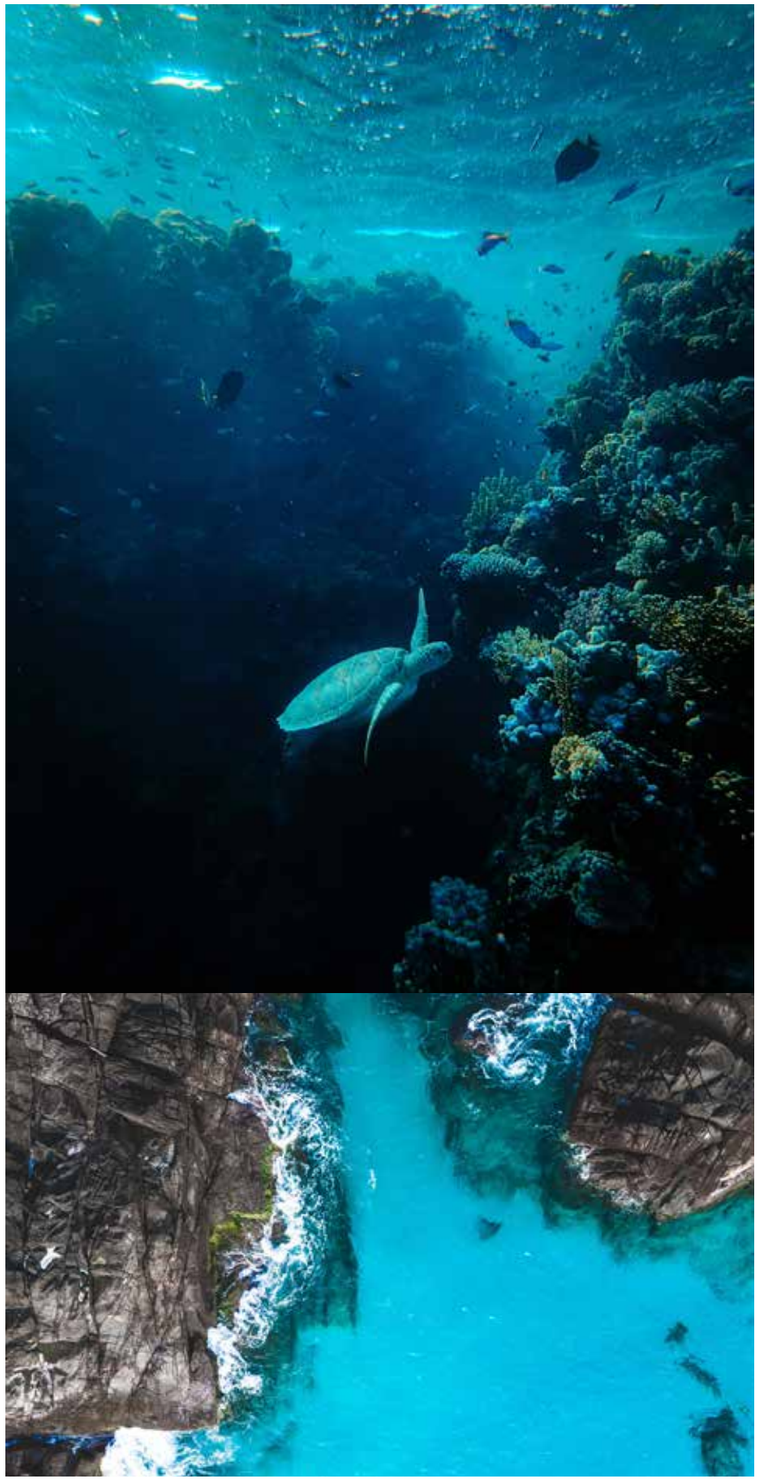
© Greg Lecoeur, WE ARE MEDITERRANEE

vigilife

/User guide to environmental DNA in

marine environments

Discovering, understanding and using eDNA to protect marine biodiversity



Authors: Carolane Giraud (SPYGEN), Pierre Boissery (Agence de l'eau Rhône Méditerranée Corse), Alicia Dalongeville (SPYGEN), Tony Dejean (SPYGEN), Julie Deter (Andromède Océanologie), Aurélie Lacoeuilhe (PatriNat), David Mouillot (Université de Montpellier)

Publication date: 9th september 2024

How to cite this document: Giraud Carolane, Boissery Pierre, Dalongeville Alicia, Dejean Tony, Deter Julie, Lacoeuilhe Aurélie and Mouillot David. 2024. User guide to environmental DNA in marine environments. Discovering, understanding and using eDNA to protect marine biodiversity. 88 pages.

Artistic direction: Here we com

Translation: Melissa Dawes, Dawes Scientific Editing

We thank all the people who kindly agreed to review this document: Andromède Océanologie: Gwenaëlle Delaruelle, Florian Holon; SPYGEN: Coline Gaboriaud, Alice Valentini; and Vigilife: Benjamin Allegrini, Albane Loiseau, Baptiste Mulot, Franck Pressiat. **We also thank the experts who agreed to contribute to this document:** Aurélien Besnard, Roberto Danovaro, Madeleine Cancemi, Cinzia Corinaldesi, Nadia Faure, Yvan Griboval, Anaïs Gudefin, Florian Holon, Jean-Baptiste Juhel, Letizia Lamperti, Gilles Lecaillon, Greg Lecoeur, Stéphanie Manel and Stefano Varrella. **Finally, we are grateful to all those who contributed to the preparation of this guide, in terms of both the written content and the images:** Laurent Ballesta, Arnaud Collin, Tangi Corveler, Régis Hocdé, Greg Lecoeur and Laure Velez.

This user guide, edited by the international alliance Vigilife, is one of the deliverables of the eREF project, which was funded by the Rhone Mediterranean Corsica Water Agency and led by the University of Montpellier in partnership with Andromède Océanologie and SPYGEN.

Partner members

Founding members

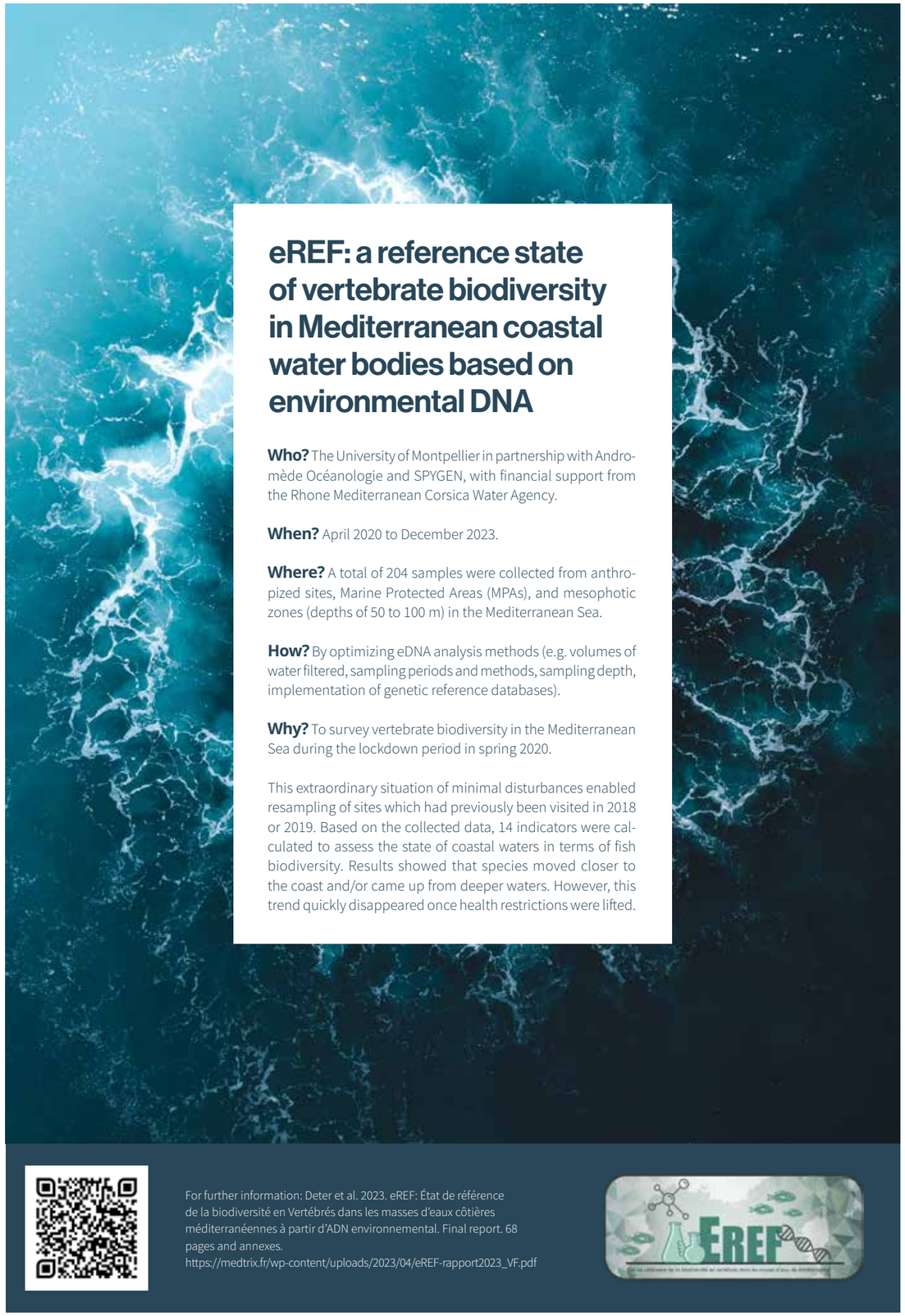
www.vigilife.org

vigilife

Our mission	Using innovation to understand and protect life.	Our vallues	Innovation, stringency, sharing, commitment

Our objectives

Unite	Bring together both public and private partners committed to increasing the number of observation sites and enabling the broader use of collected data.
Evaluate	Use standardized eDNA methods to survey aquatic and terrestrial biodiversity and monitor changes in as many ecosystems as possible around the world, especially the most vulnerable.
Monitor	Prevent the decline of the most endangered species on our planet and improve the early detection and monitoring of invasive exotic species and pathogens, particularly focusing on the primary pathways through which they arrive.
Understand	Analyze and model data collected worldwide to improve the understanding of biodiversity and to better anticipate the impact of global changes on all life forms.
Innovate	Constantly pursue research and development efforts to offer increasingly high-performing and environmentally-friendly technologies, accessible to as many people as possible.
Guide	Provide all decision-makers with reliable and up-to-date information through scientifically approved synthetic indicators to guide them in implementing conservation actions, restoration efforts and practice changes.
Train	Decentralize eDNA expertise by transferring skills and technologies, allowing each partner to process their samples locally and ensuring that each country retains control over the use of its genetic resources.
Raise awareness	Support public and private entities in better integrating biodiversity into their activities and in informing the general public of its importance and fragility.
Highlight	Highlight best practices and inspirational actions that protect life, particularly those carried out by indigenous peoples.
Ensure durability	Provide Vigilife with sustainable financial resources to enable it to pursue its mission well into the future, notably through the business world's commitment to its operation.



eREF: a reference state of vertebrate biodiversity in Mediterranean coastal water bodies based on environmental DNA

Who? The University of Montpellier in partnership with Andromède Océanologie and SPYGEN, with financial support from the Rhone Mediterranean Corsica Water Agency.

When? April 2020 to December 2023.

Where? A total of 204 samples were collected from anthropized sites, Marine Protected Areas (MPAs), and mesophotic zones (depths of 50 to 100 m) in the Mediterranean Sea.

How? By optimizing eDNA analysis methods (e.g. volumes of water filtered, sampling periods and methods, sampling depth, implementation of genetic reference databases).

Why? To survey vertebrate biodiversity in the Mediterranean Sea during the lockdown period in spring 2020.

This extraordinary situation of minimal disturbances enabled resampling of sites which had previously been visited in 2018 or 2019. Based on the collected data, 14 indicators were calculated to assess the state of coastal waters in terms of fish biodiversity. Results showed that species moved closer to the coast and/or came up from deeper waters. However, this trend quickly disappeared once health restrictions were lifted.

For further information: Deter et al. 2023. eREF: État de référence de la biodiversité en Vertébrés dans les masses d'eaux côtières méditerranéennes à partir d'ADN environnemental. Final report. 68 pages and annexes.
https://medtrix.fr/wp-content/uploads/2023/04/eREF-rapport2023_VF.pdf

Preface 00

Seas and oceans cover most of our planet's surface (70 %) and volume (98 %), but they remain vast and impenetrable in terms of visual perception and knowledge. This combination of vastness and mystery has given rise to many stories and legends, while also inspiring thrilling expeditions and discoveries. Despite many challenges (e.g. light, pressure), life thrives in these environments in surprising ways… yet we are only beginning to scratch the surface of its incredible diversity.

As human activities and their consequences have never before threatened marine biodiversity to such an extent, it is now crucial to use all available resources to understand, preserve and restore marine ecosystems. What better way to study biodiversity than through life's fundamental molecule? Each new technology comes with its own promises, and environmental DNA (eDNA) is emerging as a revolutionary step forward, revealing the underwater world in a whole new light. However, the goal is not to replace traditional methods (e.g. cameras, acoustics) but rather to complement them, providing additional insights in order to ask new questions.

In the face of the double challenge of fighting both climate change and food insecurity, oceans are emerging as a key, yet largely unknown actor. In this context, expectations regarding eDNA, a tool that some still view as mysterious or even dubious, are even higher. What can really be detected by eDNA? What are its limitations? How does it work? What equipment is needed? The aim of this user guide is to address all these questions with clear and well-documented answers.

This user guide to eDNA in marine environments is intended as an accessible and practical resource for all those who aspire to better understand, monitor, restore and protect our seas and oceans. It serves as an illustrated and well-documented overview of this innovative technology, of how it functions, and of its practical applications. With this tool, researchers, students, conservation managers, policymakers and even the general public can now explore the ocean's mysteries with unparalleled precision.

Born from a partnership between companies, public institutions and academic research organizations, this user guide also reflects the shared responsibility we have toward our marine environment and the need for cooperation among all stakeholders. By sharing our knowledge and expertise, we hope to ensure that marine biodiversity continues to inspire and amaze future generations.

Neither of us is a molecular biologist, but in eDNA we have seen another way to answer our questions. So, why not you?

> "eDNA is emerging as a revolutionary step forward, revealing the underwater world in a whole new light.

PART 01

PART 02

PART 03

PART 04

Table of contents

2024

vigilife

Summary 00

Understanding eDNA

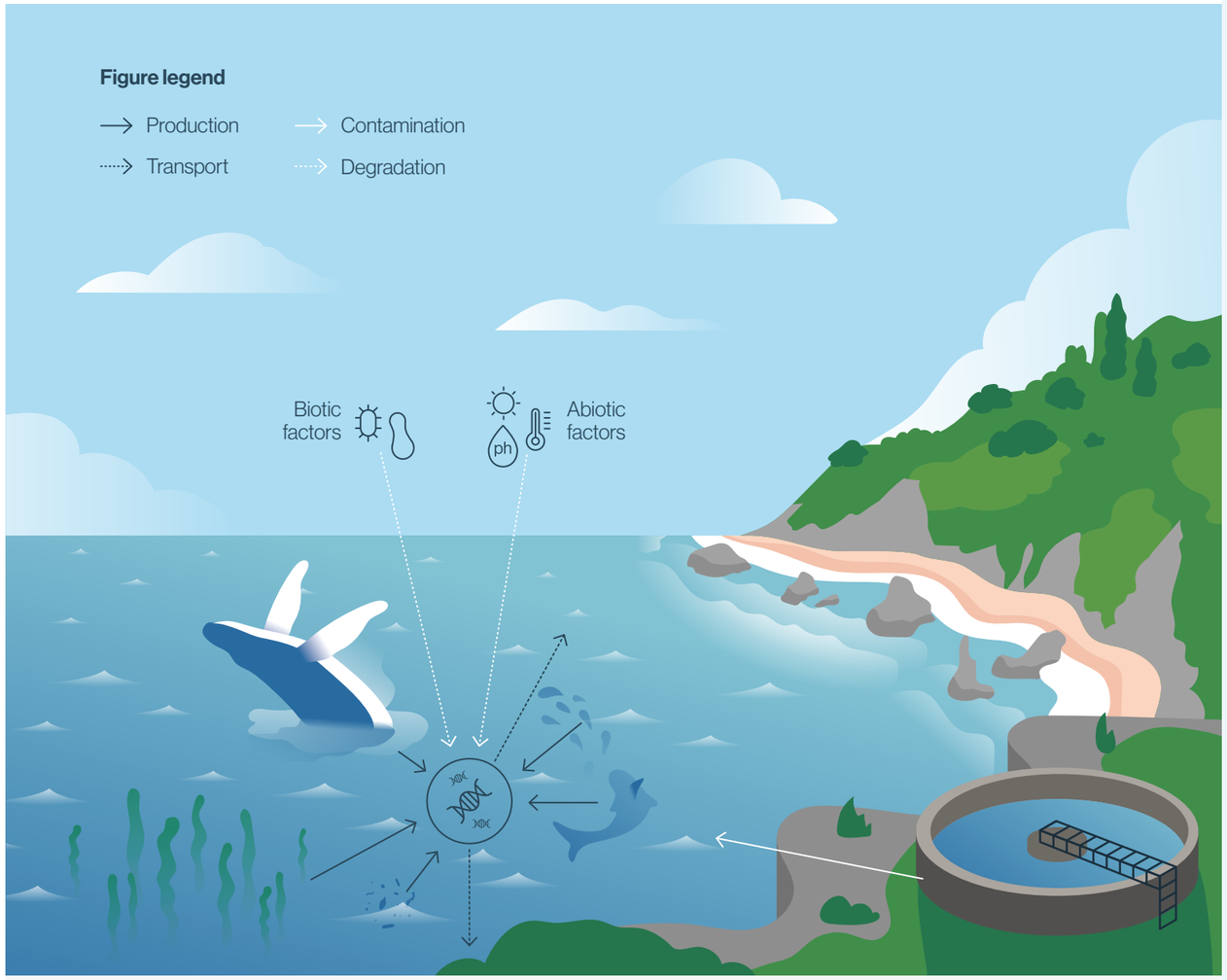
General context

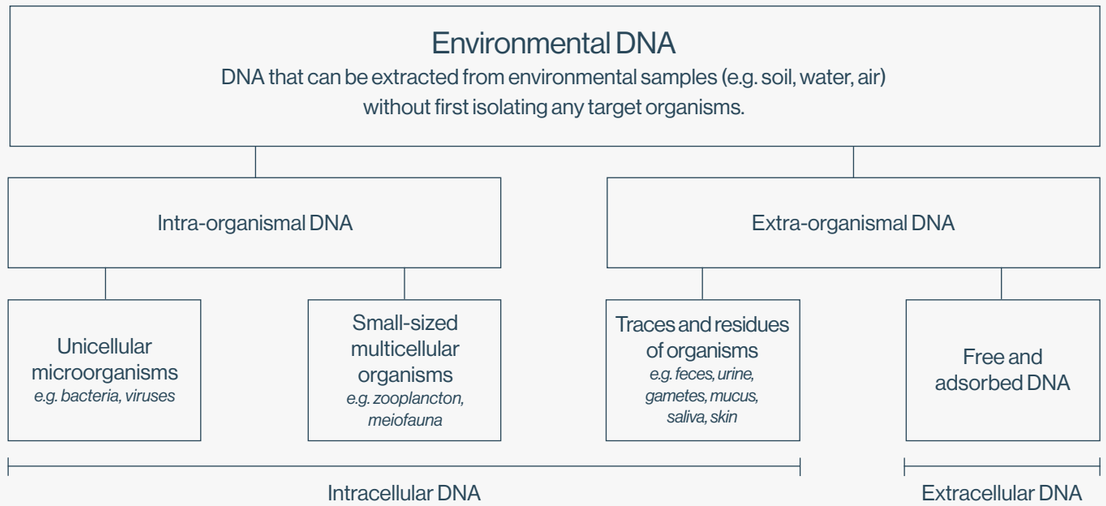
Environmental DNA, or eDNA, is defined as DNA that can be extracted from environmental samples without first isolating the target organisms[a]. Thus, through eDNA analysis, species present in a given environment can be detected by collecting and identifying the DNA traces in their surroundings. Since 2008, this innovative method has proven effective in the field of conservation biology, particularly for biodiversity inventories and monitoring[b]. By enabling, among other things, the study of all life forms and the detection of key species (e.g. protected, threatened, invasive) and those that are cryptic or invisible to the naked eye, eDNA analysis complements other methods of marine biodiversity assessment, such as capture, visual surveys and hydroacoustics.

> “Since 2008, this innovative method has proven effective for biodiversity inventories and monitoring.

eDNA ecology

The detection distance and persistence of eDNA are influenced by its ecology, meaning the composition, origin, transport and degradation of DNA molecules in the environment[c]. In marine environments, eDNA tends to be diluted in large volumes and is therefore generally present only in very small quantities in the water column. Species detectability depends on the likelihood of collecting their DNA from the environment, the preservation of that DNA during the analytical process, and the absence of contamination in the considered samples. Thus, when the user’s goal is to obtain the most comprehensive list of species possible while minimizing the risks of false negatives and false positives, it is essential to choose sampling and analytical methods that adhere to the highest possible levels of precaution. The Vigilife methods, presented step by step in this guide, have been developed with this in mind.





SUMMARY

Acquiring eDNA data

Project design

Designing an eDNA-based project involves:

- Defining the study's objective by identifying the questions asked by the user, as well as the targeted taxa and geographic areas.
- Identifying the study sponsor, sampler, data manager, and technological and ecological experts.
- Anticipating the required financial resources and timelines by consulting with the previously identified experts.
- Developing an optimal sampling strategy by choosing a method suitable for the identified objectives, determining the number of samples to collect and their spatiotemporal distribution, and identifying the applicable regulations that need to be respected.

Sample collection

Collecting samples in aquatic environments involves taking several liters of water. There are many methods available for this type of sampling, but not all are suitable for the objectives set by the user. In the context of Vigilife, samples are collected in replicates by filtering several tens of liters of water directly on-site using a motorized peristaltic pump equipped with a filtration capsule containing a membrane with a porosity of 0.2 µm. Once the filtration is complete, a conservation buffer is poured into the filtration capsule to cover the filter and thus preserve the eDNA retained on its surface.

Laboratory and bioinformatic analyses

As with sampling, there are various methods that can be deployed in the laboratory, but not all are optimized for conducting inventories and monitoring biodiversity. In the context of Vigilife, collected samples are analyzed in a laboratory that adheres to strict precautionary standards. DNA contained in the environmental samples is first extracted and then, depending on the user's needs, analyzed using either a species-specific or multispecies approach (the latter is also known as eDNA metabarcoding). The aim of the species-specific approach is to reveal the presence of a species of interest by detecting a precise sequence of its DNA. If this sequence, also called a genetic marker, is present in the sample, it can be targeted using primers, which are short synthetic DNA sequences, and then amplified using polymerase chain reaction (PCR) or a derived method (quantitative or digital PCR). Conversely, the multispecies approach enables the simultaneous and unbiased identification of multiple distinct species belonging to the same taxonomic group[d]. For this approach, the extracted DNA is first amplified by PCR using universal primers. The amplified DNA fragments are then sequenced, and the sequencing results are analyzed using bioinformatics methods. During this final step, the detected sequences are taxonomically assigned by comparing them to genetic reference databases.

© Greg Lecoeur, WE ARE MEDITERRANEE

SUMMARY

Data publication and interpretation

The eDNA data cycle

The different stages of eDNA sampling and analysis imply that there are various types of data derived from eDNA. These encompass physical data (collected samples and extracted DNA) and digital data (raw sequencing data, interpreted data and validated data). Various types of information can be associated with this set of data. This guide offers suggestions on which information to collect, on how to define data ownership, and on anticipating data storage locations and duration. The different platforms for data sharing in France and internationally are also presented.

Applications and interpretations

Testimonials from experts demonstrate how the interpretation of eDNA-derived data can lead to biodiversity indicator development, the study of benthic marine communities, the detection of rare species, and the assessment of biodiversity in harbor or tropical environments.

Taking action locally

Understanding to better protect

Like all methods of biodiversity assessment, those based on eDNA analysis face a number of limitations. However, they also offer many advantages that have already led to concrete societal actions in various regions. A few examples are provided through testimonials from researchers and environmental managers.

Limitations

- Potential production of false positives and false negatives, particularly when precautionary measures are not followed
- No identification of hybrid species or occurrences of genetic introgression
- No individual-level information or quantification of the absolute abundance of the detected species
- Limited ability to study consumed species, farmed species, genetically similar species, or those for which genetic reference databases are incomplete
- Longer turnaround times for results compared with traditional methods
- Production of single-use waste

Advantages

- Non-invasive method
- Study of all kingdoms of life from the same sample
- Detection of organisms that are difficult to observe using traditional methods (e.g. larval stages, those with a low density, those invisible to the naked eye)
- Early detection of species (e.g. invasive exotic species)
- Ease of implementation
- Reduction of biases related to unfavorable field conditions, observer experience, and variability in survey efforts
- Ability to survey protected, dangerous or polluted areas
- Potential time savings during sampling, resulting in a favorable cost–benefit ratio

BIBLIOGRAPHY

a. Taberlet et al. 2012. Environmental DNA. Molecular Ecology. https://doi.org/10.1111/j.1365-294X.2012.05542.x
b. Ficetola et al. 2008. Species detection using environmental DNA from water samples. Biology Letters. https://doi.org/10.1098/rsbl.2008.0118
c. Barnes & Turner. 2015. The ecology of environmental DNA and implications for conservation genetics. Conservation Genetics. https://doi.org/10.1007/s10592-015-0775-4
d. Bruce et al. 2021. A practical guide to DNA-based methods for biodiversity assessment. Pensoft. https://doi.org/10.3897/ab.e68634

Introduction 00

To address the magnitude and speed of biodiversity loss, a revolution in how we consider and monitor the living world is necessary. To tackle this worldwide challenge, an innovative and non-invasive biodiversity census method based on environmental DNA (eDNA) is now accessible to all biodiversity stakeholders. This easily deployable and efficient approach makes it possible to study all life forms (from bacteria to large mammals) based on a single environmental sample (e.g. water, soil), enhancing the monitoring of key (e.g. protected, threatened, invasive, exotic) and cryptic species, including those invisible to the naked eye. Major environmental issues, such as climate change, pollution, species extinction, and biological invasions, require coordinated, globally scaled, long-term actions. In this regard, Vigilife offers standardized protocols to each of its partners. They are optimized for rare species detection and scientifically validated on a large scale (90 scientific articles), facilitating effective comparisons of the collected data over time and space. Given the inherent difficulties of accessing marine ecosystems, biodiversity surveys and species monitoring could particularly benefit from this eDNA-based technology. Indeed, although they provide essential information on individual parameters (e.g. age, size, sex), traditional visual census methods, involving diving, are often costly and cumbersome to set up, and direct catch methods, such as fishing, are invasive to ecosystems and therefore ethically questionable. In contrast, eDNA analysis methods can detect organisms typically missed by visual surveys and enable exploration of inaccessible environments.

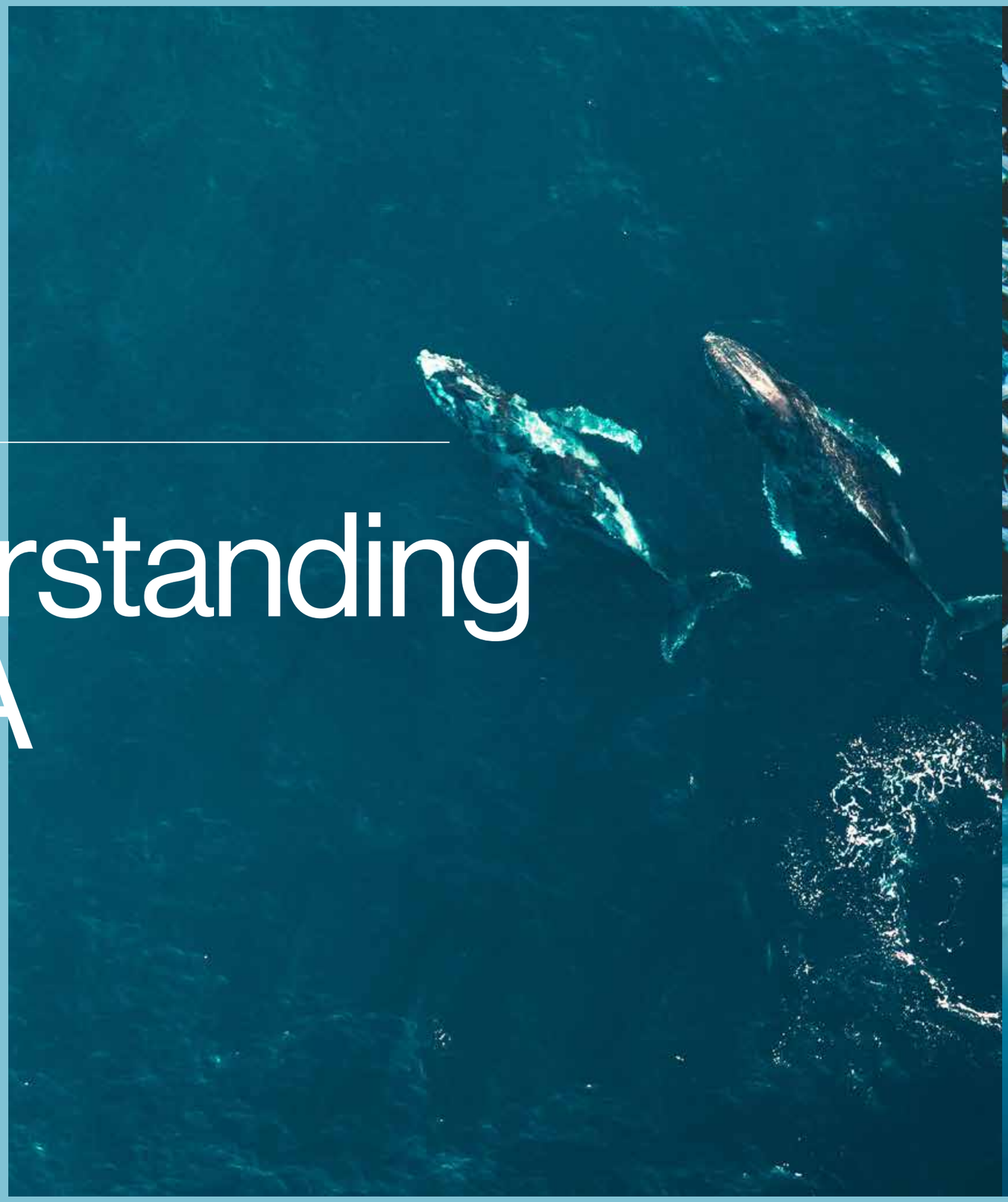
> “When one does not understand, one does not protect.
>
> Madeleine Cancemi

This document is the product of a collaborative effort involving users, researchers and experts. Our aim is to present a straightforward, step-by-step guide for the implementation of Vigilife standards for acquiring, disseminating and interpreting eDNA-derived data. Ultimately, we hope to encourage concrete societal actions in all regions.

PART 01

01

Understanding eDNA



© MarAlliance

CHAPTER 01

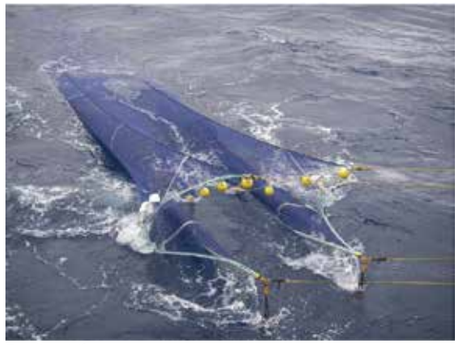
General context 01

Evolution of marine biodiversity census methods

In marine environments, conventional (or traditional) biodiversity census methods can be classified into three categories: capture methods, visual surveys and hydroacoustics.

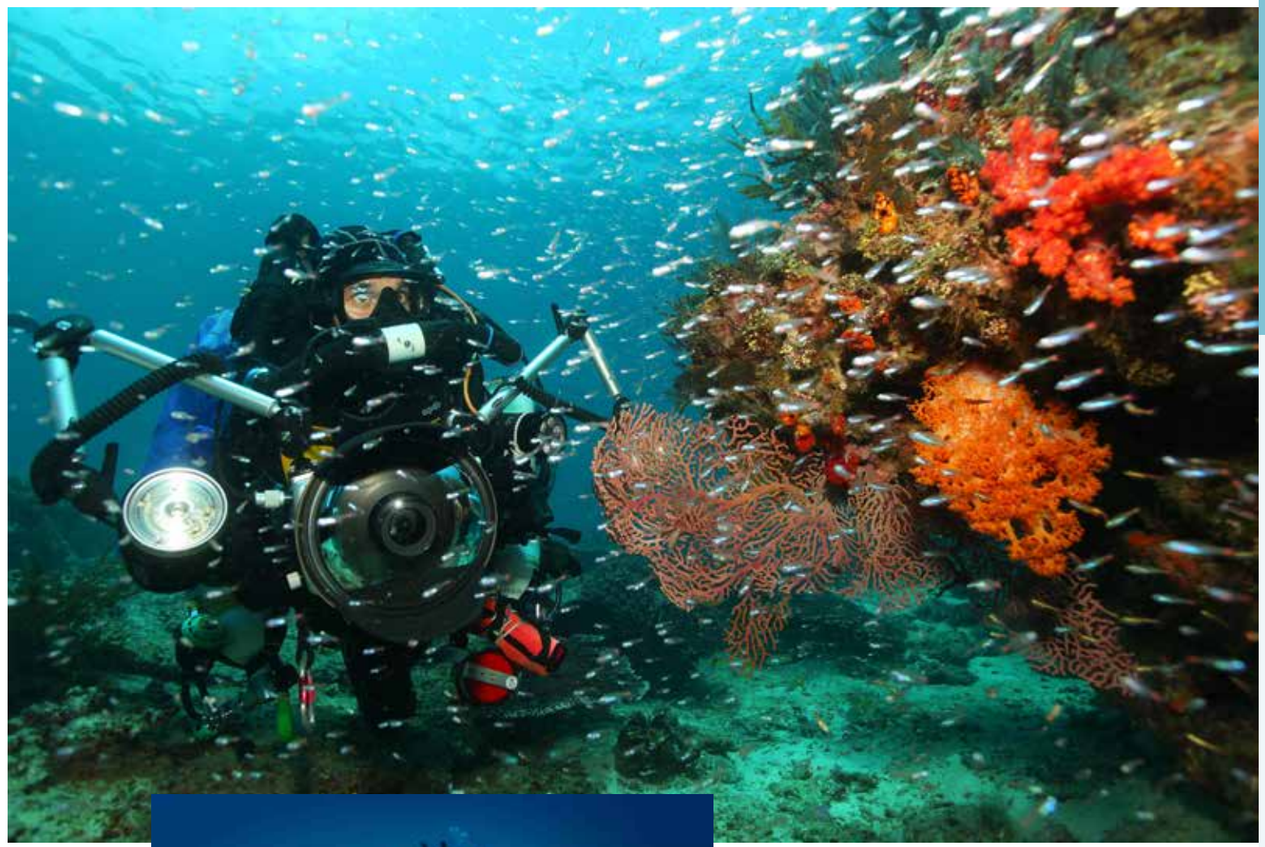
Capture methods were the first techniques to be developed for marine biodiversity surveys. They involve removing individuals from their environment for counting and identification a posteriori. This category includes various fishing techniques, such as trawling, net fishing and line fishing, as well as the use of lethal (e.g. rotenone) or anesthetic (e.g. eugenol) products. While these methods enable the collection of biological samples and provide important information on individual parameters, such as size, age, sex, health status and species abundance, they are known to be damaging to ecosystems. Additionally, they can be complex and costly to implement, depending on the density and the biological and behavioral characteristics of the target species (e.g. fishing methods are difficult to apply in coral reef environments).

> “ Capture methods were the first techniques to be developed for marine biodiversity surveys.



Trawling © Ifremer

Longline fishing



Photographic census © IRD - Gilles Di Raimondo, Lengguru 2014

Underwater visual census (UVC) © AMSA

> “ Visual surveys involve in situ biodiversity evaluations.

Visual surveys involve in situ biodiversity evaluations, typically through direct visual counts while diving (underwater visual census, UVC) or with digital tools such as videos and photos. Like capture methods, they provide essential information on individual parameters (e.g. age, size, abundance) but are influenced by certain biological and behavioral characteristics of the target species (e.g. avoidance behaviors). Their effectiveness depends on the observer’s experience level, the presence of species at the observation site, visibility conditions (water turbidity), and the specificities of the surveyed area (for example, logistical and safety challenges make direct visual observations difficult to implement in deep waters or harbor zones).

CHAPTER 01

Underwater camera observations © B. Preuss, Ifremer AMBIO project

Recently, hydroacoustic methods have emerged to address the limitations of traditional biodiversity census techniques. The aim of these methods is to minimize the environmental impact compared with capture methods and to overcome visual observation constraints, such as limited visibility. Key techniques include echo sounding (which relies on acoustic wave reflection), bioacoustics (which involves passive listening to the sound waves emitted by organisms) and telemetry (which uses acoustic wave transmitters implanted under the skin of target species). While these methods offer valuable information on organism abundances and behaviors, species-level detection can be challenging, particularly in highly diverse marine environments.

Acoustic telemetry © Andromède Océanologie

H.S.H. Albert II of Monaco in contact with scientists – Malpelo mission © Olivier Borde – Monaco Explorations

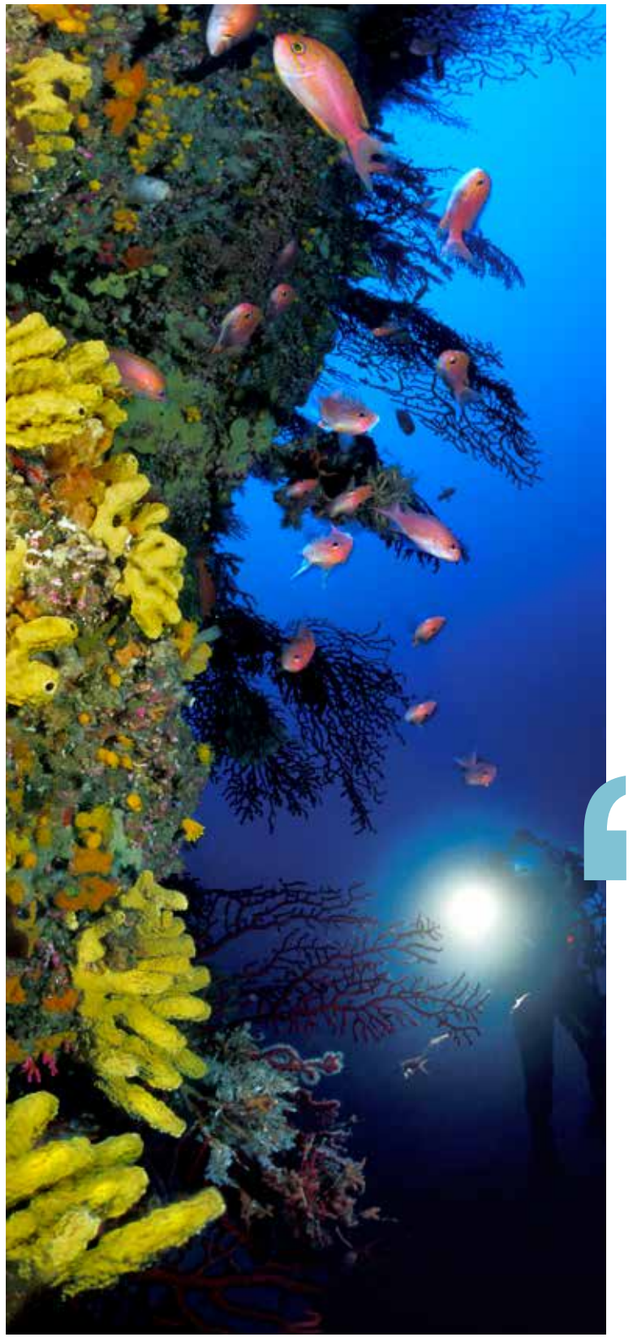
Learn more ↗

The Monaco Explorations

To improve monitoring and awareness of marine fauna, eDNA-based analyses were deployed as part of the 'Monaco Explorations', an international expedition platform initiated in 2017 by His Serene Highness Prince Albert II of Monaco. The technique was implemented in most of the sites visited (e.g. Colombia, Guadeloupe, Martinique, Western Mediterranean, New Caledonia) and led to numerous discoveries. Among them were the identification of over 100 fish species during missions to Caribbean coral reefs (equivalent to nearly 20 years of scuba diving and visual surveys) and the detection of many rare animals, such as the dwarf sperm whale near Malpelo Island.

Polanco et al. 2020. Comparing environmental DNA metabarcoding and underwater visual census to monitor tropical reef fishes. Environmental DNA. https://doi.org/10.1002/edn3.140

Juhel et al. 2020. Detection of the elusive Dwarf sperm whale (Kogia sima) using environmental DNA at Malpelo island (Eastern Pacific, Colombia). Ecology and Evolution. https://doi.org/10.1002/ece3.7057



© Laurent Ballesta, Andromède Océanologie

eDNA: Emergence, definition and applications

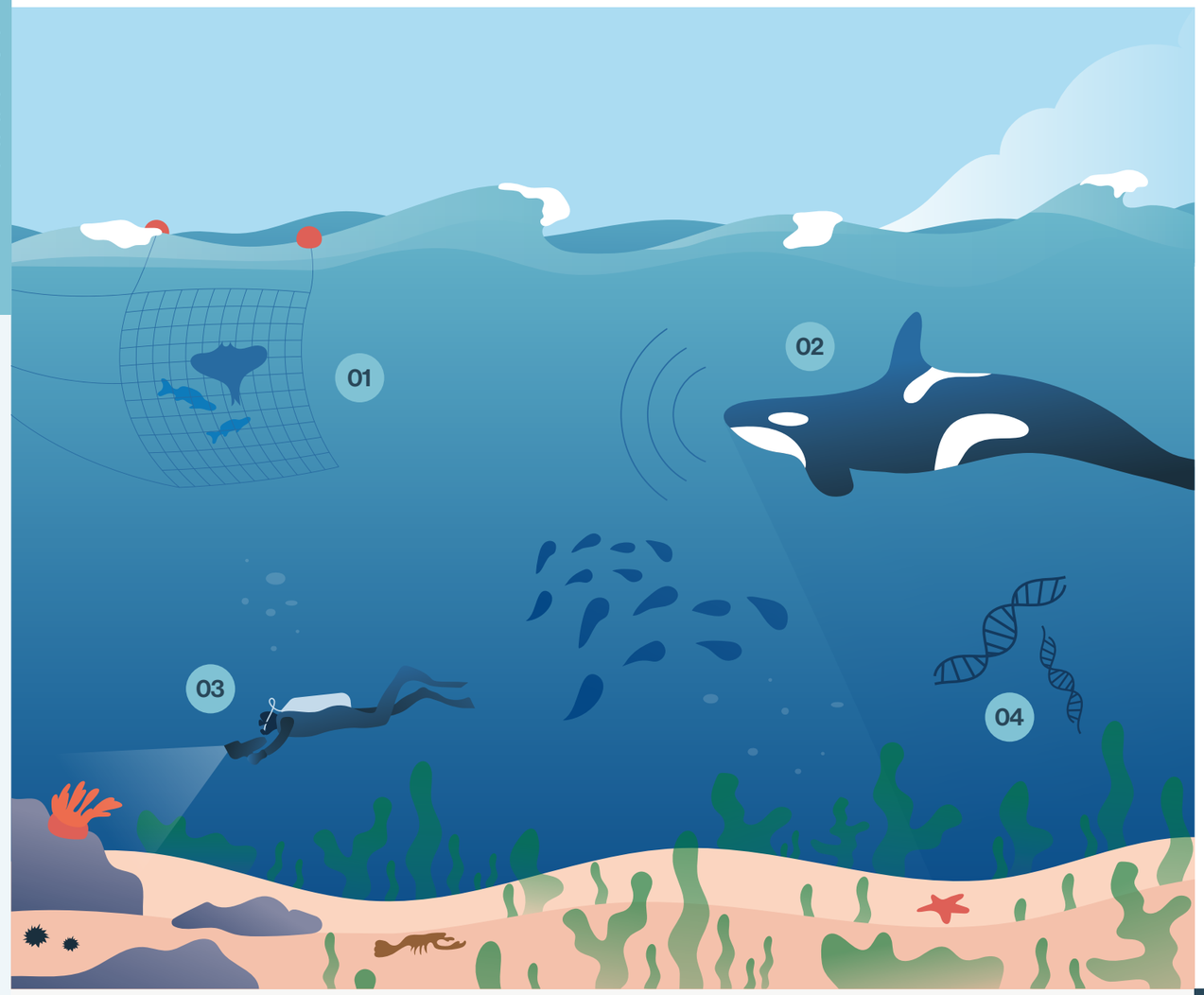
The term environmental DNA* (eDNA) was coined in 1987 to refer to microbial DNA extracted from sediment samples[1]. Initially limited to microbiology, its use broadened in 2008, when researchers from the Laboratoire d'Ecologie Alpine (LECA, a joint research unit between CNRS, University of Grenoble and University of Savoy Mont-Blanc) managed to detect the presence of an invasive species, the bullfrog Aquarana catesbeiana, in several French water bodies by identifying fragments of its DNA in the environment[2]. This pioneering study marked a turning point for eDNA-based methods, expanding their application to conservation biology[3]. Indeed, while DNA is a universal molecule common to all living beings, from bacteria to the most complex macroorganisms, it also contains the genetic information specific to each individual. Consequently, all organisms possess unique DNA sequences, and they leave traces of this material in the environment. Analysis of these 'genetic barcodes', also called markers, enables the identification of organisms present in a given environment, similar to how forensic scientists can identify a culprit through DNA traces found at a crime scene. In aquatic environments, DNA is extracted from water samples. These DNA extracts are then analyzed through two main approaches: a species-specific approach to detect a single target species or a multispecies approach called eDNA metabarcoding*.

> "All organisms possess unique DNA sequences, and they leave traces of this material in the environment.

Vocabulary

***Environmental DNA:** DNA that can be extracted from environmental samples (e.g. soil, water, air) without first isolating any target organisms[4].

***eDNA metabarcoding:** Simultaneous taxonomic identification of multiple species[4].



01 CAPTURE METHODS
e.g. fishing, traps, chemical products

- Specifically applicable to a target species + -
- Specifically applicable to a target taxonomic group +
- Simultaneously applicable to all life forms -
- Provides information regarding species abundances + +
- Provides information on individual organisms + +
- Avoids sacrificing individuals -
- Preserves ecosystem tranquility - -

02 HYDROACOUSTICS
e.g. bioacoustics, echo sounding, telemetry

- Specifically applicable to a target species + -
- Specifically applicable to a target taxonomic group + +
- Simultaneously applicable to all life forms -
- Provides information regarding species abundances +
- Provides information on individual organisms + -
- Avoids sacrificing individuals + +
- Preserves ecosystem tranquility +

03 VISUAL SURVEYS
e.g. UVC, cameras

- Specifically applicable to a target species +
- Specifically applicable to a target taxonomic group + +
- Simultaneously applicable to all life forms -
- Provides information regarding species abundances + +
- Provides information on individual organisms + +
- Avoids sacrificing individuals + +
- Preserves ecosystem tranquility -

04 ENVIRONMENTAL DNA

- Specifically applicable to a target species + +
- Specifically applicable to a target taxonomic group + +
- Simultaneously applicable to all life forms + +
- Provides information regarding species abundances + -
- Provides information on individual organisms -
- Avoids sacrificing individuals + +
- Preserves ecosystem tranquility + +

PART 01

Key point ↖

No biodiversity census method is complete when applied alone

For further information : Deter et al. 2023. eREF : État de référence de la biodiversité en Vertébrés dans les masses d'eaux côtières méditerranéennes à partir d'ADN environnemental. Rapport final. 68 pages et annexes. https://medtrix.fr/wp-content/uploads/2023/04/eREF-rapport2023_VF.pdf

eDNA-based techniques are fast, efficient, often less expensive than traditional methods considering the obtained results, and—most important—have no impact on the studied ecosystems. However, to answer certain ecological questions (e.g. about population size, sex or stage of development), more traditional census methods remain essential. For example, in the Mediterranean Sea, the eREF project demonstrated that eDNA enabled the detection of cryptobenthic (living hidden on the seabed), pelagic (living in the water column), and rare species that are difficult for divers to observe. Conversely, UVC enabled the identification of genetically similar species, i.e. those that share DNA sequences, while also providing estimates of the sizes of individual organisms and the abundances of target species. Therefore, the combination of these two approaches can be particularly interesting for surveying biodiversity and assessing the state of coastal ecosystems.

01 General context

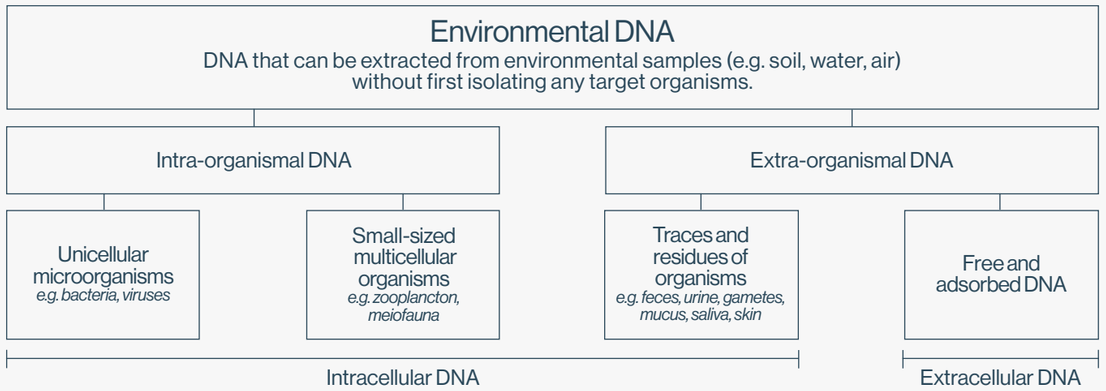
CHAPTER 02

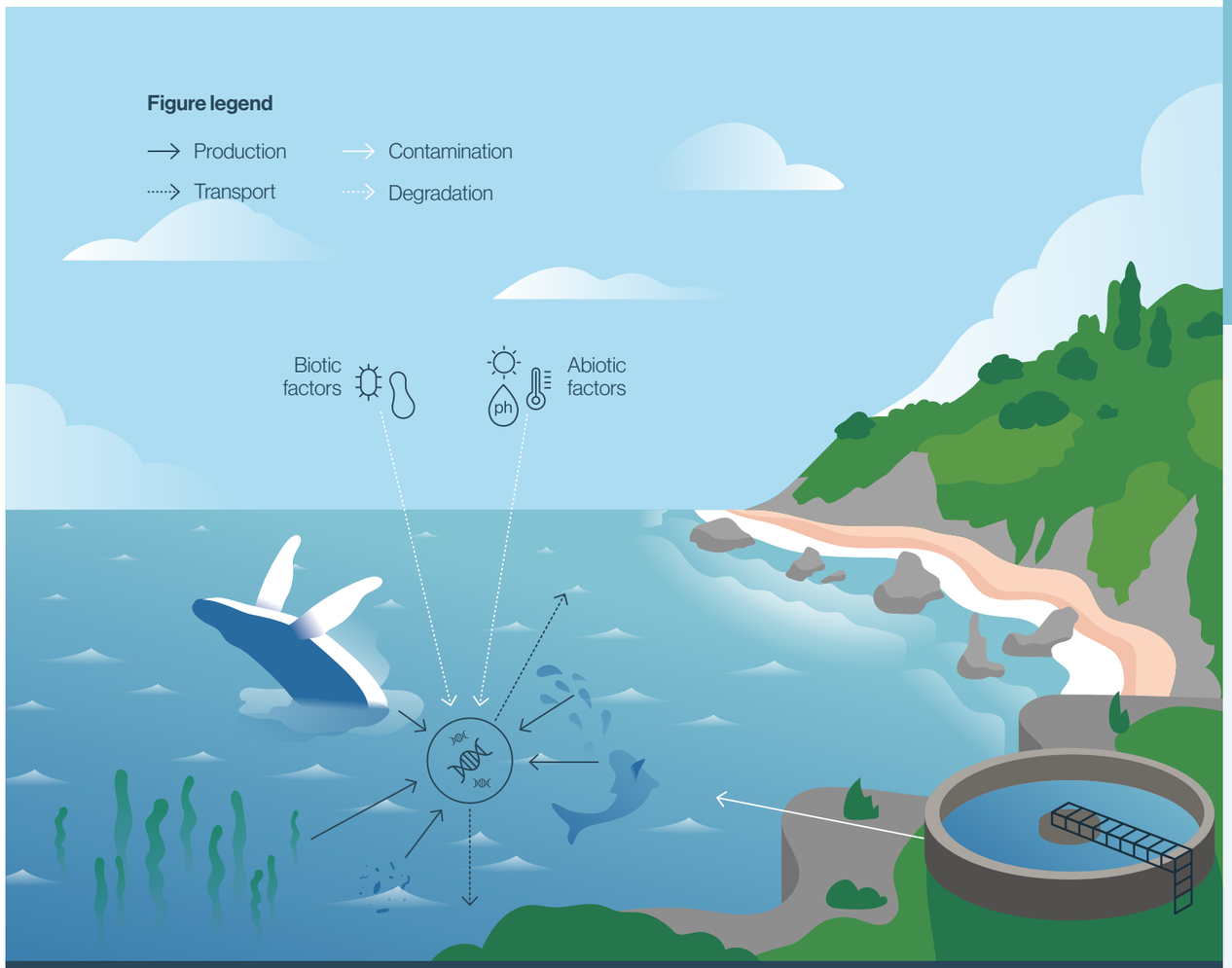
Ecology and detection probability of eDNA 02

Ecology of eDNA: composition, origin, transport and degradation

eDNA consists of free extracellular DNA, free DNA adsorbed onto organic and inorganic particles, intracellular DNA originating from microorganisms (bacteria, viruses or protists), whole small multicellular organisms (zooplankton or meiofauna), and traces and residues (e.g. feces, urine, gametes, mucus, saliva, skin) of larger organisms, such as vertebrates, invertebrates and plants[3,5]. The amount of DNA released by organisms is influenced by biological and physiological factors, such as age, developmental stage, metabolism, stress status, immune status, reproductive behavior and individual biomass, and by environmental factors, including water temperature, pH and salinity[6,7]. eDNA undergoes degradation due to a combination of both biotic (e.g. microorganisms, enzymes) and abiotic factors (e.g. UV radiation, pH, temperature). In aquatic environments, it can also be transported or sedimented and resuspended in the water column[6,7].

> Wherever life goes, it leaves traces.
>
> Nicolas Poulet & Laurent Basilico, 2019





Key point

eDNA detection distance and persistence in marine environments

In marine environments, waves and currents, coupled with eDNA degradation, result in dilution and vertical stratification of DNA molecules, leading to relatively short detection distances[8,9]. Indeed, marine eDNA is generally detectable from tens to hundreds of meters away from its source, enabling the differentiation of species community assemblages in sites located close to each other[10,11]. For instance, a study conducted on caged striped jack juveniles (biomass = 426.9 g) in Maizuru Bay (Japan) showed that eDNA detection distances decreased from 300 to 30 m just 1 hour after species removal and was undetectable after 2 hours[11]. Typically, the half-life of eDNA in marine environments ranges from a few hours to approximately 72 hours[12,13].

eDNA persistence is influenced by several factors, including environmental conditions, the amount of DNA produced (which varies with species biology), the type of environment under study, and the state (e.g. intracellular, extracellular, free, adsorbed) and size of the eDNA molecules (for instance, longer and free-floating fragments in the water column degrade more rapidly than shorter and adsorbed fragments). Further research is therefore required for a better understanding of these phenomena.

CHAPTER 02

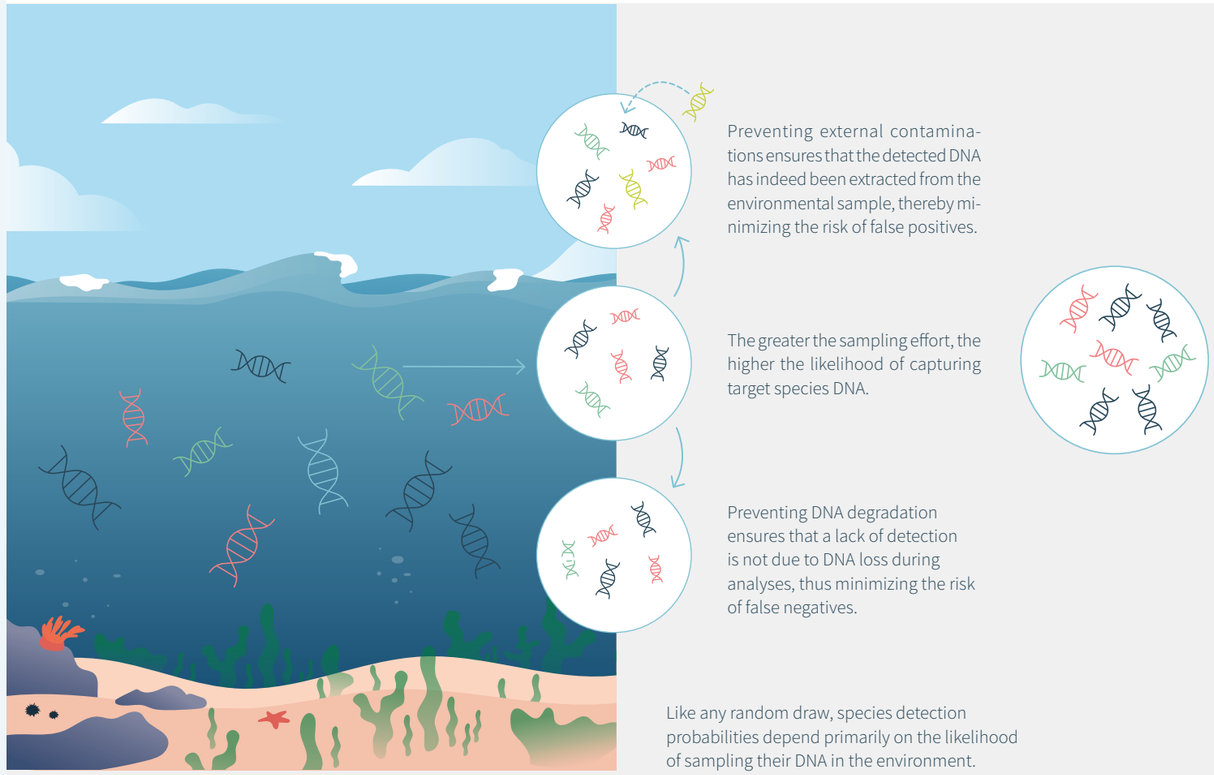
Standardized methods optimized to detect rare eDNA

As eDNA is degraded and transported in aquatic environments, it is diluted in large water volumes and therefore often present in very small quantities. The probability of detecting a species initially depends on the likelihood of sampling its DNA in the environment. Conservation of the collected eDNA is then essential to prevent its degradation and ensure that a lack of detection is not due to a loss of genetic material during the analysis process. Meanwhile, precautions must be taken to guarantee that the detected eDNA is actually extracted from environmental samples and not the result of external input (contamination). Indeed, eDNA can be transferred from one sample to another through the equipment used and/or the people handling it, which can lead to the detection of false positives*. Depending on the study objectives, these different parameters—detection probability, conservation and absence of contamination—require varying levels of precautionary measures in terms of equipment and infrastructure, as well as sampling and laboratory analyses. For example, when studying abundant bacterial communities, the required precautionary measures are not as stringent as those needed for detecting threatened and rare species leaving minimal traces of their DNA in the environment. To conduct biodiversity surveys and monitoring, it is often necessary to consider these less common species in order to obtain the most comprehensive species lists while minimizing the risks of false negatives* and false positives*, which can have significant consequences on downstream interpretations. Vigilife methods have been developed since 2011 by SPYGEN and are optimized to maximize eDNA detection probability. They are also standardized to enable data comparisons over time and space.

Vocabulary

***False negative:** Failure to detect species that were present in the studied environment.

***False positive:** Detection of species that were absent from the studied environment.



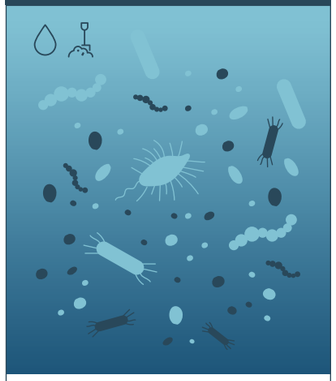
Preventing external contaminations ensures that the detected DNA has indeed been extracted from the environmental sample, thereby minimizing the risk of false positives.

The greater the sampling effort, the higher the likelihood of capturing target species DNA.

Preventing DNA degradation ensures that a lack of detection is not due to DNA loss during analyses, thus minimizing the risk of false negatives.

Like any random draw, species detection probabilities depend primarily on the likelihood of sampling their DNA in the environment.

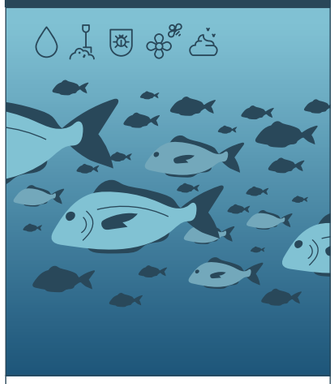
01 Caution level 1



e.g. Diatom biomonitoring, prokaryotic community analysis

For example: physical separation of laboratory analyses, wearing disposable gloves and a lab coat, performing negative controls.

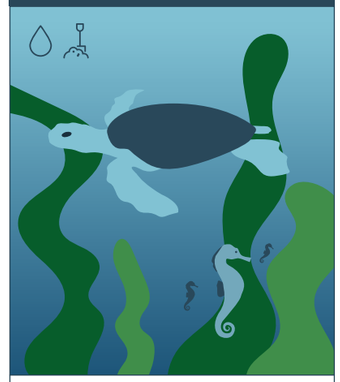
02 Caution level 2



e.g. Common species census, diet from feces analysis, floral composition of honey

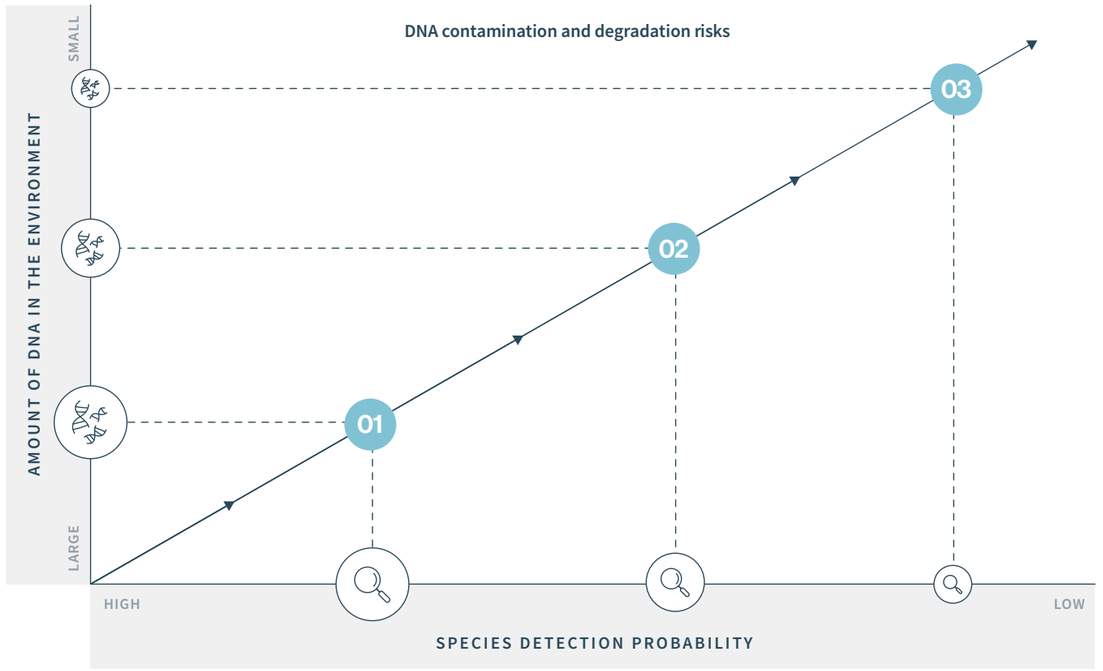
For example: dedicated room for analyzing small quantities of DNA, wearing a surgical mask during laboratory analyses, frequent decontamination.

03 Caution level 3



e.g. Analysis of rare threatened species, early detection of invasive alien species or pathogens, comprehensive community analysis

For example: dedicated room for analyzing small quantities of DNA, wearing a surgical mask during laboratory analyses, frequent decontamination.



• **TYPE OF SAMPLE**

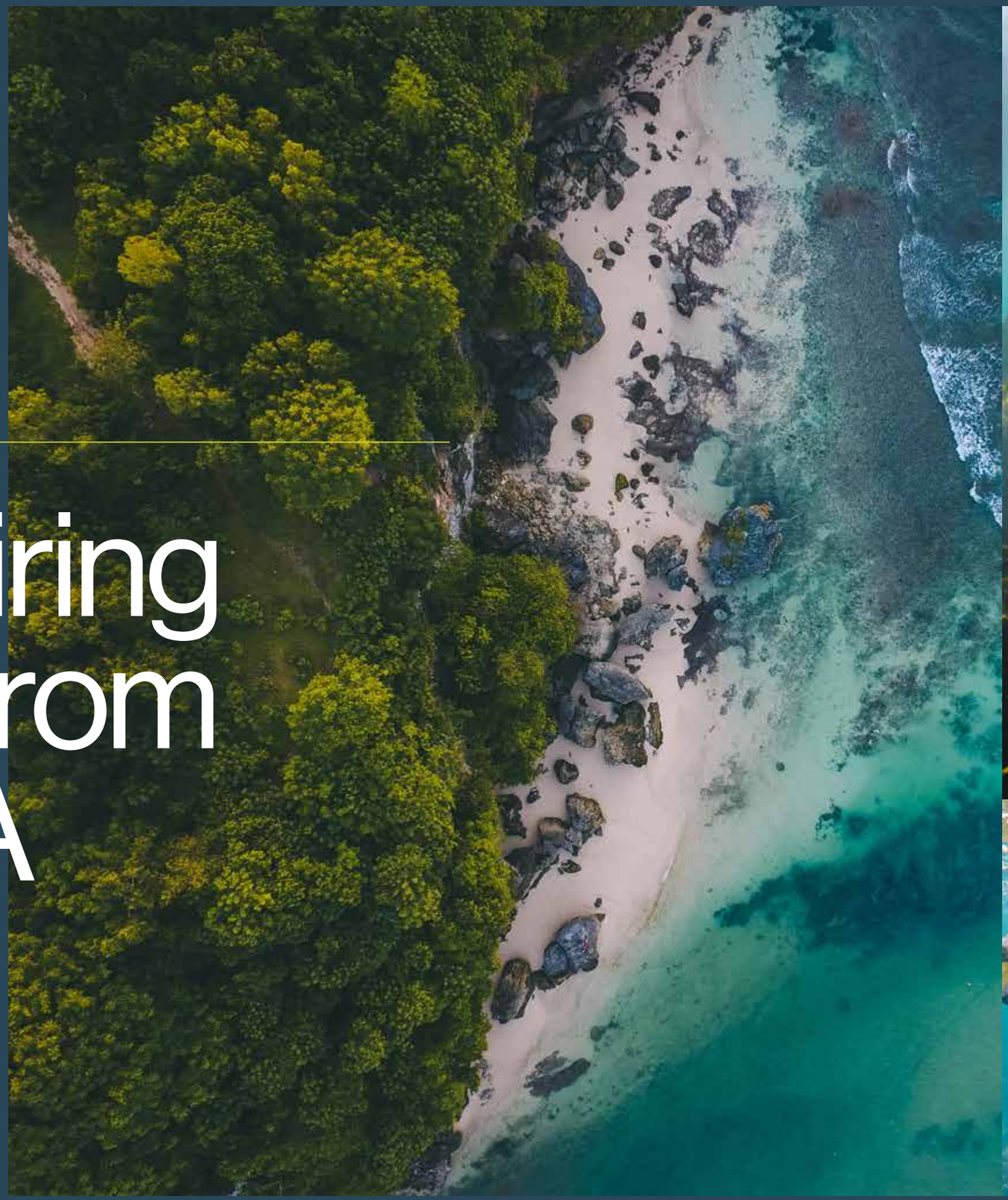
Water • Soil • Bulk* • Honey • Feces

**Whether bulk DNA can be considered eDNA is still debated within the scientific community.*

PART 02

02

Acquiring data from eDNA



CHAPTER 03

Project design 03

As with any scientific project, when designing an eDNA-based study, the users must first identify one or multiple objectives to achieve. They must then assess the required and available human, logistical and financial resources for sampling and analyses. These parameters impose technical and environmental constraints that influence, among other things, the development of an optimal sampling strategy, which can be defined as a sampling design that maximizes eDNA detection probabilities, reduces sampling efforts, and minimizes bias risks, while remaining representative of the studied environment.

Defining study objectives

The objectives of an eDNA-based study are defined according to the question(s) asked by the user, as well as by the target taxa and the geographic areas of interest. Studies may be focused on a single target species (e.g. one considered vulnerable, emblematic or invasive) or on one or more taxonomic groups (e.g. fish, crustaceans, vertebrates). They can be conducted at a local scale, involving a single site (e.g. ecological restoration area, harbor, shipyard), or at a global scale, encompassing multiple locations (e.g. a comparison of species colonizing protected vs. non-protected areas or heavily urbanized vs. non-urbanized areas).

> “A protocol is always designed to answer a question.
>
> Sordello *et al.* 2019

Identifying the stakeholders to involve

During an eDNA-based study, a single person can fill multiple roles and can be involved in different steps of the process. For example, the project manager can conduct the sample collection, transmit the obtained data to the dedicated platforms, and provide ecological expertise.

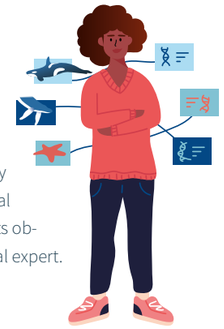
Sponsor

e.g. project manager, researcher

As the person in charge of the study, the sponsor identifies the objectives and may participate in the development of the sampling strategy. They also ensure compliance with regulations, and they oversee the technical and financial implementation of the project, as well as the publication and use of the results.

Ecological expert

They may participate in the development of the sampling strategy and may contribute to the ecological interpretation of the results obtained by the technological expert.



projet

Sampler

They are trained in and are responsible for collecting, packaging and dispatching samples, as well as acquiring information related to the sampling (e.g. location, date, time and any problems encountered).

Data manager

They ensure the quality and the structuring of the data and metadata in databases and information systems.

Technological expert

They may participate in the development of the sampling strategy, and they conduct the analyses and validate the results obtained in the laboratory. In the context of Vigilife projects, the technological expert is the company SPYGEN.

An expert’s opinion

Importance of the statistical validity of the data

Aurélien Besnard,
Director of Studies at the Ecole Pratique des Hautes Etudes (EPHE, Montpellier)

Just like when studying species distributions and factors influencing them based on simple field observations, using eDNA-based techniques requires careful consideration of the spatial sampling design. Thus, a clear definition of the research question and of the relevant statistical population is crucial to implement a rigorous spatial sampling and to make inferences in accordance with the research question. Moreover, even if the eDNA detection probability is high, false negatives are common issues, and careful consideration of how to model the eDNA detection is required to obtain unbiased parameter estimates. The definition of such robust sampling plans is crucial for data exploitation and involves applying relatively advanced statistical skills. Therefore, we encourage project leaders to work closely with eco-statisticians who can guide them in this process.

CHAPTER 03

Anticipating the allocation of financial resources and time

Budget is a critical consideration for project design. Indeed, limited financial resources compared with user needs can influence the sampling strategy by affecting the number of sites surveyed or samples collected. Thus, although it may not always be possible, we recommend developing a sampling strategy prior to requesting funding, in collaboration with the previously identified experts and ecologists. For reference, the average cost of an eDNA-based analysis for a single sample can currently reach several hundred euros. Prices vary depending on the target taxonomic groups, sampling equipment, and analyses performed by the selected technological expert. Each laboratory employs distinct methods, which may or may not be suitable for the objectives defined by the user (see Chapter 2). In general, prices communicated by technological experts do not include potential costs related to ecological interpretations of the results or the resources required to carry out the sampling at sea. However, these costs need to be factored into the final budget.

When designing a project, it is also crucial to inquire with the technological expert about the timeframe required to obtain results. This aspect mainly depends on the laboratory's workload, the number of analyzed samples, and the chosen methodological approach (single or multiple species). Consequently, the timeframe for obtaining results can extend up to several months. Finally, additional time may be required for ecological interpretation.

Designing a sampling strategy

Before samples are collected, the development of a suitable sampling strategy makes it possible to determine the number of samples that should be collected, their distribution through time and space, and the methods and regulations that need to be observed. Such a strategy should answer four key questions:

- **How should sampling be conducted?**
- **Where should samples be collected?**
- **When should samples be collected?**
- **According to which regulations should samples be collected?**

© Greg Lecoeur, WE ARE MEDITERRANEE

Key point ↖

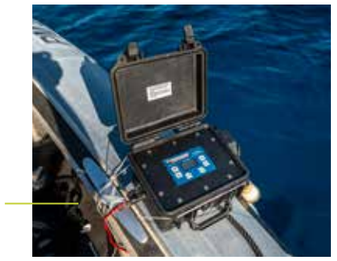
Filtration vs. precipitation

Traditionally, two methods exist for collecting eDNA from water samples: (1) DNA precipitation by ethanol and sodium acetate addition and (2) water filtration through a membrane that retains DNA. Currently, the latter method is the most commonly used[14] because it is recognized by the scientific community as more efficient[15]. Indeed, it enables the analysis of larger water volumes while limiting the use of ethanol, a flammable product under strict regulations and often costly in terms of transportation, storage and usage[15].

Off-site filtration

On-site filtration
© Greg Lecoeur, WE ARE MÉDITERRANÉE

Manual pump
© SPYGEN



Motorized pump
© Greg Lecoeur, WE ARE MÉDITERRANÉE

Open filter
© MicrobeOnline

Enclosed filter
© Greg Lecoeur, WE ARE MÉDITERRANÉE

How should sampling be conducted?

→ CHOOSING A SAMPLING METHOD

Aquatic environmental sampling involves collecting several liters of water. Although many methods exist for conducting such sampling[14], not all of them are optimized for detecting rare eDNA (eDNA present in small quantities, see Chapter 2). The main existing methods are briefly presented here.

Water can be filtered directly on-site or collected in dedicated containers before subsequent filtration in the laboratory. Off-site filtrations reduce time spent in the field. However, sample transportation and handling increase both contamination risks and eDNA degradation. To limit this second phenomenon, water samples can be kept cold or preserved by adding a buffer solution, but this entails considerable logistical and financial constraints that limit the filtered water volumes[15]. Whether they are conducted off-site or on-site, filtrations can be performed using a manual (syringe) or a motorized (peristaltic, vacuum) pump. Manual pumps are easy to use and inexpensive, but filtrations can be laborious and time-consuming, thus limiting the filtered water volumes and the number of samples analyzed[15].

> “A good sampling strategy is primarily an optimized compromise between a question and a set of constraints.
>
> Giraudoux, 2004

CHAPTER 03

Manual and motorized pumps can be used with open or enclosed filtering membranes (also referred to as 'filters' in this document) made of materials such as glass fiber, nitrocellulose or plastic polymers (e.g. polycarbonate, polyethersulfone). The material type can affect the hydrophilic and mechanical properties of the membrane, as well as its resistance capacity (e.g. ability to resist different filtration pressures, supported storage times), all of which can impact the filtered water volumes[14,15]. These volumes also depend on the filter porosity and surface. Specifically, pore sizes must be small enough to retain as much eDNA as possible while minimizing membrane clogging due to accumulation of other organic and inorganic particles. Generally, pore sizes are less than 1.5 µm, with the most commonly used filters having pore sizes around 0.22 to 0.45 µm[14,15]. In marine environments, where eDNA is often diluted and the environment is oligotrophic (low in organic matter) and slightly turbid, membranes with very small pores are recommended[15]. Open filters are less expensive but require manual handling and are exposed to the open air during filtration. Thus, their use increases the risk of sample contamination (risk of false positives). Additionally, they have smaller surface areas compared with enclosed membranes, which can lead to faster clogging and can limit filtered water volumes.

After filtration, eDNA retained on the membrane can be degraded by physicochemical processes or microbial communities. To prevent these risks, filters must be immediately stored, in cold conditions or in conservation solutions (e.g. buffer, ethanol), and protected from light. The choice of storage method depends on whether an open or enclosed filtering membrane is used. Open filters can be stored refrigerated or in conservation solutions, whereas enclosed filters typically only require conservation solutions. The choice of conservation solution depends on the DNA extraction method used during laboratory analyses.

Key point ↖

Vigilife standards

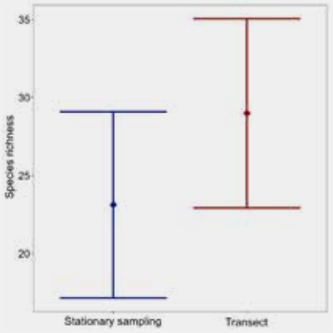
Like any random draw, species detection probabilities depend primarily on the likelihood of sampling their DNA in the environment. However, in aquatic environments, the ecology of eDNA (production, degradation and transport) means that it is diluted in large volumes and is therefore often present in low concentrations. eDNA concentrations tend to be particularly low in marine environments and in the case of rare species. As a consequence, filtered water volumes tend to be positively correlated with the amount of collected eDNA and thus with species detection probabilities[15-17]. Therefore, in the context of biodiversity surveys and monitoring, where the production of false negatives and false positives can have important consequences, we recommend filtering the largest possible water volumes. This can be achieved by increasing the number of samples taken at a given study site or the filtered water volumes (which implies using suitable equipment), or by combining these two approaches.

Vigilife methods are optimized to maximize detection probabilities of DNA traces left by organisms in the environment, including those of rare species. They involve on-site filtration of tens of liters of water (at a rate of about 1 L per minute for 30 minutes) using a motorized peristaltic pump equipped with a filtration capsule containing a membrane with a 0.2 µm pore size. Following filtration, conservation buffer is poured into the capsule to cover the entire filter, ensuring the preservation of the eDNA retained on it until DNA extraction in the laboratory.

Learn more ↗

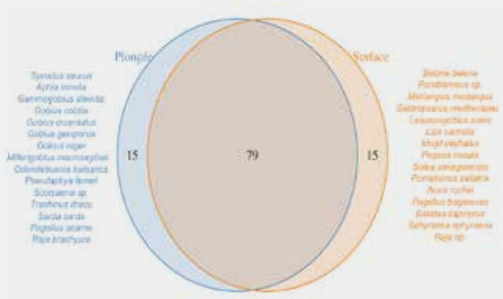
Stationary sampling vs. transects

Moving the pump during filtration makes it possible for a larger surface area to be covered. The eREF project[18] demonstrated that sampling by transect while diving (transects of a few hundred meters) enabled the identification of more species than stationary filtration. However, the identified communities were similar with the two approaches. Therefore, stationary sampling is representative of the main species present in an area, even though it limits the number of identified species compared with the transect method.



Deep vs. surface sampling

The eREF project[18] compared the fish species identified during simultaneous surface (1 m from the surface) and deep (50 cm above the sea floor, from 9 to 18 m from the surface) sampling. Both methods led to the detection of relatively similar fish communities. However, while surface sampling is sufficient to identify a large number of benthic species, deep sampling, closer to the substrate, enables the identification of more bottom-dwelling species.



Species detected by deep sampling (diving) are shown in blue, while species detected by surface sampling are shown in orange.

For further information: Deter et al. 2023. eREF: État de référence de la biodiversité en Vertébrés dans les masses d'eaux côtières méditerranéennes à partir d'ADN environnemental. Final report. 68 pages and annexes.

→ STATIONARY AND TRANSECT SAMPLING

Depending on the study's objective, the target species, the surface area, and the depth of the geographic zone of interest, filtrations can be carried out along a transect or at a fixed point, in other words, with or without moving the pump. Transect length depends on the available logistical means (motorized or non-motorized vessel, diver with underwater scooter or fins), the size of the area to be covered, and the user's objectives. For example, if users aim to precisely locate the presence of a species in a restricted area, conducting multiple short transects maximizes the detection probability and accurately locates the signal source. Conversely, if the goal is to have a general idea of the biodiversity present in a reserve spanning several square kilometers, longer transects are preferable to ensure spatial coverage of the sampling. Stationary filtrations can be suitable for studies focusing on a specific, shallow site where biodiversity survey methods other than eDNA-based analyses are concurrently deployed.

→ DEEP AND SURFACE SAMPLING

For stationary and transect filtrations, surface (less than 1 m from the surface) and deep sampling (up to 50 cm from the sea floor) can be performed from a motorized or a non-motorized vessel using a water-resistant pump or a sampling bottle (such as a Niskin bottle). In marine environments, eDNA is highly diluted and potentially subject to vertical stratification (see Chapter 2), so choosing between surface and deep sampling depends on both the target taxa and the depth of the study site. For example, filtration near the substrate is recommended for cryptobenthic organisms, which live hidden on the seafloor, while filtration in the water column is effective for identifying pelagic species. Similarly, if the studied area is shallow (e.g. coastal zone, offshore platform), surface sampling may suffice because waves and tides mix the water column, bringing eDNA to the surface. However, deep sampling is necessary if the studied site is deep (e.g. deep reef, drilling zone). Indeed, beyond 20 m depth, surface sampling does not effectively detect deep species and the identified species assemblage differs from that of deep sampling. Even with limited movement, eDNA sampling while diving can identify a greater number of species than surface sampling, likely due to the immediate proximity to the substrate and to most benthic species. Finally, sampling at different depths can be valuable for identifying all species present in an area with a bathymetric gradient (e.g. protected areas).

CHAPTER 03

→ NUMBER OF SAMPLES

In order to filter the largest possible water volumes and increase eDNA detection probabilities, multiple samples, called replicates, can be collected at the same sampling site. The number of collected replicates can be determined through a pilot study and/or according to the target taxa, environmental characteristics and study objectives. In marine environments, eDNA is highly diluted (see Chapter 2), so it is necessary to collect multiple replicates. We recommend collecting at least two field replicates per sampling site.

Where to sample?

Considering the ecology of eDNA in marine environments (see Chapter 2), the horizontal spacing between sampling sites depends on both the user's objectives and the characteristics of the area (e.g. study area size, water renewal, presence of strong currents, habitat discontinuity). For example, if the objective is to detect a specific target species, continuous sampling across the habitat (short transects placed end to end) will maximize the surveyed area. Conversely, when comparing species assemblages among multiple sites (e.g. inside and outside a protected or anthropized area), the distance between sites should be sufficient to distinguish among them. Typically, we recommend that sites be separated by at least twice the length of the transect. For stationary filtrations, sites should be separated by a minimum distance of 1 km. It is also crucial to ensure habitat and depth consistency between sites when comparing their biodiversity and to avoid potential sources of DNA contamination (by maintaining a minimum distance of 1 km from wastewater treatment plants, aquaculture discharge sites, and similar facilities if they are not relevant to the project).

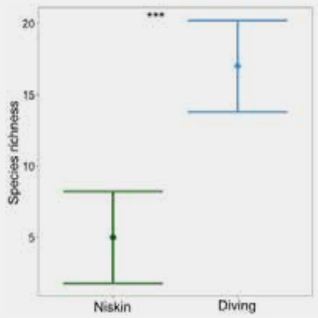
When to sample?

Ideal sampling times should be defined based on environmental characteristics and associated species. For example, we recommend sampling during species periods of breeding and/or high activity (taking care to not disturb individuals that may be encountered), as organisms excrete more DNA during these times (e.g. gamete production, birthing). Depending on species' life cycles, they may not be present in the same locations year-round (e.g. migratory flux). Therefore, obtaining prior information on the distribution and ecology of target taxa can be useful in identifying the most favorable sampling periods. In temperate zones with distinct seasons, species assemblages can vary significantly throughout the year. Therefore, when comparing biodiversity inventories, it is important to sample all sites at the same time of year. Similarly, despite current global changes, for multiyear sampling it is crucial to conduct sampling at the same time each year to ensure comparability. Ideally, to achieve a comprehensive species census in a given area, multiple samplings should be conducted in different seasons to maximize the detection of species that are only present at certain times. For example, sampling can be done in both spring and autumn.

Sampling bottle
© INRAE Thonon, JC Hustache

Learn more ↗

Deep sampling



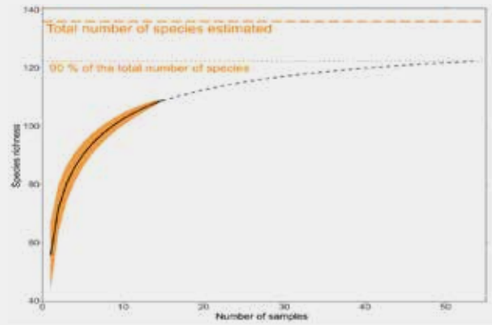
Although deep-water sampling with a Niskin-type sampling bottle does not require a diver or a water-resistant pump, the eREF project[18] showed that direct deep filtration is most suitable. Indeed, without deep filtration, samples need to be handled at the surface and complete decontamination of the sampling bottle is impossible, leading to greater contamination risks. Additionally, bottles must be open as they are lowered to the desired depth, where they are then closed with stoppers for water collection. As a result, samples are likely to contain eDNA collected from the water column during the descent

For further information: Deter et al. 2023. eREF: État de référence de la biodiversité en Vertébrés dans les masses d'eaux côtières méditerranéennes à partir d'ADN environnemental. Final report. 68 pages and annexes. https://medtrix.fr/wp-content/uploads/2023/04/eREF-rapport2023_VF.pdf

Learn more ↗

Field replicates

Results of the eREF project[18] showed that in a fully protected marine reserve, where fish diversity and density are high, more than 50 replicates of 30 L are necessary to complete a comprehensive species inventory. Therefore, sampling strategies must compromise between species detection exhaustiveness, costs and available resources.
For a biodiversity inventory, results from different replicates collected at the same site can be combined to obtain the most comprehensive species list possible. However, if statistical analyses are to be conducted subsequently, it may be necessary to analyze replicates separately to ensure robust analyses.



For further information: Deter et al. 2023. eREF: État de référence de la biodiversité en Vertébrés dans les masses d'eaux côtières méditerranéennes à partir d'ADN environnemental. Final report. 68 pages and annexes.. https://medtrix.fr/wp-content/uploads/2023/04/eREF-rapport2023_VF.pdf

Which regulations should be followed for sampling?

Before conducting a sampling campaign, it is important to ensure that all necessary permits for proper sample collection and transportation have been obtained. At the international level, access to genetic resources and the fair and equitable sharing of benefits arising from their utilization are governed by the Nagoya Protocol, adopted in 2010 at the 10th Conference of the Parties to the Convention on Biological Diversity (https://absch.cbd.int/en/). However, in metropolitan France (procedures may also apply in certain overseas territories and abroad), sampling conducted for eDNA-based studies and for biodiversity survey purposes do not fall within the scope of these agreements (source: Article L412-4 of the Environmental Code). Nonetheless, marine water sampling is regulated and requires coordination with the competent authorities in each country. For example, in France, an authorization request for Marine Scientific Research (RSM), describing the study objectives, methods employed and means used at sea, must be submitted to the Maritime Prefecture. In the case of sampling in Marine Protected Areas (MPAs), approval from the area managers must also be obtained. Abroad, any maritime activity generally requires an application to one or more ministries, depending on the country and the method used (for example, it is often easier to collect samples at the surface rather than with an immersed diver). Transport procedures for sampling equipment (e.g. lithium batteries for pumps, chemicals) and samples (e.g. filtration capsules) may also be subject to country-specific regulations.

Sampling strategy adapted to a research question

HOW TO SAMPLE?

- Standardized large water volumes
- On-site filtrations
- Motorized pumps
- Enclosed filters (0.2 µm)
- Conservation buffer
- Stationary or transect sampling
- At least two replicates

WHERE TO SAMPLE?

- Transects separated by twice the length of the transect
- Sites spaced at least 1 km apart
- Avoid areas likely to release large quantities of untargeted DNA (e.g. wastewater treatment plants, aquaculture discharges)

WHEN TO SAMPLE?

- According to species ecology, when quantities of excreted DNA are greatest
- According to seasonal and environmental conditions

REGULATIONS

- Apply for authorization to collect samples
- Inquire about the customs regulations in force

CHAPTER 04

Sample collection 04

Vigilife methods were developed by the companies SPYGEN and Andromède Océanologie, as well as the UMR MARine Biodiversity, Exploitation and Conservation (MARBEC) and the Center for Functional and Evolutionary Ecology (CEFE) of the University of Montpellier. These protocols are optimized for rare eDNA detection and require the use of sampling kits, as well as different types of filtration pumps. All these elements are presented in this chapter.

© Greg Lecoeur, WE ARE MEDITERRANEE

Filtration capsule, stoppers and conservation buffer © SPYGEN

Tube with an inlet filter and disposable gloves © SPYGEN

Recommended materials

→ SAMPLING KITS

SPYGEN offers single-use VigiDNA® sampling kits consisting of a tube with an inlet filter, a low-porosity sterile filtration capsule (0.2 µm), a conservation buffer (CL1), and pairs of disposable gloves.
In a clean and decontaminated zone, the tube must be inserted at the top of the filtration capsule, making sure to respect the indicated flow direction. The inlet filter and the outlet of the capsule must be protected (e.g. inside a disposable glove provided with the sampling kit) until water filtration.

→ PERISTALTIC PUMPS

Sampling kits must be used in conjunction with a peristaltic pump, to allow water to flow through the membrane in the filtration capsule. Depending on the desired sampling depth (see Chapter 3), different pumps can be used. Surface samples can be collected using a peristaltic pump, such as an Athena® pump (Proactive Environmental Products LLC, Bradenton, Florida, USA) placed on the vessel, while a water-resistant submersible pump is required for deep sampling and underwater filtrations.

Attaching the tube to the filtration capsule © Greg Lecoeur, WE ARE MÉDITERRANÉE

Key point ↖

Limiting risks of contamination

For all sampling, clean disposable gloves must be worn when handling the pump, filtration kits and inlet filter. Surfaces in contact with the equipment should be cleaned using a decontaminating product such as bleach (at least 2.5 % active bleach). To prevent the detection of DNA of species absent from the sampling site, we recommend avoiding filtering in vessel wakes and sampling aboard a fishing boat.

04 Sample collection

CHAPTER 04

Recommended protocols

→ SURFACE SAMPLING

During surface sampling, the pump and filtration capsule remain on board the vessel while the inlet filter, attached to the tube, is submerged between depths of 0.5 and 1 m using a weight. Water is pumped through the inlet filter and up the tube, and it flows through the enclosed filter before being discharged. Depending on the study objective, filtration can be conducted while moving along a transect or at a stationary point (see Chapter 3).

→ DEEP SAMPLING

Deep filtration follows the same procedure as surface filtration, but the pump, filtration capsule, tube and inlet filter are fully submerged. To do so, the entire setup can be lowered to the desired depth, either by a diver or using a weighted, balanced system, which is adapted to the pump and can be towed or remain stationary according to the chosen sampling method (stationary or transect, see Chapter 3). In the first case, to ensure the diver's safety, filtration times must be adjusted to limit decompression times. In the second case, with a weighted system, bathymetry and seafloor profiles must be considered to avoid damaging the equipment. The pump must closely follow the seabed contours, keeping the inlet filter sufficiently close to the substrate without making contact.

Filtration at the water surface © Abbie Trayler-Smith, Greenpeace

Filtration at the water surface © Greg Lecoeur, WE ARE MÉDITERRANÉE

Learn more ↗

Very deep sampling

Sampling in very deep environments (mesophotic or rariphotic zones, > 150 m depth) involves technical challenges, due to extreme pressure conditions and the impossibility of sending a diver to perform filtrations. To overcome these limitations, a 'very deep' pump has been developed by Andromède Océanologie and the University of Montpellier (MARBEC) to carry out filtrations directly at the sampling depth, thereby limiting contamination risks associated with steps completed at the surface. This pump is equipped with a timer and a pressure sensor to automatically trigger filtration at a given depth and with a user-defined latency. This filtration method requires a system capable of lowering and raising the pump by several hundred meters, a major technical challenge. Andromède Océanologie has developed an immersion system capable of filtering up to 1500 m deep aboard a vessel equipped with a hydraulic winch. The sampling kits used in conjunction with this pump are identical to those previously described (filtration capsule, tubes, inlet filter, conservation buffer, disposable gloves). However, the capsules and tubes must be filled with ultra-pure water beforehand to evacuate all air from the system and avoid volume contraction problems associated with the increase in pressure at depth.

© Abbie Trayler-Smith, Greenpeace

Sample conservation and data collection

Once filtration is complete, the user must wear clean disposable gloves to detach the filtration capsule from the tube, drain residual seawater, and seal one end of the capsule using one of the stoppers provided with the sampling kit. The capsule is then completely filled with the provided conservation buffer, secured with a second stopper, and vigorously shaken in all directions for at least one minute. This ensures an even distribution of the conservation solution and eliminates any bubbles introduced during the addition of the buffer. It also helps to detach organic matter present on the membrane and ensures that all captured eDNA is immersed in the conservation solution. Until laboratory analyses, capsules should be stored vertically (membrane facing downwards) at room temperature and protected from light.
Simultaneously, the user must record various sampling parameters, such as filtration duration, date, GPS coordinates and name of the sampling site (see Chapter 6). We also recommend documenting any notable events that occurred during filtration (e.g. species observations).

Deep filtration © Laurent Ballesta, Andromède Océanologie

Key point ↖

Limiting risks of contamination

Divers must position themselves above the pump and orient the inlet filter toward the seafloor to limit filtration of human DNA originating from the operator. As neoprene diving suits can also be a potential source of contamination, a dedicated diving suit must be worn when performing eDNA sampling (e.g. a diving suit that has been used for spearfishing should not be worn).

04 Sample collection

CHAPTER 05

Laboratory and bio-informatic analyses 05

© Spygen

Key point

Laboratory constraints when analyzing rare eDNA

When analyzing rare eDNA, it is crucial to ensure that the DNA extracted and amplified from an environmental sample is free from any contamination. Therefore, contracted laboratories must implement stringent methods and techniques to prevent contamination. The analysis platform should have multiple rooms to maintain physical separation of pre- and post-PCR steps (PCR, for polymerase chain reaction, is a DNA amplification step). Rooms dedicated to material preparation and DNA extraction (pre-PCR, when DNA is rare) should be maintained at positive pressures to prevent the entry of external contaminants. They should also be equipped with an entry vestibule for personnel to put on dedicated, single-use equipment (hairnets, masks, double pairs of disposable gloves, coveralls, dedicated shoes, shoe covers). Conversely, rooms designated for DNA amplification and sequencing (post-PCR, when DNA is abundant) do not require an entry vestibule and should be placed under negative pressures or isolated within separate infrastructures (e.g. separate buildings or floors) to prevent contamination of other rooms by highly concentrated DNA. This is also achieved by using appropriate equipment, such as laminar flow hoods or PCR hoods. All rooms should have regular air turnover to remove accumulated DNA, and the workflow should be unidirectional. In other words, a forward-movement system should be implemented and adhered to within the laboratory. Thus, equipment and personnel should always move from the room with the lowest DNA concentration (e.g. material preparation room) to the room with the highest DNA concentration (e.g. post-amplification room). Regular decontamination of laboratories with a DNA-destructive agent (e.g. bleach) is essential, and surfaces and equipment must be decontaminated between each set of samples. Quality checks, e.g. by including negative controls at each stage of the sample analysis process, are also critical. If all these requirements are not met, it is impossible to avoid cross-contamination and to ensure high-quality laboratory work when analyzing large numbers of samples.

PART 02

CHAPTER 05

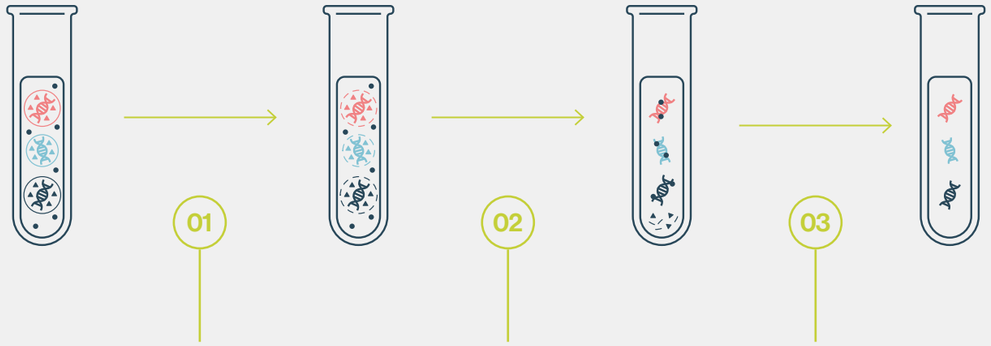
DNA extraction

The extraction process involves isolating eDNA from collected environmental samples[3] and comprises four main steps[19] (lysis, clarification, washing and elution), which can be carried out using custom protocols (such as phenol-chloroform) or commercial kits[3,14,15]. Regardless of the method applied, extracted DNA can be analyzed to detect a target species through a species-specific approach or to study multiple species within the same taxonomic group using a multispecies approach, also known as eDNA metabarcoding.

Detection of a target species with the species-specific approach

Species-specific detection involves identifying a specific DNA sequence of the target organism. Indeed, if this sequence, also called a marker region or barcode, is present in the sample, it can be targeted using primers, consisting of short synthetic DNA sequences, and then amplified by polymerase chain reaction (PCR). Other PCR-derived methods, such as quantitative PCR (qPCR) or digital PCR (dPCR), can also be used to amplify and quantify the genetic fragment of the target species.

Extraction



LYSIS

Cell membrane are destroyed by mechanical (grinding) and/or enzymatic methods (proteases).

CLARIFICATION

DNA is separated from cellular components and solubilized.

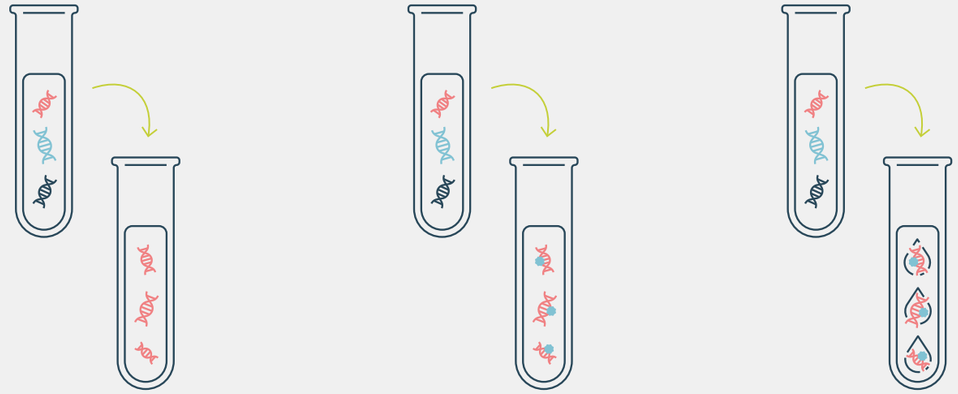
WASHING AND ELUTION

Residual organic and inorganic substances are eliminated through successive washes (ethanol or buffers). The DNA is then resolubilized.

- Cell membrane before lysis
- Lysed cell membrane
- Proteins
- Residual organic and inorganic substances

Species-specific approach

DETECTING A SINGLE TARGET SPECIES



CONVENTIONAL PCR

Amplification and detection through presence/absence of target species DNA

PCR is a method for amplifying a specific DNA fragment to obtain a quantity that is sufficient to detect and study it. In the species-specific approach, the results are visualized through the migration of the amplified DNA fragments on an agarose gel subjected to an electrical field (electrophoresis).

QUANTITATIVE PCR

Amplification and quantification of target species DNA

Quantitative PCR, or qPCR, builds upon PCR by measuring a fluorescent signal emitted during the amplification of the targeted DNA fragment. The results are visualized with a computer.

DIGITAL PCR

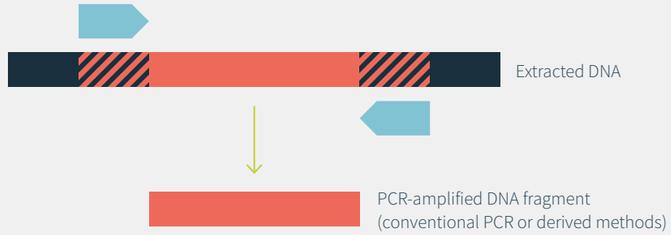
Amplification and quantification of target species DNA

Digital PCR, or dPCR, builds upon qPCR by partitioning the reaction mix into thousands of microreactions. The results are visualized with a computer.

PCR (conventional PCR and derived methods) involves several amplification cycles. Each cycle consists of three steps: DNA denaturation (double-stranded DNA is separated into two distinct strands), primer hybridization and complementary DNA synthesis.

CHAPTER 05

Vocabulary



***Barcode or marker region:** a DNA region with a precise location in the genome (e.g. a gene or a gene portion).

***Primer:** a short synthetic DNA sequence that makes it possible to target a specific marker region and initiate PCR amplification (or other derived methods).

- Genetic marker
- Primer hybridization site
- Primer

Key point

Commercial kits vs. custom protocols

Commercial kits ensure standardization and minimize contamination risks associated with the products and equipment that are used, while also reducing health and safety hazards for laboratory personnel. They are therefore recommended to ensure result reproducibility, especially in regular and high-throughput regulatory or industrial studies[15]. However, numerous kits are currently available on the market, and their effectiveness depends on several factors: filter material (open or enclosed filter), associated conservation method, target organisms and environmental characteristics (for example, residual organic or inorganic compounds may inhibit downstream laboratory steps if not properly removed)[15]. Therefore, the selection of an extraction kit should always be based on the upstream equipment used and the user's objectives. To further minimize contamination risks, we also recommend choosing methods that limit sample exposure to the open air. For example, methods without centrifugation involve using a vacuum chamber while keeping samples open throughout the extraction process.

Key point

Conventional, quantitative or digital PCR

qPCR is currently the most widely used method because it is more reliable than conventional PCR, which can be subject to interpretation bias by users. It enables the analysis of a larger number of samples within the same timeframe as dPCR[3,15,20]. Regardless of the method applied, the results can be influenced by the reagents and instruments (called thermocyclers) that are used. In qPCR, for example, the fluorescent signal required to quantify DNA strands can be emitted by a DNA intercalating molecule (such as SYBR green) or by a fluorogenic probe (a fluorochrome associated with a short synthetic DNA sequence called a probe (e.g. TaqMan), which targets a specific region of the marker; the fluorochrome is only released if the probe aligns with the targeted DNA). Fluorogenic probes reduce the risk of false positives while increasing qPCR detection and quantification capabilities[15,20].

PCR cycles

The more PCR cycles performed, the greater the number of amplified DNA molecules. For biodiversity surveys and monitoring, which require consideration of rare eDNA, we recommend conducting between 40 and 50 PCR cycles. For studies of more abundant communities, such as bacteria, 25 to 35 PCR cycles are sufficient. These values maximize the amplification of rare DNA. However, they also elevate the risks of contamination and false positives. Therefore, they require stringent precaution levels.

PCR replicates

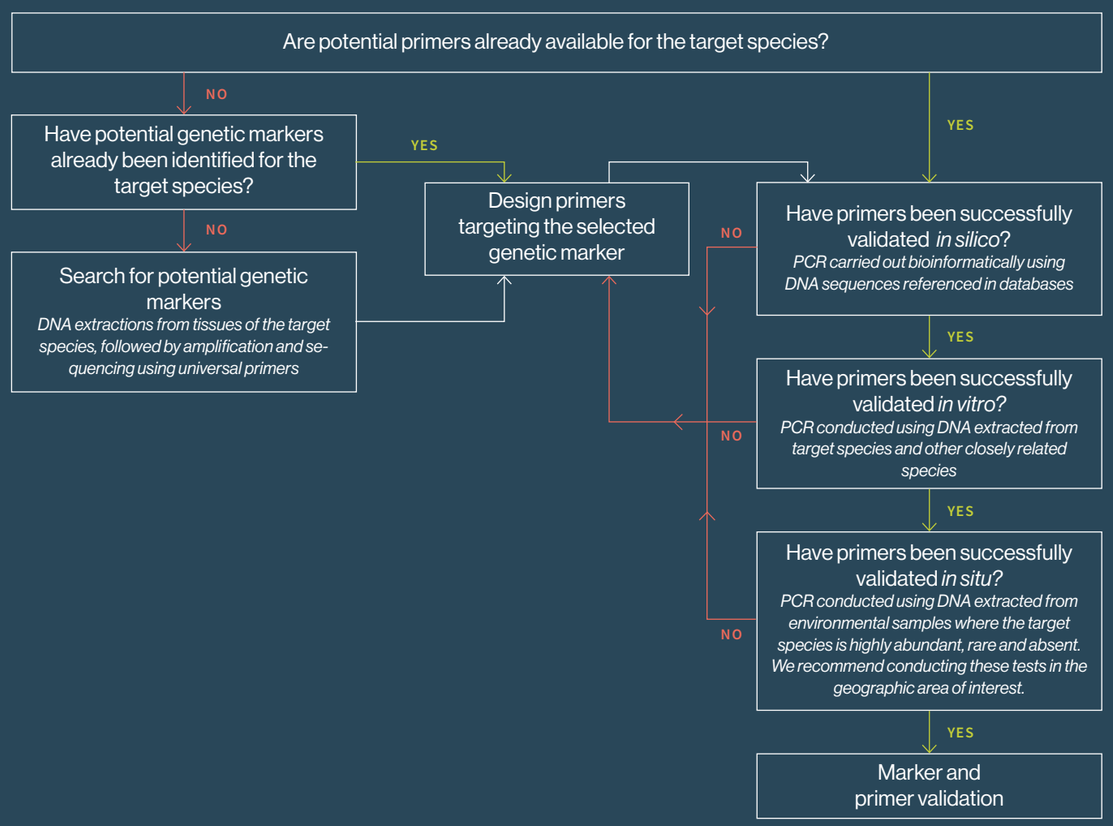
During PCR (conventional PCR or derived methods), a subsample of the total extracted DNA is used (approximately 1–10 %). Consequently, even if the targeted DNA is present in the extracted DNA, it may not be present during PCR reactions and thus may not be detected. To address this issue and increase the likelihood of detecting target species DNA, multiple PCR reactions, referred to as PCR replicates, can be performed from a single sample. It is generally recommended that a minimum of three replicates be performed per sample. However, this number should be increased to six when the species detection probability equals 0.5[21]. In the absence of this information, a minimum of eight replicates is advised. Depending on the user's objectives, especially in the case of rare eDNA analyses, increasing the number of replicates further can enhance species detectability and provide insights into the frequency and occurrence of the target species' DNA across replicates[15,21,22]. For Vigilife projects, we recommend conducting 12 PCR replicates.

CHAPTER 05

Key point ↖

Marker region and primer selection

Primers should target specific DNA hybridization sites of the target species and allow amplification of a highly conserved DNA region, but they should also be sufficiently variable to ensure that no other taxa are detected during the analysis. This is important to ensure primer specificity and thus limit risks of false negatives or false positives, which can significantly impact biodiversity inventories and monitoring (see Chapter 2). Therefore, we recommend that primers be validated according to a three-step protocol: *in silico, in vitro* and *in situ*[23,24]. In addition to targeting a specific genetic marker, primers should also amplify a short DNA sequence (typically fewer than 150 base pairs) to facilitate amplification from potentially degraded eDNA[25]. As a consequence, designing optimal primers can be a time-consuming and costly process. We also caution users that the publication of primer pairs in the scientific literature does not guarantee their quality, as some primers may amplify non-target species. Therefore, we recommend working with a trusted technological expert.



Multispecies approach or eDNA metabarcoding

The multispecies approach enables the simultaneous and unbiased identification of multiple distinct species within the same taxonomic group, from the kingdom level (e.g. bacteria, eukaryotes) to intermediate levels (e.g. fish, crustaceans)[15]. Initially, extracted DNA is amplified by conventional PCR using universal primers. The resulting amplified DNA fragments (also called amplicons) are then sequenced, and the obtained sequencing data are analyzed using bioinformatics methods. Ultimately, a table listing the identified species and the number of times the associated sequences were detected per sample is obtained and can be interpreted by ecological experts.

Vocabulary

***Tag :** a short synthetic DNA sequence added to amplified target sequences, serving as a unique identifier for each sample or PCR replicate[26]

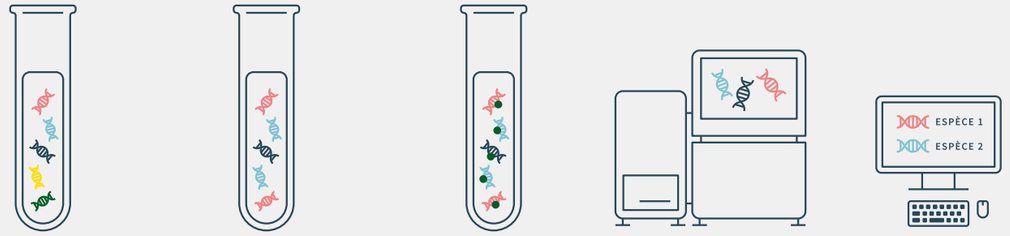
***Sequencing adapter:** a short synthetic DNA sequence added to amplified target sequences to enable binding onto the sequencing flow cell[15]

***Index:** a short synthetic DNA sequence added to amplified target sequences, serving as a unique identifier for each library[15]

***Library:** a set of amplicons (amplified target sequences) to be sequenced, with each sequence containing tags, adapters and indexes at their ends[26]

Multispecies approach

EDNA METABARCODING



EXTRACTED DNA

AMPLIFICATION

Extracted DNA is amplified by conventional PCR using universal tagged primers targeting DNA sequences of multiple species within the same taxonomic group.

LIBRARY PREPARATION

Short synthetic DNA sequences, called indexes and adapters, are added to the obtained amplicons (amplified DNA sequences). Labeled amplified DNA fragments that are ready for sequencing are grouped into sequencing librairies.

SEQUENCING

Libraries are sequenced using high-throughput sequencers (e.g. Illumina, IonTorrent) to determine the composition of the extracted and amplified DNA fragments.

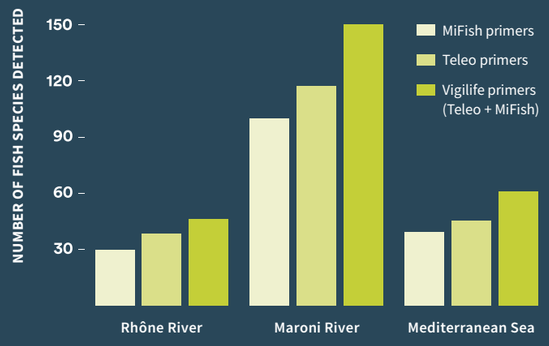
BIOINFORMATIC ANALYSES

Sequencing results are analyzed through bioinformatic pipelines. The detected sequences are assembled (aligned to reconstruct the targeted genetic marker), demultiplexed (each sequence is assigned to its original sample), cleaned (potential sequencing errors are eliminated), clustered and taxonomically assigned through comparisons with genetic reference databases.

CHAPTER 05

Key point ↖

Marker region and primer selection



As in the species-specific approach, primers used in eDNA metabarcoding should target a short genetic marker (200 to 500 bp for microorganisms and less than 120 bp for macroorganisms[3]) and must undergo prior validation *in silico, in vitro* and *in situ*[23,24]. Additionally, the targeted DNA region should exhibit sufficient variability among species to achieve optimal taxonomic resolution. Conversely, primer hybridization sites must be conserved and ubiquitous within the target taxonomic group to ensure reliable and equivalent amplification of all species. Primer choice can have a considerable effect on the species that are detected. Therefore, the number of species identified in a given sample of extracted DNA can vary greatly depending on the selected primers.

For further information: Polanco et al. 2021. Comparing the performance of 12S mitochondrial primers for fish environmental DNA across ecosystems. Environmental DNA. https://doi.org/10.1002/edn3.232

PCR cycles and replicates

The challenges associated with species-specific analysis and eDNA metabarcoding are identical; refer to the section on detecting a target species for further information.

Methods for preparing sequencing libraries

There are three methods for preparing sequencing libraries: one-step PCR, two-step PCR and ligation[26]. In one-step PCR, tags, indexes and adapters are directly added to the primers during their synthesis. This approach accelerates sample preparation for sequencing, but it is also more expensive because primers are longer (due to the addition of other sequences). Longer primers can affect amplification efficiency, potentially reducing the detection probability of rare DNA[15]. Two-step PCR involves an initial amplification step using tagged primers that target the marker of interest and include hybridization sites for additional sequences. A second PCR then adds the indexes and adapters. While this method partially addresses issues related to primer length, it is more susceptible to cross-contamination[15]. It is currently widely used, due to its cost-effectiveness and ease of implementation. However, we recommend using ligation, which involves adding further sequences after PCR amplification using enzymes called ligases. This method is the only one that maintains the original primer size, thus ensuring maximum amplification efficiency while minimizing contamination risks.

Key point ↖

Sequencing technology and depth

There are various sequencing technologies that differ in performance (analysis time), capacity (number of sequences that can be read), and error rate. Currently, due to its cost-effectiveness and relatively low error rate, Illumina® technology is the most widely used[27]. Beyond sequencer choice, sequencing depth is a crucial consideration. This term refers to the total number of expected sequence reads per sample. It depends on sequencer capabilities and on the total number of sequenced samples. Typically, studies focusing on eDNA analysis aim for a sequencing depth ranging from 50,000 to 200,000 reads per sample. However, in studies where detecting rare species is a major concern, it is advisable to increase sequencing depth beyond these values[15].

Bioinformatic analyses and genetic reference databases

There are currently numerous bioinformatic tools available for analyzing sequencing data. However, each method is likely to yield different or even ecologically inconsistent results if not chosen and used correctly. Therefore, a bioinformatic analysis must be optimized according to the targeted genetic marker, the taxonomic group of interest, and the selected sequencer. Regardless of the method selected, the resulting sequences are compared against databases of DNA sequences originating from known organisms, referred to as genetic reference databases. To identify a species based on its DNA traces, the DNA sequence of the species for the applied marker must have been previously documented in these databases. While widely used and expansive, public databases such as NCBI (National Center for Biotechnology Information), DDBJ (DNA Data Bank of Japan) and ENA (European Nucleotide Archive) may contain errors that can affect the accuracy of taxonomic assignments. To address this issue and ensure high-quality taxonomic assignments, it is possible to build a custom genetic reference database by sequencing tissues from known species (e.g. sampled in the field, from species collections, or from professional fishing landings). We recommend constructing a specific reference database based on organisms from the geographic area of interest, relying on the expertise of taxonomists, and regularly updating these data to keep pace with taxonomic developments.

Learn more ↗

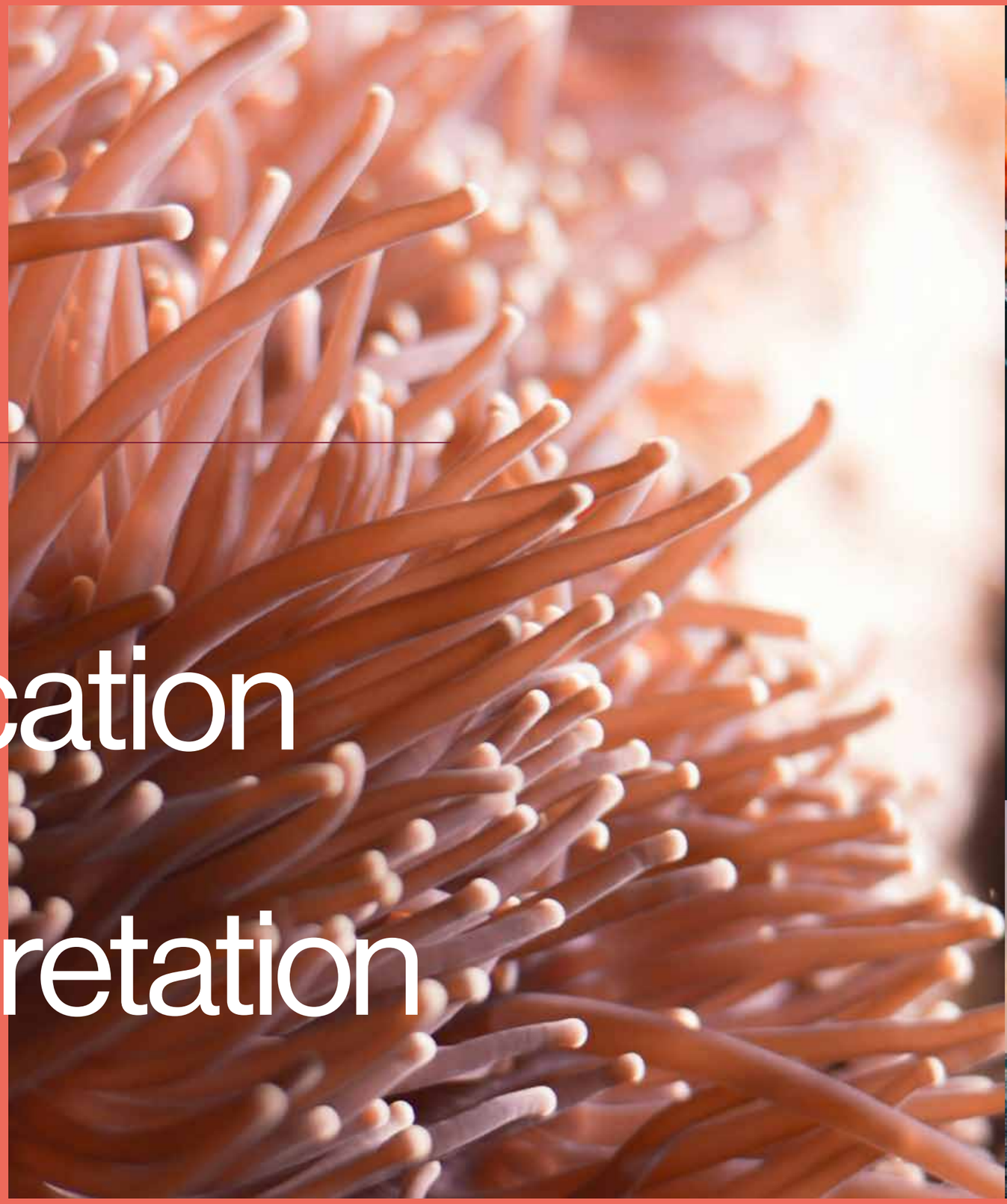
Bioinformatic analyses and sequence clusters

During bioinformatic analyses, the sequence grouping step can lead to the formation of (molecular) operational taxonomic units (OTUs or MOTUs) or amplicon sequence variants (ASVs). An OTU is a cluster of sequences that are identical to each other up to a specified threshold. In other words, an OTU consists of x sequences that have at least y % similarity. An ASV includes a main sequence and several similar, less abundant sequences considered to be errors or variants of this main sequence. Constructing ASVs thus requires an initial denoising step to model and identify sequencing errors[28].

PART 03

03

Data publication and interpretation

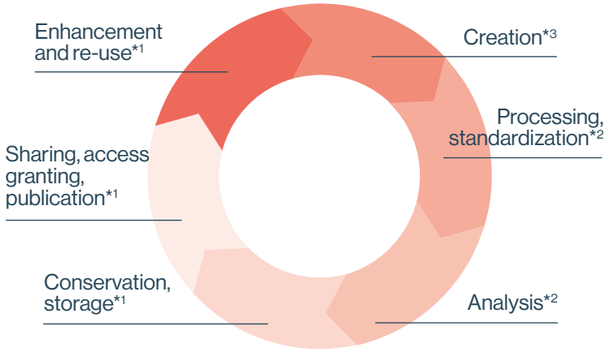


CHAPTER 06

eDNA data cycle 06

eDNA-based studies exhibit mixed practices regarding data management and sharing. Meanwhile, the FAIR principles provide guidelines to ensure that scientific data are easily Findable, Accessible and Interoperable with tools and information systems, as well as being Reusable. Consequently, it is crucial to systematically record and preserve specific information throughout the data life cycle.

.



*1 Physical and digital data
*2 Digital data
*3 Collection and laboratory analysis of samples

Definitions

→ eDNA DATA

Species occurrence data typically include essential elements, such as the species' name, location and observation date, as well as the observer's identity.

SPECIES NAME + OBSERVER + LOCATION + DATE

However, when dealing with eDNA-derived data, the observer refers to the person who collected the eDNA sample, while the location and date correspond to the sampling site and date, not a direct species observation. Therefore, it is crucial to clearly specify these distinctions and indicate that data are derived from eDNA.
eDNA-derived 'elementary exchange data' consist of the minimum set of essential information needed to ensure data quality, i.e. adhering to the FAIR principles as much as possible. This entails defining both the mandatory and the optional information to be transmitted, as well as determining whether to include them at the data or metadata level.

→ METADATA

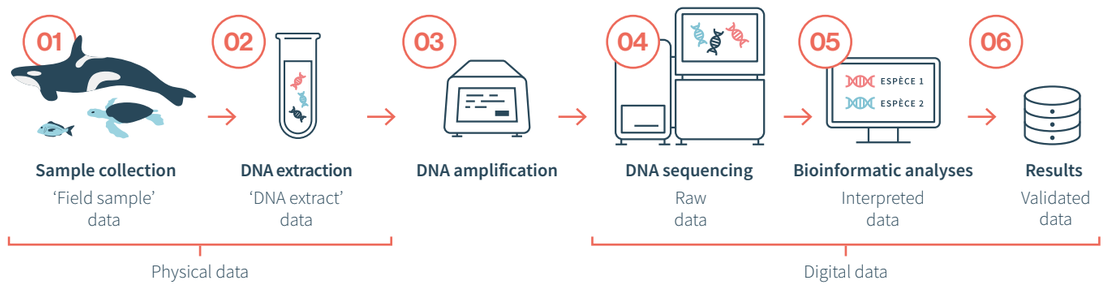
Metadata are pieces of information associated with a dataset (defined as a cohesive collection of data sharing common characteristics and derived from the same acquisition protocol). Metadata provide details about the data collection process, the individuals or organizations involved (e.g. producers and suppliers), and how data should be shared. The aim of metadata is to facilitate connections with other datasets, acknowledge contributors involved in data acquisition, and enable the reuse of data in meta-analyses.

Types of eDNA data

eDNA data can refer to several types of data, depending on the sampling and analysis steps.

SINGLE-SPECIES APPROACH :

MULTISPECIES APPROACH :



Each data type can be associated with several pieces of information related to the steps involved in the entire process, from field sampling to the determination of species names (see figure). Discussions are ongoing regarding the types of information that should be collected for eDNA-derived data. Here, we propose a non-exhaustive list to contribute to these discussions.

Learn more ↗

https://inpn.mnhn.fr/programme/donnees-observations-especes/references/qualite

https://inpn.mnhn.fr/programme/donnees-observations-especes/references/metadonnees

https://www.pndb.fr/fr/ressources/principes-fair-et-cycle-de-vie-des-donnees

https://www.ouvrirlascience.fr/fair-principles/

Some principles concerning the quality of observational data and metadata are emphasized on the National Inventory of Natural Heritage (INPN) website.
More details about the FAIR principles can be found on the National Biodiversity Data Center (PNDB) and Ministry for Higher Education and Research websites.

CHAPTER 06

→ FIELD SAMPLING

METADATA:

- Project information (e.g. project name, project objectives, project description, target taxonomic groups, project manager, financial support, partners, links to publications, links to associated data)
- Sample storage location and identifier

SAMPLING CAMPAIGN:

- Number of sites sampled

SAMPLED SITE:

- Unique identifier for each sample
- Sample type (water)
- Sampling strategy (e.g. transect, stationary site)
- Sample water volume
- Sampling date and time
- GPS coordinates, sampling depth
- Environmental characteristics (e.g. seagrass beds, coralligenous zone)
- Materials (e.g. kits, pumps, laboratory name)

Other possible information : Weather conditions, including temperature

→ DNA EXTRACTION

- Sample storage condition before and after DNA extraction
- Performed on-site or off-site
- Laboratory that performed the extraction
- Extraction protocol
- Quantity of extracted DNA
- Positive and negative controls
- DNA extract storage location and identifier

→ DNA AMPLIFICATION

- Performed on-site or off-site
- Laboratory that performed the PCR
- Species-specific approach (PCR, qPCR, dPCR)or multispecies approach
- Number of PCR cycles
- Number of PCR replicates
- Pooled or individual PCR replicates (tagging)
- Temperatures for hybridization / PCR cycles
- Thermocycler
- Barcode
- Primers and genetic reference database (universal or custom), including database version
- DNA quantity
- Blocking primers (if necessary)
- Probe used for qPCR

→ SEQUENCING

- Performed on-site or off-site
- Laboratory or company
- Method used to prepare sequencing libraries (one-step or two-step PCR or ligation)
- Sequencer name and type, paired-end or single-end
- Sequencing depth

→ BIOINFORMATICS

- Script, software, package and version of the tool
- Laboratory (and person) that performed the analyses
- ASVs / OTUs
- Genetic reference database: name, version and
- consultation date
- Similarity threshold for sequence clustering and/or taxonomic affiliation
- Name of the expert who confirmed the assigned species
- Names of validated taxa
- Quantity of data lost during bioinformatic steps (if possible, specify for each step)
- List of identified contaminants

Optional: assigned taxa names before validation

© Greg Lecoeur, WE ARE MEDITERRANEE

Storing, publishing and using eDNA data

→ DATA PROPERTY

Data ownership should be defined at the inception of the study. This involves determining who owns the data—or each type of data—and specifying the roles of each person involved in data acquisition, e.g. funders, commissioning parties, and field and laboratory technicians.

→ STORAGE TIME AND LOCATION OF FIELD SAMPLES, DNA EXTRACTS AND SEQUENCING DATA

For physical data, storage in freezers is necessary to prevent DNA degradation. Digital data, especially raw sequencing outputs, require large storage capacities. Scientific journals typically mandate authors to provide permanent links for access to raw data alongside publications, facilitating future re-analysis (e.g. with updated genetic reference databases). Online servers offer downloadable file services, while raw data can be stored in data repositories and metadata in catalogs like the National Biodiversity Data Center (PNDB). Even if data are unavailable (e.g. under embargo), ensuring metadata accessibility is crucial.

→ HOW CAN ONE PUBLISH DATA AND MAKE IT AVAILABLE?

Physical data can be stored by the owner or by the laboratory that performed the DNA extraction, depending on the agreements outlined in service or partnership contracts. Certain samples of interest may also be preserved in museums, to serve as historical records of the sampled environment at a given time. However, logistical and organizational aspects are yet to be established.
For the digital data, both raw data (sequencer outputs) and analyzed data (outputs from the bioinformatics phase) can be deposited in national or international databases (e.g. ENA, NCBI, Global Biodiversity Information Facility GBIF, National Natural Heritage Inventory INPN). Alternatively, data can be deposited on platforms, although these may face challenges related to referencing that can hinder data accessibility. To enhance their visibility, data can be highlighted through scientific publications (e.g. in a data paper) and made accessible through national and international information systems.
Validated eDNA-based data can be disseminated through 'traditional' channels via information systems, provided that their eDNA origin is clearly indicated and that comprehensive metadata accompany them. Efforts are currently underway to improve and facilitate the management of eDNA-derived data collected for research and public-policy purposes. Establishing dedicated standards and tools will be essential to address the specificities of these data, ensure their quality, and align them with FAIR principles. Similar to species data obtained through traditional methods, eDNA data that has been validated should be assigned a unique and persistent identifier, such as a digital object identifier (DOI) or a universally unique identifier (UUID), linking it to other related data types that facilitated its acquisition.

Learn more ↗

Making data available

https://inpn.mnhn.fr/accueil/index/

https://doi.org/10.35035/doc-vf1a-nr22

For French territories, eDNA data can be made available through regional platforms or through various data repositories. Recommendations are available on the INPN website.

For non-French territories, an explanatory guide is available from GBIF.

Learn more ↗

vigilife maps

Global diversity in Open Data

The Vigilife Maps platform, resulting from a collaboration among stakeholders in research, conservation and the economic sector, will soon be unveiled. Through this platform, free access to eDNA-derived data will quickly be made available to environmental managers, researchers, political decision-makers and others. It will be possible to track species distributions and abundances, to be alerted of the appearance of an invasive species, and to study the evolution over time of biodiversity at a site (referred to as a 'sentinel' site), thanks to scientifically validated synthetic indicators.

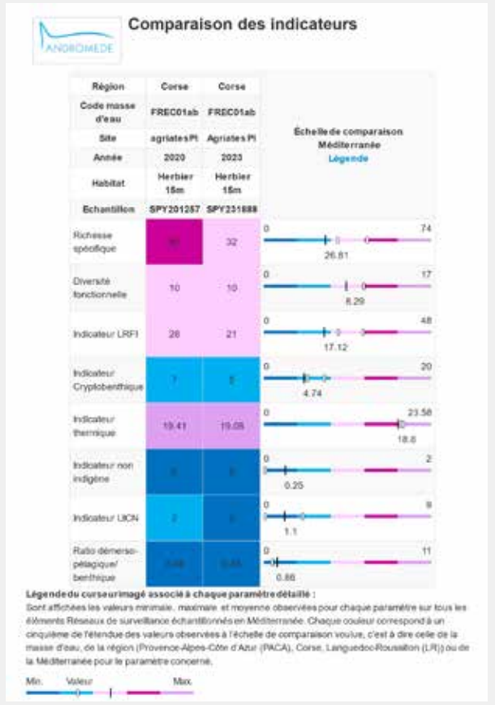
These standardized eDNA-derived data will also feed into national and international biodiversity databases. The general public will be able to learn more about and become aware of the health status of surrounding ecosystems and to assess the impact of conservation or restoration actions, such as protected areas established by Vigilife partners.

Data on the Vigilife Maps platform will be presented at high spatial resolutions, typically on the order of kilometers. Publishing rules, defined with each client or partner, will ensure that information about commercial species or those classified on the IUCN Red List is appropriately anonymized, in compliance with national policies related to access to genetic resources.

> "The Vigilife Maps platform will soon be unveiled.

Learn more ↗

MEDTRIX



MEDTRIX is an online mapping platform created in 2013 by Andromède Océanologie, with the support of the Rhone Mediterranean Corsica Water Agency, designed for monitoring coastal waters and ecosystems in the Mediterranean. Managed by the l'Oeil d'Andromède association, MEDTRIX provides access to high-resolution spatial monitoring data (depths of 0 to 130 m, mapping at a scale of 1/10,000) along the French Mediterranean coasts and certain areas in the Atlantic Ocean, Italy, Tunisia, Spain and Morocco. The platform gathers data from around 50 public and private entities and is freely available to its users (3750 registered to date) through over 40 projects grouped into 8 categories (monitoring networks, state of coastal and transitional waters, ecological restoration, coastal management, habitat mapping, observatories and workshop sites, expeditions, citizen science). Once their profile is created, users can freely access all projects and numerous platform features, such as map editing, visualization and downloading of monitoring sheets / scientific publications / study reports, graph creation, display of statistical data, site comparisons, and use of WMS flow. Additionally, they can build custom maps using data available on MEDTRIX.

One notable project on MEDTRIX is PISCIS, which aims to monitor ichthyological assemblages using eDNA-based methods. This project, supported by Andromède Océanologie and the Rhone Mediterranean Corsica Water Agency, conducts annual ichthyological assemblage characterizations at 165 stations from mid-May to late June, along the French Mediterranean coastline bordered by the three regions of Corsica, Provence-Alpes-Côte d'Azur (PACA), and Occitanie.

A station comparison tool is available within this project, enabling the comparison of various calculated metrics across different temporal and spatial scales (Mediterranean, region, coastal water body).

MEDTRIX also cultivates a community through a surveillance notebook distributed three to four times a year, along with a biennial symposium.

For further information : https://plateforme.medtrix.fr/

https://medtrix.fr/portfolio_page/piscis/

CHAPTER 07

Application and interpretation examples 07

Although eDNA-based methods offer increased analytical power, ecological interpretation of the obtained data is essential. This step ensures the accuracy, reliability and biological significance of the conclusions drawn from the analyses. While technological advancements have greatly improved the quantity and quality of available data, it is crucial to interpret them accurately and within a biologically and ecologically relevant context. In this chapter we describe some practical examples, with the aim to provide insights into interpretations based on eDNA analysis.

© Greg Lecoeur, WE ARE MEDITERRANEE

> “It is necessary to ensure the ecological validity of the obtained results.

An expert’s opinion

eDNA to develop biodiversity indicators

Alicia Dalongeville,
Research engineer, MARBEC

For further information: Dalongeville et al. 2022. Benchmarking eleven biodiversity indicators based on environmental DNA surveys: more diverse functional traits and evolutionary lineages inside marine reserves. Journal of Applied Ecology. https://doi.org/10.1111/1365-2664.14276

Coastal habitat degradation, resource overexploitation, biological invasions and climate change are causing rapid and worldwide marine biodiversity erosion, particularly in the Mediterranean, where human activities are pervasive. Marine Protected Areas (MPAs) are designated areas where human activities are regulated to stop this erosion. Fully protected zones (marine reserves), where all extraction and fishing activities are prohibited, are particularly effective in protecting fish biomass (total quantity). However, their effects on the ecological roles of species and trophic interactions within ecosystems remain unclear.

To address this issue, we analyzed 99 eDNA samples collected from both inside and outside 9 MPAs in the French Mediterranean, focusing on fully protected zones with similar habitats and depths. By sequencing the eDNA contained in these samples, we compiled a list of fish species present at each site and calculated 11 biodiversity indicators to further compare them inside and outside the MPAs.

Our analyses showed that species richness (total number of species) was unexpectedly higher outside the MPAs. This counterintuitive result is explained by an increase in the ratio of pelagic and demersal species (living in the water column) to benthic species (living on the seabed) inside the MPAs. Indeed, more demersal and pelagic species, which are typically targeted by fisheries, are found in MPAs, while cryptobenthic species, which have short life cycles and are resilient to disturbances, are more abundant in areas where fishing occurs. Our study also demonstrated that both functional diversity (ecological characteristics such as size, reproductive system and diet) and phylogenetic diversity (relatedness between species, reflecting unmeasured but evolutionarily conserved traits) were higher within the MPAs. This indicates that fish communities in fully protected zones exhibit more diverse ecological functions and genetic lineages, contributing to ecosystem balance, functioning and resilience. Conversely, in areas where fishing is allowed, the fish communities consist of closely related species with similar ecological characteristics.

The eDNA-based indicators developed in this study can serve as biodiversity monitoring tools, capable of highlighting ecosystem deterioration or recovery. Thus, eDNA offers a powerful means to diagnose the health status of marine biodiversity and to track its long-term evolution in a standardized and replicable manner.

	INDICATORS	EFFECT OF MPAs
FISH BIODIVERSITY	Species richness	-
	Functional diversity	↗
	Phylogenetic diversity	↗
FISH ECOLOGY	Ratio of demersal and pelagic species to benthic species	↗

CHAPTER 07

An expert's opinion

eDNA for benthic monitoring

Stefano Varrella
Department of Life & Environmental Sciences, Polytechnic University of Marche, Italy

Roberto Danovaro
Department of Life & Environmental Sciences, Polytechnic University of Marche, Italy

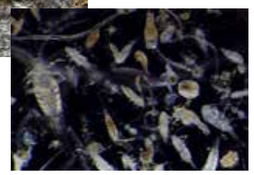
Cinzia Corinaldesi
Department of Life & Environmental Sciences, Polytechnic University of Marche, Italy

Anthropogenic pressures are increasingly threatening marine biodiversity and ecosystem functioning. There is thus an urgent need to develop tools for an accurate and rapid census of marine biodiversity, to detect changes in marine ecosystems at an early stage. eDNA is being implemented as a molecular tool for ecosystem assessments; it represents an innovative approach for studying biodiversity across a broad spectrum of taxa (from prokaryotes to large metazoans). This approach can be used at various spatial and temporal scales, addressing current knowledge gaps, and it can be applied to diverse compartments, including seawater and sediments. eDNA analysis is boosting biodiversity research in many ways: (i) it offers rapid biodiversity analysis that can be compared/associated with traditional taxonomic identifications; (ii) it involves non-destructive and non-invasive sampling approaches; (iii) it identifies the genetic signatures of rare, cryptic and larval species; (iv) it can be used to assess biodiversity shifts in relation to environmental changes; (v) it guarantees standardization and reproducibility of results; and (vi) it enhances the efficiency and cost-effectiveness of biodiversity analyses.

eDNA can be employed to detect single species through PCR amplification (e.g. qPCR or dPCR) using species-specific primers. Multiple taxa can be identified simultaneously through eDNA metabarcoding with various genetic markers (e.g. 16S rRNA, 18S rRNA), coupled with high throughput sequencing of the amplified genetic region. The key challenge often associated with metabarcoding is whether it can be employed as a stand-alone method in biodiversity studies.

Researchers at the Polytechnic University of Marche (UNIVPM) have addressed this issue in recent investigations by comparing morphological and molecular-based methods over time (in different seasons) and at large spatial scales. eDNA metabarcoding was found to outperform morphological analyses in terms of the number of identified taxa. Traditional taxonomic approaches are still needed for censuses of benthic macrofauna and meiofauna. However, when the focus is microeukaryotes, with a size between 0.2 and 20 µm, the alpha diversity detected with molecular approaches can be nearly double, as many of these microorganisms are difficult to isolate and identify with traditional approaches.

Due to several limitations (e.g. incompleteness of genetic databases and a lack of standardized bioinformatics procedures), **eDNA metabarcoding is still a complementary approach to traditional taxonomy. It is highly promising, however, and might facilitate the early identification of species (e.g. in their larval or early life stages) that have not yet reached a population size where their presence can be detected with traditional methods.**

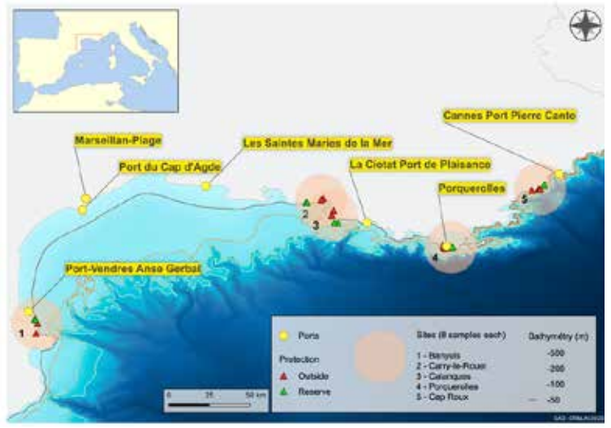


Above: coastal ecosystem, top-right: benthic ecosystem, bottom-right: plankton
© UNIVPM

An expert's opinion

eDNA to survey seaport biodiversity

Stéphanie Manel,
Stéphanie Manel, Director of studies at the Ecole Pratique des Hautes Etudes and head of the Biogéographie et Écologie des Vertébrés team at the Centre d'Écologie Fonctionnelle et Evolutive (CEFE)



Above: the port of Cannes
© David Mouillot
Right: Mediterranean port

Coastal marine areas are characterized by a great diversity of habitats that provide refuge for numerous species. These habitats are also essential nurseries for the settlement and growth of juveniles, especially those of commercially important fish species. This habitat and species diversity supports 90 % of exploited marine resources. However, it is threatened by increasing coastal artificialization, with one-third of the human population concentrated along the coastlines. Seaports in particular have replaced natural habitats and could negatively impact biodiversity, despite offering shelter from unfavorable conditions (e.g. waves, storms, temperatures), which may benefit certain species at specific life stages. Ecological engineering has recreated some of these habitats, and studies have demonstrated the effectiveness of these measures for juvenile fish. Understanding seaport biodiversity and the ecological role of artificial habitats is crucial for effective management of these anthropized ecosystems.

By sequencing 28 eDNA samples collected from 7 Mediterranean seaports and identifying species through sequences present in reference databases, we detected 122 fish species across the seaports, with an average of 65 species per site. Species richness was positively correlated with port surface area (e.g. high in the port of Cap d'Agde) and was higher in areas with rocky substrates (e.g. the port of La Ciotat) than in those with sandy substrates.

We compared these results with biodiversity data collected from 10 sites outside the seaports (including 5 samples collected in marine reserves before and during the strict lockdown of spring 2020). We identified 27 seaport-specific species, particularly small and less mobile species, such as gobies and blennies. The average number of species per sample in seaports was comparable to that in natural coastal environments outside the ports during lockdown and higher than in fished areas outside the lockdown period. However, variations in species composition among seaports were lower than those observed among natural environments, suggesting a homogenization of biodiversity within seaports.

High fish biodiversity in seaports may result from the use of artificial habitats by juvenile coastal species. Increased diversity homogenization between seaports could be explained by habitat redundancy within these areas (e.g. homogeneous substrate, shallow depth, lack of three-dimensional structuring, heavy boat traffic). This work on seaport biodiversity is ongoing as part of the BioDivMed project, supported by the Rhone Mediterranean Corsica Water Agency, and further results from additional sites will complement these initial findings.

For further information: Manel et al. 2024. Benchmarking fish biodiversity of seaports with eDNA and nearby marine reserves. Conservation Letters. https://doi.org/10.1111/conl.13001

CHAPTER 07

An expert's opinion

eDNA to study a biodiversity hotspot

Jean-Baptiste Juhel,
PhD, postdoctoral researcher at the University of Montpellier and independent consultant (octopusdatalab.com)

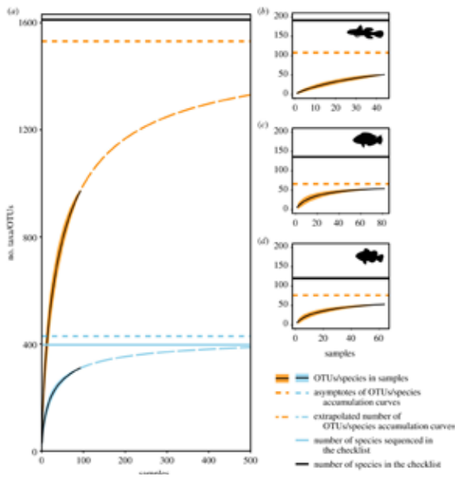


Figure 1.
Accumulation curves for assigned species (blue) and operational taxonomic units (OTUs, orange) obtained for the whole sample (a) and for the three most diverse families in the area: Gobiidae (b), Labridae (c) and Pomacentridae (d).

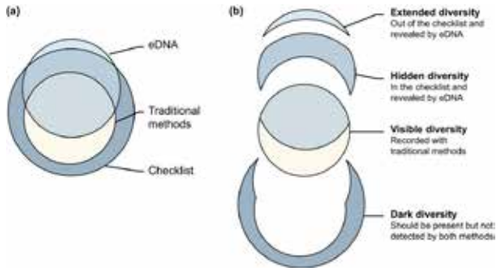


Figure 2.
Conceptual diagrams illustrating the diversity of visible, hidden, widespread and dark diversity in the context of a regional list of partially known species (a) and the description of each part of the species diversity (b).

Species surveys provide foundational data for ecology, biogeography and conservation. They are crucial for quantifying impacts of human activities and assessing conservation strategies. However, historical surveys are often incomplete, and some species remain undetected due to insufficient sampling effort, technical limitations of visual counts (e.g. diving depth), or specific functional traits (e.g. elusive behavior, small body size). eDNA metabarcoding has the potential to mitigate these biases by detecting species usually missed by traditional methods. Nevertheless, the application of this method to estimate biodiversity and complement traditional inventories needed to be evaluated and quantified.

The LENGGURU expedition in West Papua (2017, www.ird.fr), within the Coral Triangle, enabled eDNA sample collection from the world's most species-rich marine site. These data allowed a reassessment of fish species lists in the region and the development of two important analytical methods. The first method involves using accumulation curves to assess the detectability of different fish families and to determine the necessary sampling effort. This method has now become widely adopted and can be easily applied to any ecosystem. The second analytical method involves applying sampling theory to estimate the hidden diversity of species. This approach considers the occurrence of rare species through two different methods (here, eDNA and diving counts), to provide an estimate of the true species diversity within the area.

Through these two foundational studies, new modeling approaches have advanced the use of eDNA as an innovative and effective faunal survey tool.

© IRD - Régis Hocdé, Lengguru 2014

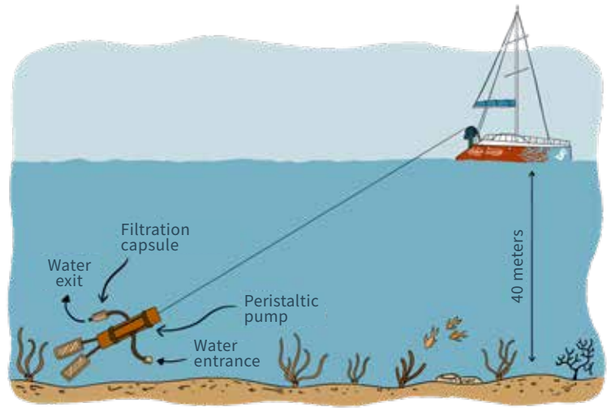
For further information: Juhel et al. 2020. Accumulation curves of environmental DNA sequences predict coastal fish diversity in the coral triangle. Proceedings of the Royal Society B. https://doi.org/10.1098/rspb.2020.0248

Juhel et al. 2022. Estimating the extended and hidden species diversity from environmental DNA in hyperdiverse regions. Ecography. https://doi.org/10.1111/ecog.06299

An expert's opinion

eDNA to detect rare species

Nadia Faure,
PhD student at Beauval Nature and the Centre d'Ecologie Fonctionnelle et Evolutive (CEFE)



© Aline et Nadia Faure

Angel shark © Laurent Ballesta, Andromède Océanologie

Studying rare marine species presents considerable challenges, due to the vast oceanic expanse and the difficulty of accessing deep waters. The emergence of innovative techniques, such as target species detection through eDNA, offers a promising solution by detecting elusive species through traces of their DNA in the aquatic environment.

We chose to use eDNA to detect the critically endangered angel shark *(Squatina squatina)* in Corsica. Frequently observed along the Mediterranean and Eastern Atlantic coasts until the early 20th century, the angel shark has become rare due to intense fishing. Given the benthic lifestyle and rarity of this elasmobranch species, eDNA-based analysis emerged as a more effective method than traditional approaches (e.g. diving counts, camera setups, trawling) for studying its current distribution.

eDNA samples were collected along 156 transects using a submersible peristaltic pump towed behind a catamaran, covering the entire Corsican coastline. These samples were analyzed in a laboratory using quantitative PCR (qPCR), which selectively amplifies target DNA. Specific primers were designed to target the mitochondrial DNA of three Mediterranean angel shark species *(S. squatina, S. oculata, S. aculeata)*, all of which are threatened with extinction. Even in small quantities, DNA could be detected through increased fluorescence associated with DNA amplification. Subsequently, the qPCR products were sequenced to identify the exact species among the three angel sharks.

The analyses revealed the presence of *S. squatina* at nine sites in Corsica, notably within the Marine Natural Park of Cape Corsica and Agriate and on the west coast of the island, where the species had never been observed before. Metabarcoding of the same samples confirmed S. squatina DNA at only four sites, highlighting the higher sensitivity of qPCR to low eDNA concentrations. Results were obtained in less than a week, enabling biologists to revisit these sites and tag two individuals for behavioral studies.

eDNA proved particularly effective for studying the distribution of a rare and difficult-to-observe species in its environment. This approach enables target species detection, facilitating early implementation of protection measures and raising awareness among stakeholders.

Barroil et al. 2023. PIAF: Poissons des fonds meubles, inventaire par ADN environnemental. Intermediate report. Project call "Renforcer la connaissance des habitats de fonds meubles en Méditerranée" co-financed by the Rhone Mediterranean Corsica Water Agency, the French Biodiversity Office, and the Corsican Environment Office. 42 pages.

Barroil et al. 2024. ANGE: Connaître et faire connaître le dernier refuge (Corse) de l'Ange de mer commun (Squatina squatina) en France. Final report. Funded by the Rhone Mediterranean Corsica Water Agency, the French Biodiversity Office, the Marine Natural Park of Cape Corsica and Agriate, and the Prince Albert II Foundation.187 pages.

For further information: Faure et al. 2023. An environmental DNA assay for the detection of Critically Endangered angel sharks (Squatina spp.). Aquatic Conservation: Marine and Freshwater Ecosystems. https://doi.org/10.1002/aqc.3954

CHAPTER 07

An expert's opinion

eDNA to assess the impact of anthropogenic pressures on marine biodiversity

David Mouillot,
Professor at the University of Montpellier, UMR MARBEC

Marine Protected Areas (MPAs), especially reserves that prohibit all fishing activities, are the main management tools to curb the erosion of marine biodiversity. However, they do not necessarily represent a reference state, as fishing pressure around their perimeters and noise pollution from tourism within their boundaries can affect their conservation effectiveness. As alternatives, isolated or near-pristine areas could provide a more appropriate reference point. However, such areas are virtually absent in coastal regions, including those in the Mediterranean Sea, which have been densely populated and exploited for millennia. As part of Vigilife, we aim to establish a baseline for marine biodiversity in order to assess conservation and restoration strategies in coastal areas.

In August 2023, we sampled the last relatively intact areas of the northwestern Mediterranean, in the Tyrrhenian Sea to the east of Corsica (Italy). The island of Monte Cristo is one of the few 'no-entry' marine reserves, where all human access is prohibited, from fishing to tourism. We conducted visual counts through scuba diving to estimate fish abundance, and we filtered eDNA to detect fish and crustacean biodiversity. These sites will remain central to our strategy in the coming years to better understand the long-term dynamics of coastal marine ecosystems exposed to minimal direct human influence but still affected by climate change. We will carefully assess the capacity of these last sanctuaries to serve as climate and anthropogenic refuges.

The suspension of most 'outdoor' human activities during the early stages of the COVID-19 pandemic constituted a unique 'natural experiment' to better understand the influence of human presence on wildlife and to evaluate the potential rebound of biodiversity in the case of reduced disturbances. Inaccessible beaches, banned tourism and decreased fishing led to a significant reduction of human pressures on the sea and coastline between March and June 2020. This unprecedented event provided a new baseline for assessing fish biodiversity loss and the capacity of MPAs to maintain this biodiversity in coastal ecosystems typically dominated by humans. By filtering eDNA inside and outside the reserves during this period and by comparing the results with those from previous years, we demonstrated a 30 % increase in biodiversity during the suspension of human activities in spring 2020. These findings suggest that reserves do not represent an absolute reference state and that human pressure, beyond fishing, erodes biodiversity more than previously expected.

Sampling conducted in the Monte Cristo Marine Nature Reserve

Monte Cristo Marine Nature Reserve © Ewann Tregaro

PART 04

04

Taking action locally

CHAPTER 08

Limitations and advantages of eDNA 08

Limitations

→ CONVENTIONAL CENSUS METHODS AND eDNA: COMMON LIMITATIONS

As for all existing traditional census methods, eDNA-based tools are prone to producing false positives and false negatives. These biases can arise from methodological choices that do not align with user needs or from inadequate practices leading to sample contamination and degradation. Indeed, each step of the process, from sampling to bioinformatic analysis, offers a wide range of methods that may or may not be optimized to meet the initial objectives (e.g. target taxonomic group, study site of interest, applications).
For example, false negatives may occur due to insufficient sampling effort or to an inappropriate sampling plan that fails to capture target species DNA. They can also result from DNA degradation if handling and storage precautions are not observed. Conversely, false positives may be caused by external contaminants introduced during sampling or analysis. Therefore, as with traditional survey methods, results from eDNA analyses may require validation by an ecological expert or verification using a complementary biodiversity census approach.
Moreover, neither traditional nor eDNA-based methods can currently identify hybrid species or detect genetic introgression within populations. These scenarios require comprehensive genetic analyses conducted on tissue samples from the organisms in question and validated by an expert, preferably a taxonomist.

© Greg Lecoeur, WE ARE MEDITERRANEE

→ BIOLOGICAL LIMITATIONS

eDNA analyses rely on the detection of DNA traces in the environment rather than on direct observations of organisms in their habitats. As a result, data on individual organisms, such as age, sex, size or health status, cannot be obtained. Similarly, an absolute quantification of the detected species cannot be achieved. The amounts of DNA produced and released by organisms vary both between taxonomic groups and between individuals, due to various biotic and abiotic factors (e.g. stress level, behavior, age, environmental temperature). However, there is a positive correlation between the biomass of all individuals of a given species in the sampled environment and the concentration of genetic material that is collected. Thus, eDNA analysis can, in some cases, serve as an indicator or proxy for species abundance.
It can be equally challenging to study consumed or farmed species, as their DNA traces can be widespread in the environment as a result of anthropogenic discharges (e.g. sewage treatment plants, aquaculture facilities). Beyond economically important species, certain taxa may be difficult to study because of an insufficient taxonomic resolution of the selected marker (e.g. the inability to distinguish genetically close species with standard primers) or incomplete genetic reference databases.

© Greg Lecoeur, WE ARE MEDITERRANEE

→ TECHNICAL LIMITATIONS

Due to the multiple steps performed in the laboratory, eDNA analyses take longer than traditional methods (see Chapter 3).
Accurate detection of specific species is critical for biodiversity surveys and monitoring. It is therefore essential to ensure that all equipment is free from contamination. Consequently, eDNA analyses require numerous single-use consumables, leading to waste accumulation. Future challenges will include reducing this waste and promoting a recycling industry to minimize the environmental impact of a tool designed to protect biodiversity.

Advantages

→ ECOLOGICAL ADVANTAGES

Despite some limitations, eDNA-based methods have proven highly effective in aquatic environments for simultaneously detecting species within various taxonomic groups using a single water sample. Thanks to these sensitive methods, it is now possible to study organisms that are difficult to observe using traditional methods because they are rare (very low density), small or only present in larval stages. eDNA-based methods are particularly valuable for species that are difficult to capture and that require laborious, costly, dangerous or invasive sampling. Additionally, eDNA-based analyses can serve as an important tool for the early detection of potentially invasive non-native species that are difficult to distinguish with the naked eye. This is especially crucial in a context where there is a shortage of expert naturalists and taxonomists compared with the biodiversity threats we are facing.

→ TECHNICAL ADVANTAGES

Beyond the comparatively high sensitivity of eDNA-based methods, these techniques are easy to implement and only require access permits to sampling sites, unlike other more invasive survey methods. They help reduce biases due to potentially unfavorable field conditions (observation conditions), errors associated with the observer's level of experience, and variability in survey efforts. Further, the personnel conducting the sampling can be trained in just a few hours. Field periods can be longer over the season and more flexible throughout the day. eDNA sampling is non-invasive and can be performed without physically touching the organisms, thereby minimizing potential stress and pathogen transfer. Consequently, it is possible to sample in areas where diving and fishing are prohibited, such as MPAs, or where there are safety concerns or high pollution levels (e.g. seaports, offshore wind farm sites). Thus, eDNA-based methods ensure greater safety for the involved personnel. Time and energy can be saved in the field, resulting in a cost-to-benefit ratio that is often more favorable than traditional census methods.

“ Beyond the comparatively high sensitivity of eDNA-based methods, these techniques are easy to implement.

© Greg Lecoeur, WE ARE MEDITERRANEE

CHAPTER 08

An expert's opinion

Advantages and limitations of eDNA tools compared with traditional methods

Pierre Boissery,
Coastal waters and Mediterranean coastline expert at the Rhone Mediterranean Corsica Water Agency

eDNA is a recently developed tool to serve biodiversity. As with any new tool, people's reactions vary from enthusiasm to skepticism. Enthusiasm is fueled by the new possibilities this analytical technique offers: surveys can be conducted rapidly, in complex environments, and without invasive or destructive methods, meaning that they can serve as a preliminary screening approach. eDNA is reassuring. It involves chemistry. It is often perceived as more precise than direct observations of living organisms. There is no longer a need to recognize all species on-site. No need to spend hours, sometimes hiding out of sight, hoping to compile the most exhaustive species lists. Collecting and analyzing water samples in the laboratory and comparing the obtained DNA sequences to a genetic reference database is enough to quickly identify species. Easy and reassuring for a manager of an aquatic environment, isn't it?
However, naturalists are much more reserved. Machinery and chemistry cannot replace expert knowledge. Some consider the method unreliable because reference databases are incomplete and because its deployment and analyses are costly. Ultimately, why use a questionable method instead of relying on much more competent naturalist experts? eDNA should not be prioritized. It should not compete with naturalists' expertise. There is some truth to this.
With some perspective, one can easily apply eDNA as a first screening tool, complementary to naturalists' expertise. This is especially true because, as in all chemistry, having a list of chemical molecules and concentrations does not constitute an environmental diagnosis. The machine can't do everything. Someone must interpret the results of the analysis, and this is the role of naturalists. In these terms, eDNA is a new and complementary tool that can be easily deployed, perhaps as a preliminary step before more extensive fieldwork. Especially as eDNA is currently limited to qualitative assessments. It does not provide information on size classes or numbers of individuals, whereas the human eye can count.
eDNA is a complementary tool to naturalists' expertise. Let's take a few examples. A good specialist in Mediterranean fish can easily recognize an average of 60 species while diving. The Mediterranean hosts around 17,000 fish species. The current genetic reference database contains eDNA signatures of 600 fish species. If I combine my diver's expertise with my eDNA analyses, my fish survey will undoubtedly be more comprehensive, especially as a diver cannot remain indefinitely underwater or explore deep-sea bottoms. For deep-sea exploration, fishing is always an option. This technique is somewhat effective, but it is becoming increasingly less sensible to destroy nature to study it. Indeed, fish surveying using eDNA technology has a cost, but it may not be higher than that of trawling at sea or surveying with a diving team. Ultimately, sampling water in the sea remains a simple manipulation, achievable by a technician.
eDNA actually highlights the need for naturalists. Indeed, with the multitude of inventories that can be easily conducted, the need for naturalist experts will become greater. It is undoubtedly an opportunity for this expertise, which has been somewhat in the background in recent years, to return to the forefront by training new specialists capable of using eDNA technologies. Today, we cannot afford to miss out on a complementary tool that offers promising performance and a wealth of prospects to improve our assessments and, ultimately, our ability to define corrective measures.

© Greg Lecoeur, WE ARE MEDITERRANEE

© Aurélie Lacoeuilhe

> "eDNA is a complementary tool to naturalists' expertise."

© Greg Lecoeur, WE ARE MEDITERRANEE

CHAPTER 09

Understanding to better protect 09

eDNA-based technologies are unlocking new horizons in our understanding of life. These innovations provide unparalleled and reliable insights that are crucial for societal decision-making and actions. They offer unprecedented perspectives across temporal and spatial scales, spanning the entire spectrum of living organisms and enabling exploration across all branches of the phylogenetic tree. Thus, it is important to translate this knowledge into tangible actions by aligning users' needs with appropriate scientific responses.

An expert's opinion ↙

eDNA for the census of non-indigenous (alien) species

Stefano Varrella
Department of Life & Environmental Sciences, Polytechnic University of Marche, Italy

Roberto Danovaro
Department of Life & Environmental Sciences, Polytechnic University of Marche, Italy

Cinzia Corinaldesi
Department of Life & Environmental Sciences, Polytechnic University of Marche, Italy

The globalization of maritime transportation and trade promote the proliferation of non-indigenous species (NIS). Certain NIS can settle in a new area and become invasive, leading to negative consequences for local biodiversity and ecosystem functioning. Most NIS can be introduced by shipping, and commercial and touristic ports therefore represent the checkpoint for the introduction of NIS into new marine environments and their diffusion into the surrounding areas. There is increasing awareness of the importance of the timely detection and identification of early developmental stages of NIS, which are sometimes difficult for taxonomic experts to recognize, because the likelihood of eradicating NIS is minimal once they become established.

The Polytechnic University of Marche (UNIVPM) and the Italian Institute for Environmental Protection and Research (ISPRA) have developed new protocols for NIS detection through multimarker eDNA metabarcoding of seawater and sediment samples collected in various Mediterranean ports on a seasonal basis. These results are then compared with those from traditional taxonomic approaches. This protocol is based on a standardized procedure, from sampling to bioinformatic analysis, and it has been shown to be reliable for detecting marine species. Overall, eDNA metabarcoding using multiple molecular markers (i.e. 18S rRNA, COI and rbcL) detected the presence of twice as many species as morphological analyses, while only a few species (around 5 %) were detected with both approaches. Narrowing the comparison to results obtained with the two approaches for NIS identification, the percentage of commonly detected species only increased to 8 %. More than half of the NIS were exclusively identified through eDNA metabarcoding, including some new taxa never before detected in Italian coastal environments and others never reported in the entire Mediterranean Basin. eDNA metabarcoding was a highly sensitive tool, also for identifying the presence in seawater of genetic signatures of NIS of both hard and soft substrates during their breeding season. However, to have a detailed picture of the NIS present in hotspot areas, an integration of morphological and molecular approaches is required. The combination of high-tech and traditional approaches is essential for promptly detecting and mapping NIS on a large scale. This integration is crucial within national and international directives, as it facilitates the implementation of more effective prevention strategies while minimizing ecological impacts.

CHAPTER 09

An expert's opinion

Managing protected natural areas

Madeleine Cancemi,
General Director of the Marine Natural Park of Cape Corsica and Agriate

Island of Giraglia - réserve naturelle des îles du Cap © Eric Volto

Angel shark © Laurent Ballesta, Andromède Océalonogie

Since its establishment in 2019, the Marine Natural Park of Cape Corsica and Agriate, overseen by the French Office for Biodiversity (OFB), has been dedicated to marine environment protection and sustainable maritime activities. Grounded in a deep understanding of ecosystems, the Park conducts awareness-raising actions targeting sea users.

From the outset, we turned to eDNA-based methods to detect deep and cryptic species, which are challenging to observe while diving. An initial study, conducted between 2019 and 2020, highlighted refuge zones that support a diverse array of elasmobranch species, including critically endangered ones such as the blonde ray and the eagle ray. These analyses also led to the detection of an invasive species previously unknown in the region, the Senegalese sole.

Building on this initial experience, we conducted a second study within the Park and beyond (in the Eastern Plain), using a species-specific approach to target the angel shark, a critically endangered shark species that has disappeared from French continental waters. The results demonstrated a positive correlation between the presence of this emblematic species and the reproductive behavior of picarel fish. This highlighted picarel spawning grounds as potential feeding sites for angel sharks, thus revealing the existence of crucial refuge zones in Corsican waters.

Our efforts extend beyond scientific research to practical and educational dimensions, with the aim to raise awareness among fishermen and other sea users about the importance of these species and to advocate for responsible fishing practices to minimize accidental captures and promote bycatch release.

Looking ahead, the Park plans to continue its research by conducting deep-sea sampling (1500 m depth) to confirm the presence of another rare species, Cuvier's beaked whale, for which traces of feeding activity have been observed at submarine mounts. Concurrently, we are initiating a project to assess the genetic status of the resident population of bottlenose dolphins in the Park. Filtrations will be carried out in the wake of identified individuals to conduct haplotypic DNA analyses from the collected eDNA. Finally, the application of eDNA-based techniques holds promise for managing and preserving marine biodiversity. These methods could enable the precise localization of species of interest that are subject to prefectural decrees, such as groupers and corbs.

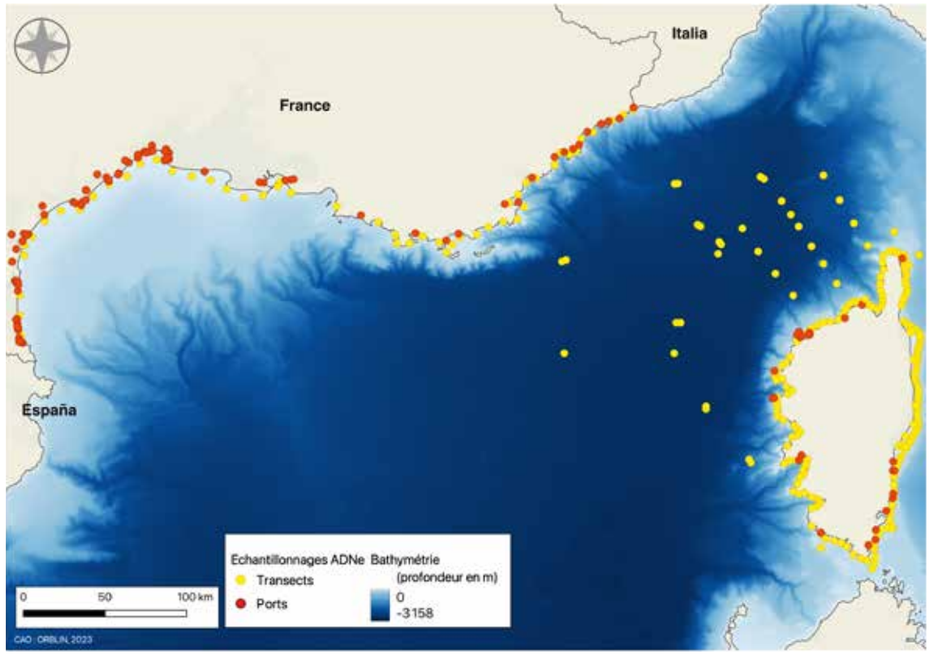
An expert's opinion

Mapping Mediterranean biodiversity – BioDivMed

Pierre Boissery,
Coastal waters and Mediterranean coastline expert at the Rhone Mediterranean Corsica Water Agency

Oceano Scientific team collecting eDNA aboard the Vanguard-Suzuki vessel from the LOVE THE OCEAN catamaran, which is equipped with the filter pump and the base developed by Yvan Griboval.

Characterizing coastal water biodiversity is a key ambition for the Rhone Mediterranean Corsica Water Agency. Until now, this ambition has only been realized through annual regional studies, which have resulted in a complete assessment of the entire coastline only every three years, with environmental conditions changing from year to year. Field investigations are logistically challenging, both in terms of material and human resources, and require, for instance, long periods of underwater diving for in situ data acquisition. The use of eDNA as a screening tool for biodiversity, combined with effective coordination of three oceanographic campaigns during the BioDivMed 2023 operation, enabled a comprehensive assessment of all French Mediterranean coastal waters with just two months of sampling. Thus, for the first time, a complete and 'instantaneous' image of coastal biodiversity will be established. By 2024, we will have an unprecedentedly comprehensive assessment of marine life, thanks to the 700 sampling stations operated by the three partners Andromède Océanologie, We are Méditerranée, and OceanoScientific. The analyses conducted by SPYGEN and the interpretation carried out by the University of Montpellier will establish a globally important baseline for characterizing the marine biodiversity of a Mediterranean country.



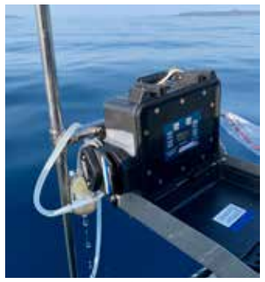
eDNA samples collected along transects are displayed in yellow. eDNA samples collected in seaports are shown in red. Bathymetry (depth in m) is visualized with a blue gradient (from light blue for shallow areas to dark blue for deep areas)

An expert's opinion

Yvan Griboval,
CEO of OceanoScientific and SAS LOVE THE OCEAN

Presentation to Professor David Mouillot (left) of the 104 samples of eDNA collected by the OceanoScientific team (four members shown here) under the direction of Yvan Griboval (second from left). © OceanoScientific

eDNA sampling at Cap de Saint-Tropez: biologist Léni Guillotin puts the inlet filter in the water and Yvan Griboval drives the Vanguard-Suzuki. © OceanoScientific



Pump operating on the base developed by OceanoScientific to guarantee the same collection quality in all situations. © OceanoScientific

eDNA sampling along the Mediterranean coast were primarily carried out at daybreak, to take advantage of flat seas. © OceanoScientific

"How can we preserve something we don't know about in detail?" When Pierre Boissery (Rhone Mediterranean Corsica Water Agency) and Professor David Mouillot (MARBEC, University of Montpellier) explained the advantages of using eDNA for a better scientific understanding of fish and crustacean species, I realized the importance of collaborating on the project that would become the BioDivMed mission. The more scientifically precise our knowledge of nature becomes, the more its conservation and preservation become possible, enabling us to move from wishful thinking to engaging in a concrete process with guaranteed results.

Our first action, even before collecting 104 samples from Menton to Gruissan—which involved sailing our catamaran LOVE THE OCEAN without CO2 emissions across 52 stations over 23 days in July 2023—was to design a sampling device to duplicate sample collections with maximum rigor. The experience I have gained since 2006 with eminent scientists—especially during our solo sailing trip around the world, during which we conducted an unprecedented 60-day campaign under the 40th parallel south, under the three great continental capes (Cape of Good Hope, Cape of Leeuwin and Cape Horn)—has taught me that the quality of a scientific study depends foremost on the rigor applied to sample collection.

Therefore, we designed and developed a device that facilitates precise sample collection, i.e. always at the same depth and reducing sample contamination risk by rigorously controlling the use of the inlet filter that collects seawater. At the end of the BioDivMed 2023 mission, we improved this equipment. It is now ready for us to conduct 'BioDiv missions' along any coastline.

The most important reason for our commitment is the opportunity to participate in scientific research, not only to promote nature conservation, specifically marine biodiversity, but also to work within the BioDivMed consortium for the benefit of humanity. Once marine biodiversity sentinel sites (SSBM) are in place, scientific knowledge of biodiversity will allow us to quantify each identified species. We will then concretely promote "sustainable coastal fishing for sustainable food with short supply chains".

An expert's opinion

Greg Lecoeur,
Photographer,
CEO of We Are Méditerranée

As a naturalist photographer specializing in the underwater world, I have always dreamed of bridging the gap between photography and the scientific world. The association We are Méditerranée was created to bring together a community of marine experts and enthusiasts to achieve environmental conservation, particularly conservation of the Mare Nostrum. This association combines photographic vision, a scientific approach, and an educational dimension to accomplish four main missions: explore, study, protect and raise awareness.
Our commitment is reflected in our participation in the BioDivMed project through the Pelagos Expedition. This initiative combines naturalist expeditions with scientific missions to deepen the knowledge and protection of the Pelagos sanctuary, which is dedicated to preserving marine mammals and their habitats.

In 2023, we crossed the Mediterranean Sea during May and June, collecting 25 samples to perform eDNA-based multispecies analysis. eDNA is a non-invasive, innovative and revolutionary tool that allows us to uncover the mysteries of biodiversity without disturbing ecosystems. While this technology can still be improved, especially in detecting genetically similar dolphin species, it already facilitates the detection of species that are often difficult to observe, such as the monk seal or the great white shark, and it simplifies ecological monitoring.

Beyond its increasingly recognized scientific value, eDNA is also a powerful communication and awareness tool. As demonstrated by the BioDivMed project, eDNA dramatically changes the spatial scale of biodiversity studies. The scope of the expeditions attracts interest and curiosity from a wide audience, allowing us to reach many people.

The photographic work, coupled with results of the scientific expeditions, produces striking visuals and conveys strong, understandable and impactful messages to the general public.

CHAPTER 09

An expert's opinion ↙

Surveillance

Florian Holon,
General Director of Andromède Océanologie

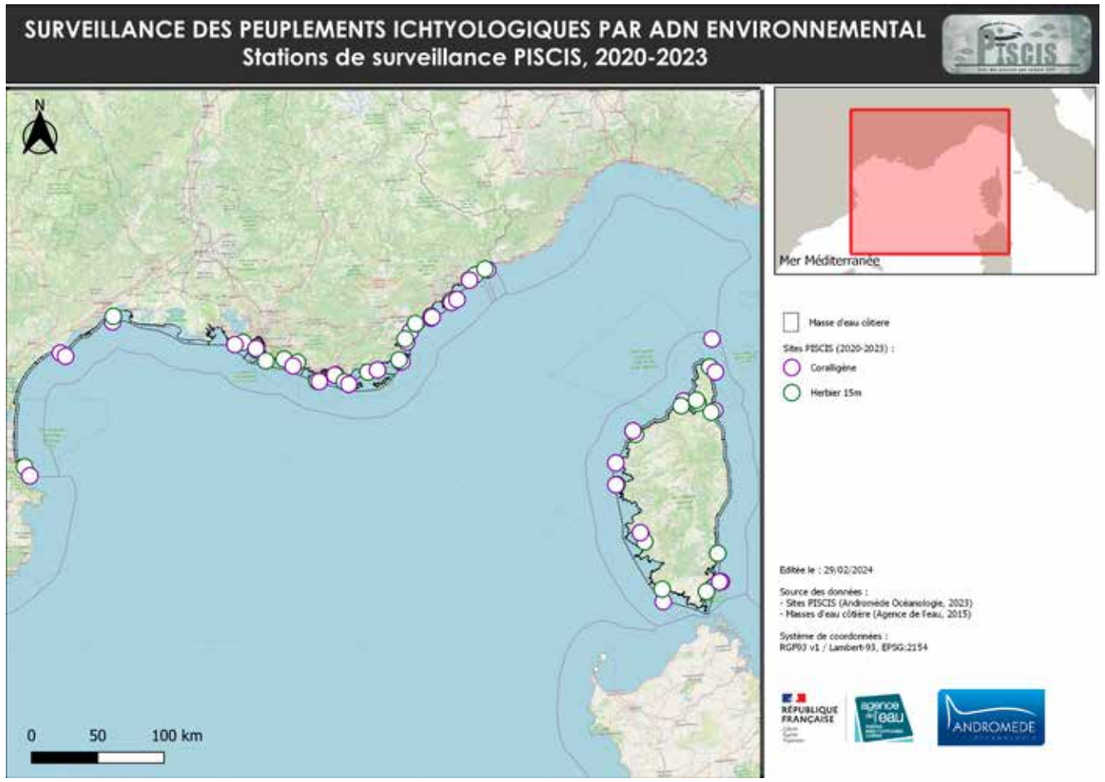
Herbier © Andromède Océanologie

Andromède Océanologie uses eDNA for its professional activities and works on improving this technique and the associated sampling methods. In 2016, Andromède Océanologie partnered with the University of Montpellier to create the joint laboratory InToSea. Within the framework of this joint laboratory, a prototype of a waterproof and submersible pump was developed to directly sample eDNA up to 150 m below the surface. This prototype and its improved versions have been used annually in the French Mediterranean for various biological surveillance projects (PISCIS), explorations (Gombessa 5 and 6 scientific expeditions), and scientific research (e.g. ANGE and PIAF projects), with each protocol being optimized according to the specific scientific questions: static deep sampling, deep transects over several kilometers, eDNA/camera coupling, development of a specific barcode for the angel shark, etc. In 2021, a new prototype dedicated to deep-sea environments was developed, leading to several hundred filtrations being conducted from the surface to 1500 m deep.

Using eDNA for coastal water surveillance has been one of our most notable experiences. Supported by the Water Agency, the PISCIS network has been operational since 2015 to characterize fish assemblages. Each year, regional campaigns covering the entire French Mediterranean coastline across three regions are conducted between mid-May and late June. From 2015 to 2019, we evaluated fish assemblages by video acquisition with 360° cameras. Since 2020, we have been using eDNA-based methods. In 2023, we sampled 75 sites across 3 regions of the French Mediterranean. Among these sites, 37 corresponded to Posidonia seagrass habitats (TEMPO sites) and 38 to coral reef habitats (RECOR sites). Under the biological surveillance contract for coastal waters of the Rhone Mediterranean Corsica Water Agency, eDNA-based fish population evaluations will continue in 2024, 2025 and 2026 for 58 PISCIS sites.

eDNA samples are collected by filtering 30 L of water through a filtration capsule with a 0.2 µm pore size. For each surveillance site, two VigiDNA ® kits are used (resulting in two samples per site). Samples are collected close to the seabed using a waterproof submersible pump. Extensive descriptions of these methods are available on the MEDTRIX platform as part of the PISCIS project.

The MEDTRIX platform for monitoring coastal waters and ecosystems in the Mediterranean (www.medtrix.fr) hosts and disseminates all results obtained within the PISCIS network. Beyond the 174 fish species detected so far by eDNA, 8 descriptors and diversity indices have been computed: species richness, functional diversity, the large reef fish indicator (LRFI), the cryptobenthic indicator, the thermal indicator, the non-native species indicator, the IUCN indicator, and the ratio of demersal/pelagic to benthic species. These indicators are computed at different scales: site (combining two habitats), site-habitat (habitat for each site) and habitat (all sites combined). For most indicators, results show that seagrass sites at a depth of 15 m exhibit similar or slightly higher values than coral reef habitats. Thus, species richness, functional diversity and LRFI are generally higher at seagrass sites. Since 2020, the PISCIS network has detected rare species and those with a high conservation status (IUCN). Numerous chondrichthyan species have been inventoried: thresher shark *(Alopias vulpinus)* in 2021, common smooth-hound *(Mustelus mustelus)* in 2022, and white skate *(Rostroraja alba)* in 2023, all three endangered, as well as the critically endangered eagle ray *(Aetomylaeus bovinus)* in 2023. Emblematic Mediterranean species have also been inventoried, such as the dusky grouper *(Epinephelus marginatus)*, the brown meagre *(Sciaena umbra)*, and the angel shark *(Squatina squatina)*.

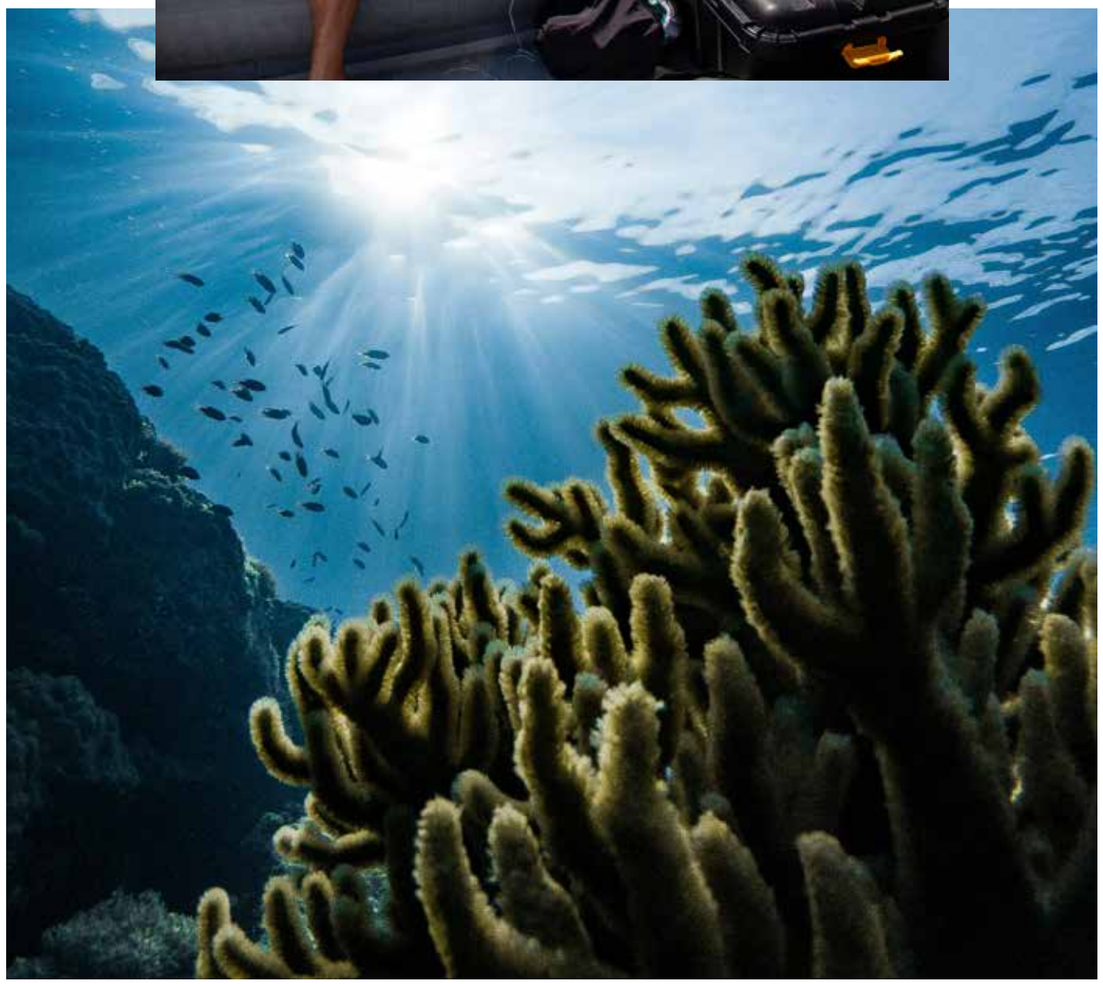


Surveillance of ichthyological assemblages with environmental DNA. PISCIS surveillance stations, 2020–2023.

Coralligenous habitat © Andromède Océanologie

All reports and associated publications are available of the PISCIS project page.
https://medtrix.fr/portfolio_page/piscis/

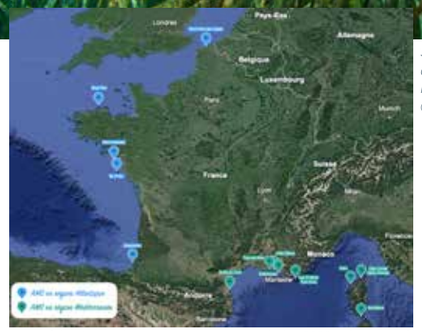
Sampling carried out as part of the Sentinel Marine Areas program © David Mouillot



An expert's opinion

The Vigilife program Sentinel Marine Areas

David Mouillot,
Professor at the University of Montpellier, UMR MARBEC



Sampling carried out as part of the Sentinel Marine Areas program © David Mouillot

Map of the Sentinel Marine Area sites sampled in 2023. Atlantic and Mediterranean SMAs are marked in blue and green, respectively. © David Mouillot

The Sentinel Marine Areas program, co-financed by Electricité de France (EDF) and the Rhone Mediterranean Corsica Water Agency, has set three goals to address the knowledge gap in current marine biodiversity monitoring: (1) conducting near-exhaustive and standardized inventories across numerous taxonomic groups (bony and cartilaginous fish, crustaceans and marine mammals), (2) embedding these inventories in a long-term strategy to assess the impacts of global changes (climatic and anthropogenic), and (3) involving local partners in the collection, analysis, interpretation and promotion of the data.

A Sentinel Marine Area (SMA) must therefore be of interest for monitoring and/or conserving marine species. It consists of several sites; some are 'treated' sites that have undergone human intervention, whether positive (restoration or protection) or negative (impactful or disruptive), and others are 'control' or 'reference' sites that have not experienced such human intervention but are comparable to the treated sites (same habitat or environment). Some SMAs include marine reserves and their nearby surroundings, while others are offshore wind farms (e.g. St. Nazaire), which are considered interventions in an already anthropized natural environment. At this stage, it is unclear whether these interventions have a negative ecological impact (on habitat) or a positive one (by reducing fishing pressure). Finally, restoration actions, such as the establishment of artificial reefs, will also be monitored using the SMA approach.

The program began in 2023, with around 15 SMAs strategically placed from the English Channel to Corsica, covering the French metropolitan coasts. The aim is to provide early signals of the arrival of non-native species and to assess the local dynamics of threatened species (extirpation or recolonization). By involving local partners, such as wind farm or marine reserve managers, the program is also intended to unite a community of stakeholders who will eventually be able to conduct their eDNA sampling independently, share their data via a mapping platform, and contribute to the development of relevant indicators of marine ecosystem health.
This SMA program is designed to expand to sites outside metropolitan France and abroad, to extend this long-term monitoring of coastal waters with standardized multitaxa protocols.

For further information:
https://www.vigilife.org/nos-programmes/

PART 04

Conclusions and prospects

Conclusions

eDNA analyses involve collecting and identifying traces of DNA left by organisms in their environment. Since 2008, this innovative method, which complements conventional biodiversity survey methods, has proven effective in the field of conservation biology, particularly for conducting inventories and monitoring biodiversity. However, in aquatic environments, and especially in marine environments, eDNA concentrations tend to be very low in the water. They can even be minimal for species with a low abundance (e.g. threatened, cryptic). Species detection probability depends on the likelihood of collecting their DNA in the environment, the preservation of this DNA during the analytical process, and the absence of sample contamination. Therefore, when the user's objective is to obtain the most comprehensive species list while minimizing the risks of false negatives and false positives, it is crucial to choose sampling and analysis methods that adhere to the highest precaution levels possible. Vigilife relies on eDNA-based methods, which have been developed with this mind, to build a collaborative approach in favor of biodiversity. The purpose of this guide was to provide a simplified synthesis of commonly used methods, as well as operational instructions for implementing Vigilife methods. It is intended to be regularly updated, in line with technological improvements and scientific research advancements, which, given the increasing importance of eDNA analysis in biodiversity studies, are likely to be numerous in the coming years.

> "It is crucial to choose sampling and analysis methods that adhere to the highest precaution levels possible.

Flabellina affinis © Laurent Ballesta, Andromède Océanologie

Prospects

→ TECHNICAL PROSPECTS

• DETECTING NEW TAXA

eDNA analyses offer the considerable advantage of detecting a wide range of organisms, from bacteria to large mammals, including plants. Thus, these innovative approaches open new perspectives for studying taxonomic groups that have been underrepresented or even overlooked by traditional survey methods. However, the literature reveals disparities in taxa targeted by molecular biology techniques in marine environments. Indeed, while some groups, such as fish and mammals, are well studied, other crucial organisms, such as corals and algae, are considered less often[14]. Developing sampling methods, along with creating new primers and genetic reference databases tailored to these species, could provide essential complementary data, leading to a better understanding of biodiversity and ecosystem functioning.

Sea urchin © Laurent Ballesta, Andromède Océanologie

• OBTAINING QUANTITATIVE DATA

Although eDNA analysis is not suitable for characterizing population status or providing information on individual organisms, it does offer promising prospects for estimating species biomass. Indeed, given the positive correlation between eDNA concentration and species abundance in the environement[29], eDNA-based methods can determine relative organism abundance. A recent study, conducted on fish populations, involved combining eDNA metabarcoding with total eDNA quantification using the same primers. Results suggest that this approach could enable an approximate estimation of fish absolute abundance[30].

• EXPLORING NEW ENVIRONMENTS

New sampling methods conducted by autonomous underwater vehicles (AUVs) are currently being tested by Vigilife partners. These technological advancements could enhance, both spatially and temporally, the scalability of eDNA analyses for biodiversity studies.

• ANALYZING ENVIRONMENTAL RNA

Beyond eDNA, environmental RNA (eRNA) is increasingly being considered for biodiversity analyses. Analyzing RNA molecules in ecosystems offers interesting prospects for developing functional genetic markers and assessing biosafety risks (e.g. for pathogen detection)[31,32]. However, RNA degrades faster and has a shorter lifespan in the environment than DNA, which implies additional constraints for sample conservation and analyses (e.g. maintaining the cold chain, working with ice blocks in the laboratory).

→ APPLICATION PROSPECTS

Initiatives such as BioDivMed and Sentinel Marine Areas (see Chapter 9) illustrate perfectly the potential of eDNA analyses to carry out large-scale biodiversity conservation projects. This potential was further strengthened by the recent unveiling of the French national biodiversity strategy in November 2023. This strategy includes the implementation of a comprehensive national inventory to survey the biodiversity of the territory, using eDNA-based methods among other techniques.

An expert's opinion

eDNA and artificial intelligence

Letizia Lamperti,
PhD student at the Ecole Pratique des Hautes Etudes

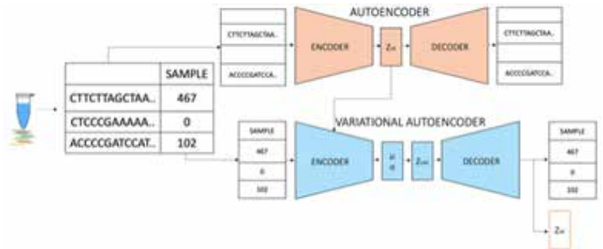


Figure 1.
Diagram of the variational autoencoder (VAE) method applied to data derived from eDNA.

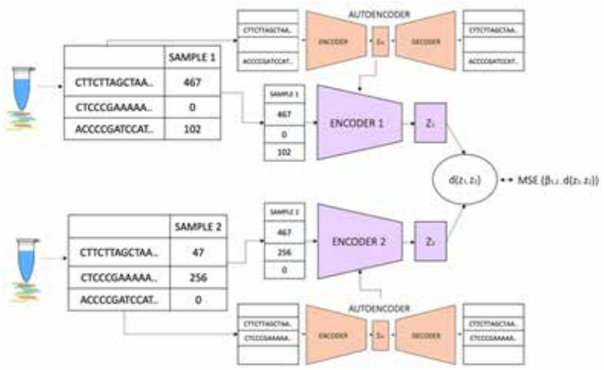


Figure 2.
Diagram of the deep metric learning (DML) method applied to data derived from eDNA.

eDNA has become a crucial resource for understanding marine biodiversity and terrestrial ecosystems. However, eDNA analyses generate considerable amounts of complex and multidimensional data, requiring innovative approaches to extract relevant information. In this context, artificial intelligence (AI) plays an essential role by offering advanced methods to analyze and interpret eDNA data.

Deep neural networks, a form of AI, have emerged as a promising solution to organize and visualize eDNA samples in a reduced two-dimensional space. By combining different neural network architectures, these methods provide an accurate representation of various biodiversity indicators, thus enhancing the ecological interpretation of eDNA data.

eDNA metabarcoding offers an effective method for monitoring biodiversity in various ecosystems. This technique involves retrieving and analyzing DNA naturally excreted by organisms in their environment, providing valuable taxonomic, functional and phylogenetic information. However, eDNA metabarcoding produces a large amount of sequencing data, requiring dimensionality reduction to extract relevant features. Traditional dimensionality reduction techniques, such as principal component analysis (PCA), are not always suited to the complexity of eDNA data.

To address this challenge, we have developed two new deep-learning-based methods (variational autoencoder VAE and deep metric learning DML) that combine different types of neural networks to organize eDNA samples and visualize ecosystem properties in a two-dimensional space. The strength of our new methods lies in the combination of two inputs: the number of sequences found for each detected MOTU and their corresponding nucleotide sequence.

Using three different datasets, we demonstrated that our methods accurately represent several biodiversity indicators in a two-dimensional latent space: MOTU richness per sample, α sequence diversity per sample, and Jaccard and sequence β diversity between samples. We showed that our nonlinear methods are better at extracting features from eDNA datasets while avoiding the major biases associated with eDNA analyses. Our methods outperformed traditional dimensionality reduction methods.

By using AI, it is possible to overcome challenges related to eDNA-derived data analysis, thus opening new perspectives for understanding and preserving ecosystem biodiversity. These advances could improve the ability to monitor ecosystems, to better understand their responses to environmental disturbances, and to design appropriate management and mitigation measures to preserve biodiversity.

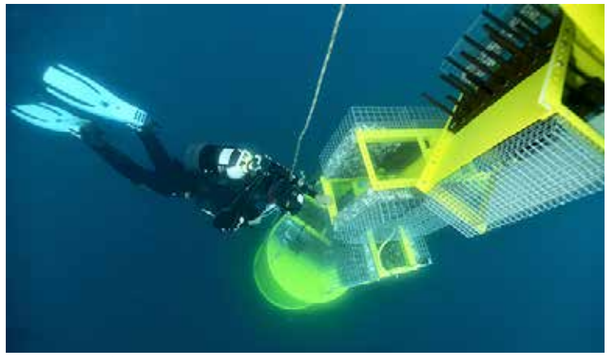
For further information: Lamperti et al. 2023. New deep learning-based methods for visualizing ecosystem properties using environmental DNA metabarcoding data. Molecular Ecology Resources. https://doi.org/10.1111/1755-0998.13861

An expert's opinion

eDNA in offshore environments and prospects for improvement

Gilles Lecaillon,
General Director at Ecocéan

Anaïs Gudefin
Scientific Leader at Ecocéan



BoB buoy © Ecocéan

BoB buoy © Ecocéan

OCGdata buoy © Ocergy

Knowledge about life in the open sea, especially regarding species that can settle on offshore structures, remains limited. We have used eDNA, alongside other commonly used tools such as visual and camera monitoring, in the context of research and development projects conducted on offshore floating buoys (BoB and OCG-Data buoys), located 15 and 30 km off the coast of Leucate in waters ranging in depth from 80 to 100 m. As these buoys are less accessible than coastal habitats, diving periods are limited, and cameras require both technical maintenance and logistical setup, it was of interest to supplement the list of species around and on the buoys by using a relatively simple sampling method.

Three eDNA surveys were conducted at different times on the buoys (one on BoB and two on OCG-Data) in the upper few meters of water. These surveys were jointly conducted with the University of Montpellier and SPYGEN. All eDNA results showed much larger species lists than any other monitoring method, thus successfully supplementing the species list around the buoys. This was especially the case for highly mobile species, such as pelagic fish, which are difficult to observe during diving.

However, eDNA results also revealed bottom-dwelling species, such as Uranoscopus scaber or mullets, and coastal species, such as swallowers. While it is possible that this results from the capture of eggs or larvae present in the water column, it still raises questions about the calibration of offshore sampling. Indeed, the issue of distance and depth of capture in these open and highly turbulent environments is substantial and must be addressed to make the best use of this tool. Additionally, some species known to be present on the buoys, such as blennies, were not detected by eDNA. To address this issue, filtration duration was increased in subsequent samplings, improving the detection of these more cryptic species.

Therefore, while this eDNA technique is relevant as a complement to more traditional monitoring methods, it still requires protocol adaptations in offshore environments to improve result interpretations.

Glossary

- **Amplicon:** DNA fragment amplified through polymerase chain reaction (PCR) or other derived methods.
- **Amplicon sequence variant (ASV):** Group of sequences consisting of a main sequence and several other similar but less abundant sequences, considered errors of the main sequence.
- **Autonomous underwater vehicle (AUV):** Autonomous underwater robot.
- **Barcode or marker region:** DNA region with a precise location in the genome (e.g. a gene or a gene portion).
- **Bioinformatic analyses:** Set of computer analyses performed on sequencing data derived from the analysis of eDNA extracted from the considered samples.
- **Contamination:** Introduction of exogenous DNA into a sample.
- **Control:** Sample whose genetic material is absent or known. A negative control is a sample without genetic material, used to check that the analysis is free from contamination. A positive control is a sample containing known genetic material, used to check that the analysis step (extraction or amplification) was carried out correctly.
- **Decontamination:** Steps to remove/clean DNA traces from sampling equipment or from surfaces that may come into contact with samples in the field or in the laboratory.
- **Digital PCR (dPCR):** Method derived from quantitative PCR (qPCR), where the reaction mixture is partitioned into thousands of microreactions.
- **Deoxyribonucleic Acid (DNA):** Universal molecule, common to all living beings but containing the genetic information specific to each individual.
- **DNA extraction:** Process of isolating DNA from environmental samples.
- **Environmental DNA (eDNA):** DNA that can be extracted from environmental samples (e.g. soil, water, air) without first isolating any target organisms[4].
- **False negative:** Failure to detect species that were present in the studied environment.
- **False positive:** Detection of species that were absent from the studied environment.
- **Filtration capsule:** Capsule enclosing a filtering membrane.
- **Genetic reference database:** Collection of DNA sequences originating from known organisms.
- **Index:** Short synthetic DNA sequence added to amplified target sequences and serving as a unique identifier for each library[15].
- **Library:** Set of amplicons (amplified target sequences) to be sequenced, with each sequence containing tags, adapters and indexes at their ends[26].
- **Multispecies approach or eDNA metabarcoding:** Simultaneous detection of multiple species belonging to the same taxonomic group[4].
- **Operational taxonomic unit (OTU):** Cluster of sequences that are identical to each other up to a specified threshold. The term molecular operational taxonomic unit (MOTU) is also used.
- **Polymerase chain reaction (PCR):** Method for amplifying a specific DNA fragment to generate a quantity that is sufficient for detection and analysis.
- **Primer:** Short synthetic DNA sequence that makes it possible to target a specific marker region and initiate PCR amplification (or other derived methods).
- **Quantitative PCR (qPCR):** PCR-derived method involving the measurement of a fluorescent signal when the targeted DNA fragment is amplified.
- **Replicates:** Samples collected at the same sampling site at the same time or analyzed under identical experimental conditions.
- **Read:** Sequence detected by the sequencer.
- **Sampling strategy:** Sampling design, which determines the number of samples to collect and their spatial and temporal distribution, as well as the methods and regulations to follow.
- **Sequence:** Succession of nucleotides.
- **Sequencing:** Determination of the sequence constituting a DNA fragment.
- **Sequencing adapter:** Short synthetic DNA sequence added to amplified target sequences to enable binding onto the sequencing flow cell[15].
- **Sequencing depth:** Total number of sequence reads expected per sample.
- **Sequencing library:** Set of amplicons (amplified sequences of interest) to be sequenced, with tags, sequencing adapters, and indexes at their ends[26].
- **Species-specific approach:** Detection of the presence of a target species by detecting a precise sequence of its DNA.
- **Tag:** Short synthetic DNA sequence added to amplified target sequences, serving as a unique identifier for each sample or PCR replicate[26].
- **Underwater visual census (UVC):** Underwater visual census carried out by divers.

Bibliography

1. Ogram *et al.* 1987. The extraction and purification of microbial DNA from sediments. Journal of Microbiological Methods. https://doi.org/10.1016/0167-7012(87)90025-X

2. Ficetola *et al.* 2008. Species detection using environmental DNA from water samples. Biology Letters. https://doi.org/10.1098/rsbl.2008.0118

3. Pawlowski *et al.* 2020. Utilisations de l'ADN environnemental pour la surveillance et l'évaluation biologiques des écosystèmes aquatiques. https://doi.org/10.5167/uzh-187800

4. Taberlet *et al.* 2012. Environmental DNA. Molecular Ecology. https://doi.org/10.1111/j.1365-294X.2012.05542.x

5. Pawlowski *et al.* 2020. Environmental DNA: What's behind the term? Clarifying the terminology and recommendations for its future use in biomonitoring. Molecular Ecology. https://doi.org/10.1111/mec.15643

6. Stewart. 2019. Understanding the effects of biotic and abiotic factors on sources of aquatic environmental DNA. Biodiversity and Conservation. https://doi.org/10.1007/s10531-019-01709-8

7. Barnes & Turner. 2015. The ecology of environmental DNA and implications for conservation genetics. Conservation Genetics. https://doi.org/10.1007/s10592-015-0775-4

8. Marques. 2020. Nécessité, potentiel et limitations de l'approche en Unités Taxonomiques Moléculaires pour analyser la biodiversité de l'ADN environnemental des poissons. https://ged.biu-montpellier.fr/florabium/jsp/nnt.jsp?nnt=2020MONTG039

9. Canals *et al.* 2021. Vertical stratification of environmental DNA in the open ocean captures ecological patterns and behavior of deep-sea fishes. Limnology and Oceanography Letters. https://doi.org/10.1002/lol2.10213

10. Harrison *et al.* 2019. Predicting the fate of eDNA in the environment and implications for studying biodiversity. Proceedings of the Royal Society B: Biological Sciences. https://doi.org/10.1098/rspb.2019.1409

11. Murakami *et al.* 2019. Dispersion and degradation of environmental DNA from caged fish in a marine environment. Fisheries Science. https://doi.org/10.1007/s12562-018-1282-6

12. Collins *et al.* 2018. Persistence of environmental DNA in marine systems. Communications Biology. https://doi.org/10.1038/s42003-018-0192-6

13. Scriver *et al.* 2023. Harnessing decay rates for coastal marine biosecurity applications: A review of environmental DNA and RNA fate. Environmental DNA. https://doi.org/10.1002/edn3.405

14. Takahashi *et al.* 2023. Aquatic environmental DNA: A review of the macro-organismal biomonitoring revolution. Science of The Total Environment. https://doi.org/10.1016/j.scitotenv.2023.162322

15. Bruce *et al.* 2021. A practical guide to DNA-based methods for biodiversity assessment. Pensoft. https://doi.org/10.3897/ab.e68634

16. McClenaghan *et al.* 2020. Harnessing the power of eDNA metabarcoding for the detection of deep-sea fishes. PLoS One. https://doi.org/10.1371/journal.pone.0236540

17. Bessey *et al.* 2020. Maximizing fish detection with eDNA metabarcoding. Environmental DNA. https://doi.org/10.1002/edn3.74

18. Deter *et al.* 2023. eREF : État de référence de la biodiversité en Vertébrés dans les masses d'eaux côtières méditerranéennes à partir d'ADN environnemental. Rapport final. 68 pages et annexes. https://medtrix.fr/wp-content/uploads/2023/04/eREF-rapport2023_VF.pdf

19. Ali *et al.* 2017. Current nucleic acid extraction methods and their implications to point-of-care diagnostics. Biomed Research International. https://doi.org/10.1155/2017/9306564

20. Purcell *et al.* 2016. Comparison of standard, quantitative and digital PCR in the detection of enterotoxigenic Bacteroides fragilis. Scientific Reports. https://doi.org/10.1038/srep34554

21. Ficetola *et al.* 2015. Replication levels, false presences and the estimation of the presence/absence from eDNA metabarcoding data. Molecular Ecology Resources. https://doi.org/10.1111/1755-0998.12338

22. Biggs *et al.* 2015. Using eDNA to develop a national citizen science-based monitoring programme for the great crested newt (Triturus cristatus). Biological Conservation. http://dx.doi.org/10.1016/j.biocon.2014.11.029

23. Herder *et al.* 2014. Environmental DNA a review of the possible applications for the detection of (invasive) species. http://dx.doi.org/10.13140/RG.2.1.4002.1208

24. Thalinger *et al.* 2021. A validation scale to determine the readiness of environmental DNA assays for routine species monitoring. Environmental DNA. https://doi.org/10.1002/edn3.189

25. Taberlet *et al.* 2018. Environmental DNA : For biodiversity research and monitoring. Oxford University PressOxford. https://doi.org/10.1093/oso/9780198767220.001.0001

26. Bohman *et al.* 2021. Strategies for sample labelling and library preparation in DNA metabarcoding studies. Molecular Ecology Resources. https://doi.org/10.1111/1755-0998.13512

27. Piper *et al.* 2019. Prospects and challenges of implementing DNA metabarcoding for high-throughput insect surveillance. Gigascience. https://doi.org/10.1093/gigascience/giz092

28. Antich *et al.* 2021. To denoise or to cluster, that is not the question: optimizing pipelines for COI metabarcoding and metaphylogeography. BMC Bioinformatics. https://doi.org/10.1186/s12859-021-04115-6

29. Rourke *et al.* 2022. Environmental DNA (eDNA) as a tool for assessing fish biomass: A review of approaches and future considerations for resource surveys. Environmental DNA. https://doi.org/10.1002/edn3.185

30. Pont *et al.* 2022. Quantitative monitoring of diverse fish communities on a large scale combining eDNA metabarcoding and qPCR.Molecular Ecology Resources. https://doi.org/10.1111/1755-0998.13715

31. Cristescu. 2019. Can Environmental RNA Revolutionize Biodiversity Science? Trends in Ecology & Evolution. https://doi.org/10.1016/j.tree.2019.05.003

32. Miyata *et al.* 2022. Comparative environmental RNA and DNA metabarcoding analysis of river algae and arthropods for ecological surveys and water quality assessment. Scientific Reports. https://doi.org/10.1038/s41598-022-23888-1


vigilife