for matrix pipetting. 1-Step PCR primers already include sequencing adapter, index and PCR adapter.
- Samples will be run in triplicates (pooling before quantification).
- Per PCR plate, 2 positive controls (e.g. *Filippovia knipovitchi*, *Psychroteuthis glacialis* [20-80 ng/µl]), 1 mock control (1:1 mix of both pos ctrl) and 1 negative control (PCR grade water) are added in duplicates ⇒ equals 8 controls in total (pooling before quantification).
- Extraction blanks (=Lab blanks) are treated as samples.
- For PCR plates, all samples are completely randomized.
- Avoid repeated freeze/thaw cycles as it can damage eDNA samples and primers.

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2.1 PCR: 1-step-PCR for Ceph18S

Target	Primer	Direction	Sequence	Amplicon size [bp]	Reference
Cephalopda nuclear 18S rRNA region (V2)	Ceph18S_F	forward	CGCGGCGCTACATATTAGAC	140-190	De Jonge et al. 2021
	Ceph18S_R	reverse	GCACTTAACCGACCGTCGAC		De Jonge et al. 2021

Ceph18S Primer Information

MasterMix Ceph*18S* rRNA gene

(A)	Taqman environmental Mastermix 2.0 (Thermo Scientific™)	10 µl
	PCR grade water	8 µl
(B)	Primer F (Ceph18S) 5 µM	1 µl
	Primer R (Ceph18S) 5 µM	1 µl
(C)	(e)DNA template	5 µl
	Total Volume	**25 µl**

PCR program *Ceph18S* rRNA gene: Touchdown PCR

Denaturation	10 min	95°C	
Denaturation	30 sec	94°C	8 x (-1°C after each cycle)
Annealing	30 sec	**70°C**	
Elongation	1 min	72°C	
Denaturation	30 sec	94°C	32 x
Annealing	30 sec	**62°C**	
Elongation	1 min	72°C	
Final elongation	5 min	72°C	
Cooling	∞	4°C	

1. Gently thaw reagents and samples at 4°C; work on ice.
2. Clean bench with 70 % EtOH and RNase Away.
3. Prepare master mix under **DNA-free clean bench** in RNase/DNase free tube (one “plate” ⇒ mastermix x 96 (+ 5%) = 1008 µl TaqMM + 807 µl water).

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(A) Pipet 18 µl of Mastermix per well. Use 0.2 mL sterile 8-well-stripes with caps (use two racks for 96 wells with one empty column in between stripes).

4. From this step on, never have more than one tube open at a time.
   (B) Add 1 µL forward and 1 µL reverse primer (unique combinations) in each well.
5. Change to **DNA clean bench**.
   (C) 5.1. Add 5 µl PCR grade water to negative ctrl wells.
   5.2. Add 5 µl eDNA template to sample wells.
   5.3. Add 5 µl DNA to positive ctrl and mock wells.
6. Briefly vortex and spin down plate in centrifuge, then start PCR program.

2.2 Gel electrophoresis check

1. Run 2% agarose gel with 1x TAE buffer; add LoadingDye and GelRed to sample. 2% agarose is appropriate for short amplicons as typical in eDNA metabarcoding, but needs to be adjusted for longer amplicons.
2. Check all controls and a few random samples on the gel directly after each PCR.
3. Storage: store plates at -20°C if not further processed within 3 days (then 4°C).

2.3 Pooling

1. Once all plates for a primer are finished and quality checked, pool technical replicates in a new plate. Store plates at 4°C and only take out the one in use.
2. Now 1 sample/control = 1 well.

2.4 Quantification

1. Quantify libraries with Qubit high sensitivity kit (max 24 samples at a time), 2 µl per sample.
2. Normalize libraries to 20-80 ng DNA/sample and pool in RNase/DNase free Eppendorf tube (use max. 7 µl sample and 1 µl controls).
3. Quantify concentration of **library pool** with Qubit high sensitivity kit.
4. Storage: store at -20°C if not further processed within 3 days (then 4°C).

3 Purification

3.1 Gel preparation

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1. Prepare 2 % agarose gel with 1x TAE buffer containing 5 % GelRed and broad gel wells. 2% agarose is appropriate for short amplicons as typical in eDNA metabarcoding, but needs to be adjusted for longer amplicons.
2. Add roughly 10 µl 6X (orange) LD (loading dye) per 200 µl library pool directly to Eppendorf with library pool. The color should be light green.
3. Add 3 µl Gene ruler LD + 2 µl 6x orange LD to first and last gel well.
4. Pipet 10 µl of library pool per well in ~4-6 wells, then repeat until everything is divided between wells (max 40 µl per well).
5. Run gel with 1x TAE running buffer at 80 V, 4 W and 55 mA for about 1.5 h. Settings may be adjusted according to gel concentration, size, buffer, apparatus and dye used.

3.2 Recovery of DNA with Zymoclean Gel DNA Recovery Kit (Zymo Research)

1. Excise bands and transfer to RNase/DNase free Eppendorf tubes (weigh tubes before and after, max weigh of agarose piece = 350 mg).
2. Add 3x the volume ADB to agarose and incubate for 10 min at 50°C or until gel is dissolved.
3. Divide and transfer liquid to several Zymo-Spin column and centrifuge for 1 min at 12,000 rpm.
4. Discard the flow-through and place into new 2 ml collection tube.
5. Add 200 µl DNA wash buffer and centrifuge for 30 sec at 12,000 rpm.
6. Discard the flow-through and repeat washing step.
7. Elute DNA with 6-12 µl pre-heated elution buffer (60°C). Incubate for 10 min before centrifugation for 1 min at 12,000 rpm.
8. Pool all extracts in to one **NGS pool** tube.

3.3 Quantification

- Quantify DNA concentration of **NGS pool** with high sensitivity Qubit (ng/µl).
- Storage: store sample at -20°C or proceed to step 4 immediately.

4 Sequencing

- Fill out sample information with correct indices and sequencing taqs and bring NGS pool to sequencing center on ice.

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Appendix 2 – Program of the 2025 EU TWILIGHTED Scientific Training School at GEOMAR Helmholtz Centre for Ocean Research Kiel, Germany (EU TWILIGHTED Milestone M3.1)

The 2025 EU TWILIGHTED Scientific Training School for early career and experienced researchers and technicians at ARDITI and partner institutions took place from June 10 – 20, 2025, on the premises of GEOMAR Helmholtz Centre for Ocean Research Kiel, Germany. The comprehensive 2-week scientific workshop served as a formal introduction to methods that have proven powerful to improve understanding of deep-sea biodiversity, biological interactions and food web structure and functioning. It was focused on three methods: imaging (workshop Theme 1), stable isotope analysis (Theme 2) and environmental DNA analysis (Theme 3). Training included the theoretical foundations, introduction to the sampling and analysis protocols, hands-on practical work with actual sample sets in the molecular and biochemical laboratories at GEOMAR, and the subsequent work (statistical analysis, synthesis, visualization and interpretation) with resulting data sets.

The workshop program included a combination of lectures, field and laboratory work, data analysis, group work and discussion sessions:

Day	**Activity**
Tuesday, June 10 9:00 – 12:00	Welcome and lecturer introduction round Introduction to GEOMAR; Workshop overview Lab intro and safety instruction
Tuesday, June 10 13:00 – 17:00	Tour of GEOMAR facilities Start of workshop Theme 1: imaging - Setting out of lander - Start lectures
Wednesday, June 11 – Thursday, June 12	Theme 1: imaging (continued) [Thursday 12.6. parallel meeting outreach & media and transfer teams; project managers]
Friday, June 13 Morning	Curation of image data Data management Sample management

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Friday, June 13 13:15	FB3 seminar on MARE-Madeira research by Joao Canning-Clode, opportunity for exchange GEOMAR - MARE-Madeira afterwards
Friday, June 13 Afternoon	Cruise organization primer: Marine Facilities Management (MFP) International legal frameworks: Nagoya Protocol Primer Tour of Technical and Logistics Center of GEOMAR
Saturday, June 14	Excursion Day: Baltic Sea ecosystem
Monday, June 16 – Tuesday, June 17	Theme block 2: stable isotope analysis (SIA)
Wednesday, June 18 – Thursday, June 19	Theme block 3: eDNA-analysis
Friday, June 20	Perspective on deep-sea research Participant presentations on future project ideas Synthesis End of event

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