

Review

Evaluating eDNA for Use within Marine Environmental Impact Assessments

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Abstract: In this review, the use of environmental DNA (eDNA) within Environmental Impact Assessment (EIA) is evaluated. EIA documents provide information required by regulators to evaluate the potential impact of a development project. Currently eDNA is being incorporated into biodiversity assessments as a complementary method for detecting rare, endangered or invasive species. However, questions have been raised regarding the maturity of the field and the suitability of eDNA information as evidence for EIA. Several key issues are identified for eDNA information within a generic EIA framework for marine environments. First, it is challenging to define the sampling unit and optimal sampling strategy for eDNA with respect to the project area and potential impact receptor. Second, eDNA assay validation protocols are preliminary at this time. Third, there are statistical issues around the probability of obtaining both false positives (identification of taxa that are not present) and false negatives (non-detection of taxa that are present) in results. At a minimum, an EIA must quantify the uncertainty in presence/absence estimates by combining series of Bernoulli trials with *ad hoc* occupancy models. Finally, the fate and transport of DNA fragments is largely unknown in environmental systems. Shedding dynamics, biogeochemical and physical processes that influence DNA fragments must be better understood to be able to link an eDNA signal with the receptor's state. The biggest challenge is that eDNA is a proxy for the receptor and not a direct measure of presence. Nonetheless, as more actors enter the field, technological solutions are likely to emerge for these issues. Environmental DNA already shows great promise for baseline descriptions of the presence of species surrounding a project and can aid in the identification of potential receptors for EIA monitoring using other methods.

Keywords: eDNA; environmental impact assessment; marine environment; reactive transport; occupancy models



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1. Introduction

In principle, any species can be detected by traces of genetic material left behind by individual organisms in their environment. This DNA is called environmental DNA (eDNA, or e-DNA; [1]) today. Initially, during the 1960s and 1970s, these extracellular DNA molecules were considered only in terms of their concentrations detected in environmental samples, like seawater [2,3]. Studies of the base-pair sequences in this DNA began in earnest during 1980s a few years after sequencers were available commercially [4]. At this time, microbiologists began sequencing DNA extracted from water and soil samples (e.g., [5–7]. Their interest was motivated by the realization that a significant portion of the microbial community composition was not being studied because strains could not be cultured in the laboratory [8,9]. Under the impetus of calls to inventory global biological diversity, this led to the idea that species identifications could be made more efficient by developing a method to ‘barcode’ characteristic sequences of all organisms [10,11]. Environmental DNA was soon being tested successfully for species surveys to detect the presence of invasive

and rare species [12,13], in difficult to access aquatic environments [14], or to reconstruct past environments using DNA recovered from sediment cores (e.g., [15]).

Since the mid-2000s, an impressive variety of different studies using eDNA have been published [16]. The technique has made rapid, undeniable progress across all fields of ecological sciences. There have been at least 105 review articles published since 2012, and 50 of these appeared in the last two years alone (Figure 1). Post 2008, eDNA has been integrated into biodiversity research and management for a plethora of questions regarding past and present distributions of species in all environments (terrestrial, aquatic and marine). Some examples are: community surveys (e.g., [17]), monitoring ecological restoration projects (e.g., [18]), spatial planning [19], for sustainable management of forests (e.g., [20]), as a tool for pathogen detection (e.g., [21]) or invasive species detection [13] and as a tool for environmental management with the objectives of detecting changes in communities as a function of land-use patterns [22–24] or the presence of chemical contamination [25]. The creation of a new journal in 2019 (Environmental DNA, Wiley Science, see Appendix A.1) will certainly enhance these trends.

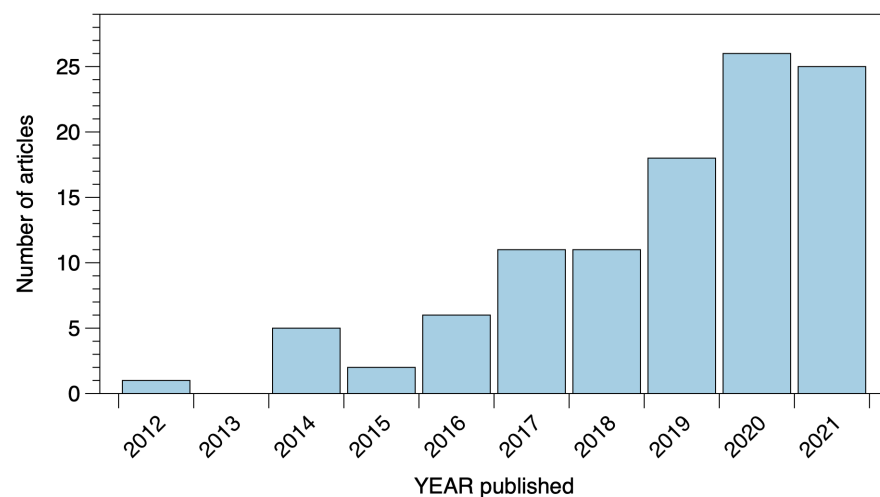


Figure 1. Number of review articles published each year about eDNA between 2012 and 2021, Web of Science database. Searched on 27 November 2021; $n = 106$. On plot, $n = 105$, because one article had no year assigned. The search is described in Appendix A.1.

Despite all the research and applications in conservation biology and environmental protection, until 2016 [26], eDNA had not been confronted with the regulatory frameworks used in environmental impact assessments. Impact assessment stands out for its obligation to meet standards of proof for describing projected changes made to ecological systems. Environmental Impact Assessment (EIA) is defined by the International Association for Impact Assessment (IAIA) as:

“... the process of identifying the future consequences of a current or proposed action. The “impact” is the difference between what would happen with the action and what would happen without it.” [27]

EIA is part of a formal process regulated by networks of institutions, agencies and supra-national organizations (e.g. the European Commission). The details vary depending on where the proposed action or project will be developed. In a recent report, the United Nations Environment Programme [28] highlighted strong differences in EIA compliance between countries. This also suggests a need exists for new methods that are workable in a wide range of environments, contexts and analytical capacities. In parallel, a handful of studies have indicated that eDNA could become a feasible and robust method within impact assessment [29–33], and specifically for marine systems [34].

The aim of this review is to explore the potential for eDNA as a tool within the framework of an EIA. This review does not detail the state of eDNA technologies, questions

about PCR sequencing, and protocol validations. These issues are covered exhaustively in a critical review by Cristescu and Hebert [4], in a new initiative to validate PCR assays [35], in the review articles published since 2020 (e.g., [36–38]) and in the first textbook [39] on eDNA. Instead, our goal here is to focus on how eDNA measurements can provide estimates of potential impact on receptors as described in EIA. Receptors are the objects for which the impact is assessed; the state(s) of the designated receptor(s) in the project area is(are) required to be known both before and after project implementation. Receptors can be species populations, communities, habitats, as well as diverse socio-cultural assets that could be affected by a proposed project [40]. Typically, the receptor is the impacted fraction of habitats, species (i.e., sub-populations associated with the project area) or assemblages of species (i.e., sub communities associated with impacted habitats or particular impaired functions), and which are often subject to some level of protection or regulation. We review and discuss the statistical challenges involved in detecting a potentially significant impact on species or assemblages of species in a project area, using eDNA data. For marine environments, in particular, this leads us to consider how well the transport and fate of DNA in water masses is known. Finally, we discuss the advantages eDNA data offers for different stages of the EIA process, especially for baseline surveys.

2. The Main Elements of Environmental Impact Assessment

Environmental Impact Assessment is a formal, regulated process that aims to identify the potential impacts a development may have on the environment in which it will be installed. The overall objective is to be able to anticipate where and when a future development would impact some part of the environment. The specific goal of an EIA is to identify potential impacts on receptors, then to propose and implement solutions to minimize these potential impacts. The evaluation of a potential impact therefore rests on having capacity to forecast the response(s) of an ecological system to a perturbation. Both the strength and difficulty of the EIA depends on the development of a comprehensive baseline understanding of the designated impact receptors [41].

The specifics vary, but an EIA is usually structured into five basic steps or stages (Table 1; see [42]). Screening and scoping tasks are when existing information on the project site is compiled and the precise components of the impact assessment are defined, taking into account relevant regulations in force and the project requirements. At this stage, a project area is defined that corresponds to the geographic domain which could be impacted during the project installation and operation. During the baseline evaluation, receptors' states are evaluated before the project installation; this step may include collecting new data if data gaps are critical. Potential impacts on the receptors are considered by comparing receptor states inside and outside of the project area and mitigating measures are proposed.

The taxonomic information from eDNA could offer new capabilities for EIA (Table 2). For instance, establishing a baseline (i.e., providing a reference state) currently requires a considerable investment to collect information on the species and habitats present within the project area. Environmental DNA sequencing, could provide a more comprehensive inventory of taxonomic groups with a smaller sampling effort. The results could also, however, enlarge the scope of an assessment if a previously unknown rare or protected species, or a pathogen, is detected. For example, Johnson et al. [43] characterized dynamic patterns in airborne eDNA surveys performed for a restoration project, detecting not only seasonal variations, but also the effect of changes in human activities which were outside of the project objectives. But, use of eDNA could permit early detection of fast and transient phenomena, such as harmful algal blooms [44,45] during monitoring, which would offer significant advantages to managers, decision-makers and stakeholders [29].

Table 1. Basic steps of Environmental Impact Assessment.

Steps	Description
Screening	Preliminary identification of potential impacts created by a proposed project and description of alternative propositions, can be an iterative process
Scoping	Potential impact receptors are specified, determination of the extent and scales of the impact assessment study, includes definition of a project's zone of influence and identification of data gaps
Baseline	<i>Part of Scoping</i> , Describes baseline measurements needed to estimate impacts
Impact Assessment	Evaluation of significance and consequences of the potential impacts on receptors identified during Scoping
Monitoring	Measurements done during project installation and operation to compare the predicted and actual impacts and the effectiveness of the mitigation measures taken

A molecular approach could change fundamentally the notion of another aspect of EIA: cumulative impacts. Currently, cumulated impacts are inconsistently assessed [46,47]; the significance of cumulated impacts can be based on expert opinions [46] or conceived as sets of indices summed and extrapolated over a region of interest (e.g., [48]). Neither style of cumulative impact evaluation treats the complex dynamics of ecological systems. In addition, it is possible that repeated eDNA sequencing in impact assessments could lead policy-makers and stakeholders to require databanking of eDNA for pattern analysis. For instance, since first suggested in the 1970s [49], numerous studies have been examining the hypothesis that human activities act as a selective pressure on populations (see [50]). Recognition that rapid evolution of populations is possible on short time-scales has already inspired a proposition [51] to evaluate the potential for evolutionary impacts be introduced into impact assessments.

Table 2. What new capabilities could e-DNA introduce in the EIA process?

EIA Stage	New Capabilities Introduced
Screening	Rare, endangered, or protected species presence detections
Scoping	Enables objective comparisons with earlier studies on project area, changes notion of cumulative impact
Baselines	Potential for high frequency sampling of sensitive receptor(s) throughout project area, high-throughput screening methods could produce lists of groups potentially present with less effort, possible to store information on organisms present in situ for analysis post-project
Impact assessment	More sensitive detection of changes allowing more responsive management; ability to assess changes at an assemblage level
Monitoring	Potential to increase speed and reduce the cost of biological surveys, could use smaller field teams, could collect information on wider range of species simultaneously

Nonetheless, as indicated in Kelly et al. [29], all aspects of eDNA information should comply with the requirements of existing EIA frameworks. As defined in Table 1, EIA is constrained by the strict boundaries of the project area and the set of receptors identified during scoping. Receptors are compulsory elements of EIA methodology and are potentially subject to regulatory constraints (e.g., invasive, threatened and endangered species)

or are considered of special interest regarding project acceptability among stakeholders. Potentially impacted receptors are often subject to mitigation measures, for example to limit expansion of conditions that could permit harmful or pathogenic species to increase, or to decrease the economic value of exploited habitats (such as shellfish or fish aquaculture installations, or traditional harvesting grounds). There is, therefore, great interest in using eDNA to provide evidence that any actions taken to mitigate and reduce impacts on project sites have been effective.

3. The Fate of DNA in Aquatic and Marine Environments

DNA has its own dynamics once released in the environment ([52]; Figure 2). Any environmental sample contains a mixture of DNA molecules all having different sources and ages that have been exposed to biotic and abiotic environmental gradients, including: heterotrophic interactions, UV light, temperature, pH and salinity [53–55]. In marine and other aquatic environments, eDNA will disperse, degrade and interact with other organisms, solutes and particles in the water column and sediment and DNA molecules can undergo a number of complex biotic and abiotic transformations, which alter their molecular characteristics [52]. However, important uncertainties remain because insufficient experimental work has been done on these processes ([53]; see Appendices A.2 and A.3). Nonetheless, for EIA studies, these processes must be understood to be able to simulate DNA behaviour under the environmental conditions of a project site. This information is needed to guide and inform decisions about sampling protocols, analyses and other interpretations.

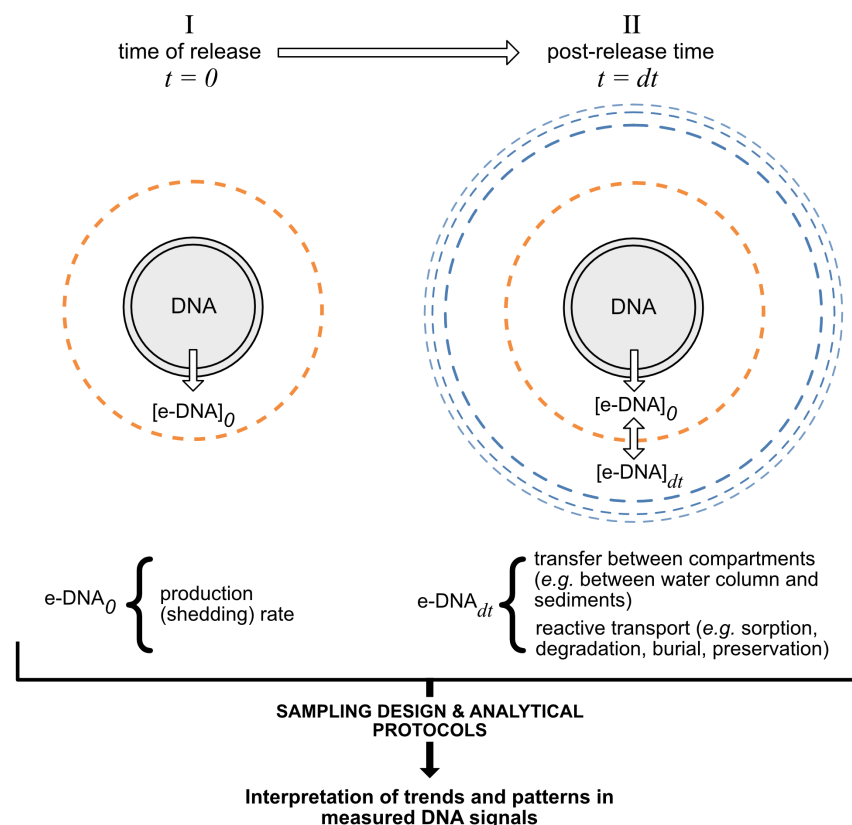


Figure 2. While the DNA signal may be easily detectable in a given sample, the meaning and interpretation are the outcome of dynamic, partially known processes. These processes can be thought of in terms of release conditions (I., left hand side) where e-DNA is subject to environmental conditions within immediate surroundings of an individual organism (gray-filled circles), and longer-term conditions (II., right hand side) where e-DNA is subject to complex dynamics of continuously changing environmental conditions that produce reaction gradients.

The fate (dispersion, degradation, solubility) of the DNA molecule is what happens when the genetic material is separated from the organism. Lack of information about the dynamics of eDNA fragments in environments has been invoked regularly to explain difficulties in interpreting eDNA data [37,53]. In addition to the large number of unknowns about the fate of DNA, other authors have noted that there is not yet enough methodological validation of sample processing steps (i.e., filtration, preservation, amplification and sequencing) before sequences are read and aligned [4,35]. Presently, frequently cited problematic issues in eDNA studies are the lack of standardization of the methods, incompleteness of referenced sequence database, and lack of appropriate contamination controls, or sensitivity measures [36,56,57]. Together these create many theoretical and analytical challenges for the interpretation of eDNA results within EIA. Studying the dynamics of eDNA in the environment, hence the state of eDNA when sampled, should help to tackle the sample processing issue and improve the explanatory power of eDNA results. Four major areas of concern are evoked in the sections that follow: shedding rates, decay and degradation, transport and dispersion, and burial and preservation.

3.1. Sources of DNA: Shedding Rates

The primary source of DNA in the environment is through “shedding”. Genetic material is shed in different forms from living organisms, for example as: excretions (e.g., feces), secretions (e.g., mucus), and exfoliation (e.g., epidermal cells). Each of these sources can have different shedding rates, transport and degradation characteristics in an environment [52]. Allan et al. [58] report shedding rates vary, not only by species, but also by life stage, size, and environmental factors such as pH, temperature, and the presence of predators [59–64]. Shedding rates are also affected by the behavior of organisms [65] and by the stress they are submitted to [66], which is more relevant for EIA. In aquatic environments, whether marine or continental, the passive or active movement of organisms can blend eDNA signals; this was shown theoretically for animal species that migrate vertically in the water column [67]. In aquatic environments, however, most studies have focused on fish [58] and the knowledge of other taxa is limited.

In addition to environmental, physiological and specific factors that affect shedding rates, the total quantity of DNA shed is presumed to be a function of the unknown number and biomass of organisms present in an unspecified distribution volume [60,64]. The problem of quantifying shed, or released, DNA has led authors to develop a concept of DNA “persistence” in the environment [52]. This conception would remove the dynamic process and replace it with an indicator of DNA stability without consideration of the variability in underlying processes [59,68,69]. However, Barnes and Turner [52] emphasize that “persistence” is a balance between what is added (shed or imported) and what is lost (exported, degraded or transformed). Therefore, it is impossible to interpret changes in “persistence” without a better understanding of these fundamental reactive transport processes. In EIA, preliminary tests of receptor species shedding, in the range of environmental conditions in which organisms will be submitted to an impact, is justified and recommended.

3.2. DNA Degradation

There are many mechanisms of degradation, and one of the most common factors described is temperature. Temperature increases have long been known to alter the molecular structure of DNA [70], making it more flexible and changing its folding. In studies of eDNA, temperature increases are reported to enhance microbial activity and DNA degrading enzymes released by them [52]; this is presumed to explain changes in observed degradation rates under different conditions [71–73]. Collins et al. [74] estimated experimentally, variations in eDNA loss rates under typical marine water column conditions (inshore/offshore, winter/summer). They reported faster loss under artificial inshore conditions and suggest that degradation rates in natural offshore marine waters are expected to be lower. In the Mediterranean Sea, Salter [75] reported a seasonal dependence on the persistence of DNA

in the water column associated with bacterial degradation of eDNA under phosphate limiting conditions.

Allen et al. [58] in a laboratory study of DNA degradation rates, examined differences between four different animal forms across four temperature/light treatments. They concluded that decay rates were likely not constant. These authors also point out that decay rate studies have been performed in specific conditions, mainly in sunlit waters, and few were performed in waters less than 10 degrees Celsius. This is obviously a problem for eDNA studies of deep and cold waters, a condition which includes most of the oceanic basins. At the same time, Jo et al. [59] has described a more complex interaction between the DNA fragment size, mitochondrial vs nuclear DNA and temperature in experiments on the degradation of fish genetic material and the apparent persistence of DNA signals.

The interplay of biotic and abiotic factors in aquatic and marine systems affecting mechanisms of decay and transport implies that an emphasis on the particular conditions in which species were sampled and detected is needed [76]. A recent proposition to use the ratio between eRNA and eDNA instead [77] could improve degradation rate estimates. However, at this time, the use of single decay constants is not recommended, and more complex functions will be needed.

3.3. Transport and Dispersion

As is the case for any other particle or molecule, genetic material undergoes a variety of transport processes, including, settling, mixing, advection and burial [53]. Globally, the chemical reactivity of a DNA fragment would depend on conditions encountered during transport in the water column including: solute content, pH, temperature, and sunlight [78–82]. Transport will depend on the state of the DNA fragment (sorbed or dissolved), particle size and the hydrodynamic regime. Most eDNA studies have examined DNA transport in aquatic systems, like streams and rivers, and these provide some insights into possible issues to consider in marine environments. For example, the eDNA from two invertebrate species (a mussel and a crustacean) in a lake-river system was investigated by Deiner and Altermatt [79]. They found traces of eDNA of the mobile crustacean species over 10 km downstream, while for the second, immobile bivalve, they detected its DNA over shorter distances. Jerde et al. [83] found that substrate types (sandy or rocky) in streams do not induce systematic differences in the transport of eDNA. However, they reported a large variability in DNA transport which remains unexplained.

Several studies have examined indirectly the question of transport and dispersion, by studying how well eDNA signals describe species distributions (e.g., [84–86]). Friebertshauser et al. [87] have shown with an experimental approach that filter-feeders can decrease the quantity and significantly alter the state of eDNA associated with suspended particles in the water column. Pont et al. [88] showed that eDNA behaves like a small particle and the transport distance will depend on the sedimentation process. There have also been several studies that have detected substrate-induced differences in the quantity of eDNA detected [85,89–91].

For marine systems, knowledge of DNA transport and reactivity dynamics is not well-studied [32]. A more synthetic view of the dynamics of eDNA molecules at sea was proposed by Andruszkiewicz et al. [78]. These authors modeled eDNA transport in an coastal oceanic system, based on the following general equation, to which a source of eDNA was added:

$$\frac{\partial C}{\partial t} + v \nabla_H C - w \frac{\partial C}{\partial z} - K_H \nabla_H^2 C - \frac{\partial}{\partial z} \left(K_V \frac{\partial C}{\partial z} \right) = \alpha_l - \mu C \quad (1)$$

where C , in $M \cdot L^{-3}$, is the eDNA concentration, v , in $L \cdot T^{-1}$, a vector of horizontal velocity components, w , in $L \cdot T^{-1}$, a settling (vertical) velocity, K_H and K_V , in $L^2 \cdot T^{-1}$ are dispersion rates, in the horizontal and vertical dimensions respectively, α_l , in $M \cdot L^{-3} \cdot T^{-1}$ an eDNA shedding rate function of the spatial location, l , and μ , in T^{-1} , a global eDNA

decay rate. The ∇ operator represents divergence of C in the horizontal (∇_H) or vertical (∇_V) dimensions.

With this type of model and coupled with a hydrodynamic model to calculate velocities and water height, they were able to predict the place of origin and time it took for the eDNA to arrive at the sampling site [78]. They also revealed that depending on the location of the source (α_I), eDNA can be displaced over long distances (tens of kilometers) in short times (days), and settle far from their production source location. Several types of eDNA fragments from different sources and sizes can be represented in this type of model [58,67,78], which can also include ecogeochemical interactions. This approach should help specify what can be obtained with different sampling strategies, but this requires parameterization of all processes.

In point of fact, Andruszkiewicz et al. [78] assumed a minimal effect of eDNA transport due to horizontal water movement in the open ocean and focused mostly on vertical variations. They emphasized whether the eDNA is present as a particulate or dissolved form. This raises an ancillary issue about the size of DNA molecules in natural waters that was of concern in earlier research (see Appendix A.2). Paul et al. [92] were one of the first to try classifying eDNA according to the size of particles, setting a threshold between dissolved and particulate states at $\varnothing = 0.2 \mu\text{m}$ (averaged equivalent diameter). The classification was set for technical reasons (i.e., water filtering technique used), but led to a more ecological classification: the particulate fraction was assumed to be composed of living organisms and divided in picoplankton ($\varnothing < 1.0 \mu\text{m}$) and microplankton ($\varnothing \geq 0.2 \mu\text{m}$). Dissolved DNA is divided into a bound fraction, and a soluble fraction. This classification was questioned later. Based on a series of experiments, Moushomi et al. [93] found that eDNA has a large range of sizes, which mainly depends on the degradation state of the eDNA molecules. Therefore, for marine environmental questions, DNA should be considered within a reactive transport framework of the ecosystem, in which it can be sorbed on particle surfaces [94], change states, be subject to mixing, aggregation, diffusion and degradation according to site conditions. Adopting systematically this approach implies developing sampling and experimental plans that conform with this type of framework.

3.4. DNA Burial and Preservation

In the reactive transport equation above, the settling rate conditions the processes of degradation that affect preservation and burial rates. For example, during settling, DNA particles and fragments would be accessible to other organisms, like bacteria [75], in the water column. The half-life of DNA in seawater is not well known [74], but the presumption is that some fraction of DNA from the surface and water column will accumulate in benthic environments, even on the abyssal ocean floor. This has been tested recently [95]; these authors found that abyssal marine sediments do contain genetic material from a range of water depths, even if the majority is from benthic species.

While there have been studies of preserved eDNA from cores to reconstruct past ecosystems [15,96,97], once material accumulates in sediments, buried DNA is not necessarily immobile and inert. Any event that involves soil or sediment excavation, whether of natural or of human origin, will redistribute the buried DNA [98]. In marine systems, bioturbation, tides, current scouring, floods, debris flows, and slumps can displace and redistribute sediments and their associated eDNA. Dredging, drilling and mining are examples of activities that cause sediment displacements. The imminent introduction of mining operations into the deep-sea environment is motivating surveys of seabed eDNA and eRNA (e.g., [95,99,100]) in preparation for the assessment of potential impacts. Environmental DNA has already been used to detect changes in microbial communities after sand-mining operations [101] at a coastal site off South Korea. It is, however, difficult to generalize as the dynamics and spatial scales vary considerably between sites and projects.

In summary, all these considerations suggest that eDNA has a high potential to efficiently survey biological diversity for EIA. However, the decision procedure that evaluates the potential impact on one or more receptors would have to treat eDNA separately, since

it can have a dynamic that is not conditioned by the impact. This could occur if a factor affects the shedding process, causing eDNA to have a dynamic that evolves separately from the receptor organism. Without a robust link between the states of receptors and states of eDNA, eDNA can only be used as a proxy of receptors for EIA at this time.

4. Exploring eDNA as a Proxy for an EIA Receptor

The main question of our review is to determine if eDNA could be considered as a reliable proxy for a receptor of concern focusing on marine environments. The receptor is within the project area and is subject to the impact, hence its state could change significantly once the project is implemented. When the receptor is observed, the quantification of the state (and the impact) is direct, and defined by the effect of the stressor on the receptor [102,103]. This is not the case for eDNA, which is subject to many additional processes (Section 3), which are a function of environmental factors that are not necessarily linked directly with the impact. Based on our review of the extant knowledge of eDNA, there is no reason to set identical properties for eDNA and receptors. The only exception would be for small organisms (e.g., bacteria in water or sediments), for which intracellular DNA is measured. As a result, the only way to make the receptor 'observable' using eDNA is to quantify the processes that generate, propagate and degrade eDNA in the environment.

The second issue is that, in many cases, impacts are small quantitative changes in state without a complete disappearance of the species [47]. Conversely, most eDNA analyses are restricted to the detection of presence (actual or past) of the species, and hence can be insensitive to the impact. As a consequence, a predicted change in an impacted receptor can be undetectable by eDNA analysis. Alternatively, a change in eDNA detection can be distinct from the dynamics of the receptor. This was shown by Carraro et al. [76] using simulations to demonstrate that non-uniform patterns can be generated even when taxa are uniformly distributed. The results of Carraro et al. [76] have important consequences for sampling and suggest that spatial and temporal replication challenges have not been adequately explored. One of the few studies to have looked at temporal replication [104] has found significant but unexplained dissimilarities between samples taken only one week apart. Another group looking at mussel eDNA within a river system was able to successfully combine data collected on the eDNA shedding rate for the species of interest with transport modeling to create a population monitoring tool [81]. Recently, Kozoil et al. [105] have reported that environmental signals may be biased by the affinity of a particular organism (and the related eDNA) for the substrate it was extracted from. This emphasizes further the importance of gathering species-level information on both DNA shedding rates and population dynamics to better sample and interpret eDNA results.

Another challenge is how to evaluate sampling uncertainties ([4], Appendix A.3). The sampling strategy of the receptor within EIA is usually designed carefully in such a way that the impact can be assessed without ambiguity [106]. It leads to determining precisely the spatio-temporal scale of the impact and optimizes the sampling plan. Nothing indicates that this type of optimization can be applied to eDNA sampling. Taberlet et al. [39], in the sampling section of their book, discusses the difficulty of providing a universal methodology and suggests that sampling eDNA is more about designing a sampling plan for environmental constraints rather than the source population.

Finally, one of the few experimental studies to address the effect of an event on deep-sea communities with eDNA was a recolonization experiment where repeat eDNA and faunal samplings were done before and after mechanically clearing surfaces at a hydrothermal vent site [107]. These authors found good year-to-year reproducibility of eDNA taxa identification across the study area. However, these authors also highlight the need for traditional taxonomic information to interpret the results of the experiment in terms of ecological interactions. It therefore appears challenging to use eDNA as a proxy for a potential impact without having sufficient information about the receptor state.

5. The EIA eDNA Sampling Conundrum

The sampling conundrum means that to design an optimal sampling plan for EIA estimates, it is necessary to have information about the source(s) and state(s) of the eDNA while the distribution of the receptor is not yet identified. As discussed above in Section 4, the distribution of eDNA cannot be assigned directly to the distribution of the receptors because it is a trace of evidence shed from the receptor, and thus has its own dynamics.

By definition, the environment is sampled, not eDNA. An environmental sample contains a mix of eDNA, coming from different sources [54,55]. Determining whether eDNA comes from a particular species of interest in a project area can only be expressed in terms of probability. In other words, if a volume (of an environment) is sampled close to living organisms that were well identified, there is only a chance, hence no certainty, that the sample contains fragments of DNA of the organism that can be further analyzed [108].

In designing a monitoring program, the first planning task is to define the sampling unit. This is defined as the smallest subdivision of the object investigated, which, in the case of eDNA sampling, is a set of DNA fragments in the medium. As we have seen earlier, there is no standardized protocol to define the sampling unit; this will depend not only on the environmental conditions, but also on the species or assemblage of species of interest and the preservation state of the eDNA. Although the sampling unit should be defined by estimating the distribution mode of eDNA in the environment [32,98], it is usually done as a compromise between several contingencies [109], mainly: the sampling effort, sample collection method, storage capacities, technical limitations and overall cost. Regarding any water column sampling in an aquatic system, the prevailing idea is the larger the better, attempting to increase both the speed and efficiency of filtration techniques [110].

The second task is to design a sampling plan based on the distribution of the sampling unit. Because eDNA concentration can be considered to generate a gradient from the source outward, the optimal sampling strategy would be a systematic sampling plan [111]. However, when considering that the source is not known (i.e., investigated at the relevant time lag), it is very difficult to optimize a systematic sampling plan, defining an adequate orientation and dimension of the grid with a suitable resolution along and across the gradient. Again, this emphasizes the need to have good information on the origin and fate of eDNA [32] to be able to better estimate sampling outcomes. To overcome this issue, the use of automated sampling systems is being considered to maximize the spatio-temporal resolution and coverage without optimizing it [21,109,112].

The purpose of a baseline survey is to create a robust basis for comparisons between states of variables during and after the project implementation. In EIA, the sampling for the baseline and for monitoring are linked and condition the statistical precision obtained in impact assessment. This principle should be applied to eDNA sampling, which implies that the link between the receptor and its corresponding eDNA must be quantified. The strength of the structure of eDNA distributions can be estimated by modeling the eDNA quantity (Equation (1), “C”) using this typical reactive transport equation within the space-time window of the project. Variances associated with estimates are minimized by a systematic sampling which can then be optimized, and they should decrease when the structure of the distribution of the regionalized variable is strong. For example, it would be particularly interesting to test this approach for projects where large amounts of sediment are re-suspended that could interact with DNA in the water column, as well as release DNA buried in the sediments. Even if the macroscopic steady-state can be established in the time frame of the impact assessment (i.e., the impact is assessed between two steady-state values calculated from before and after project implementation), the time delay between the state of the receptor and eDNA must still be accounted for explicitly.

6. The Puzzling Statistics of eDNA Results

The value of eDNA as a technique is that it provides a means to ‘measure’, or evaluate the presence of a species independently of having actually ‘seen’ the organism. With traditional surveys, the expert asserts they have observed the presence of the organism in

a sample, and there is no uncertainty associated with this observation of presence. With eDNA, we have an indirect inference that generates a complicated set of probabilities. In other words, for every sample analysis, when the analysis detects a species (or an operational taxonomic unit (OTU)), the actual presence of the organism in the environment can only be expressed as a probability.

Schmidt et al. [113] formulates this problem in terms of the site occupancy probability. Site Occupancy-Detection Models (SODM) were developed originally for ecological research to confront the notion of presence (and conversely absence), with the notion of detection (conversely non-detection), and introduces the concept of probability detection [114]. Schmidt et al. [113] were the first to propose that the SODM be applied to eDNA data. They suggested that the occupancy probability can become an estimate of the probability to identify a species from a series of environmental samples. Most of the time, these results are converted to presence/absence, not abundances.

In SODM, the “site” is assimilated to the actual sample. With no uncertainties, the site occupancy probability can be estimated by:

$$\psi = \frac{n_d}{n_T} \quad (2)$$

where n_T is the total number of sampled sites and the n_d is the number of sampled sites where specimens of a species were detected. However, when a sampling is done, the detection of specimen can fluctuate randomly. Therefore, the occupancy probability must be corrected from the probability, p to detect specimens of the targeted species:

$$\psi = \frac{n_d}{n_T p} \quad (3)$$

This adds a requirement to perform repeated samplings (i.e., a “visit”, like a repeated draw) of the same sites to estimate the detection probability. The new occupancy probability can be estimated by:

$$\psi = \frac{n_d}{n_T p^*} \quad (4)$$

where p^* is the cumulative probability to detect the species, estimated from t successive samplings of the same site.

Using this model leads to an inherent difficulty in eDNA methodology. The presence/absence assessments of species in samples are associated with four probabilities [115]:

- p_{11} , is the probability of the species to be detected when really present in the medium (true positive),
- the associated probability p_{00} is defined as the probability to not be detected while absent in the medium (true negative),
- the third probability p_{10} defines the probability of a species to be detected in the sample when not present, hence being a false positive result, and
- p_{01} defines the probability of a species to not be detected in the sample while actually being present in the medium and is a false negative result.

This set of probabilities $\{p_{11}, p_{10}, p_{01}, p_{00}\}$ quantifies the reliability of the estimates of eDNA. In other words, each time a sample is taken, a species might be detected or not. When it is not detected, it can be absent (a true negative) or can be present, but not detected (a false negative). Conversely, there are also possibilities to detect species that are not present. False positives were neglected in early studies because their causes were difficult to identify and occurring at different steps of the protocol, from contamination to detection errors. However, as Ficetola et al. [116,117] pointed it out, they should not be neglected. In consequence, the only way to ensure that a species is actually present is to reject the hypothesis that a positive result is false.

The statistical reasoning described in [116] has led to consider whether to replicate, or repeat samples. It has been suggested that repeating the entire sample treatment process

(amplification through sequencing) on sub-aliquots of each sample not be regarded as pseudo-replicates, but as Bernoulli experiments in which presence is a success, and absence is a failure [117]. Then, on the cumulative distribution of the number of successes, it is theoretically possible to identify the quantile corresponding to the risk taken to reject a false positive when it is. However, to calculate the probability that a detection is a false positive, it is necessary to know the false positive rate. Without knowing this, when performing a series of sample analyses for which some detect a species and others do not, presences and absences can be either true or false. This apparently circular reasoning can be solved either by replicating k times each sample and the amplification and detection process for each of the replicates, or by modeling the probability distributions, or by applying a model of a site occupancy-detection that accounts for the different types of errors [113,115,118].

The consequence for EIA applications is that it is therefore necessary to identify changes in the detection probability distribution that is estimated from sets of probabilities for one or more receptors. A significant effort is required to validate such a relationship because it is likely to vary as a function of the receptor state and would be subject to all previously described environmental conditions (Section 3) associated with shedding, transport and degradation, and even if an estimate of DNA quantities in a sample can be made, this value is not directly linked to the corresponding receptor quantity.

In addition, there are analytical issues involved that can affect the probabilities of detecting species from DNA in an environmental sample. In particular, differential specificities of primers for sequences may lead to over-estimating the presence of species and under-estimate the presence of others [119]. The q-PCR method has taxonomic limits, but the quantification feature is useful to target specific taxa for which functions are important in terms of health or environmental disturbances [61]. It has also been shown to be more cost effective when only trying to detect a few species [120,121].

7. Opportunities and Challenges for EIA with eDNA

Few articles explicitly link the use of eDNA with EIA, but the adoption of molecular methods in areas of environmental management and regulation can be expected to generate many new opportunities and challenges (Table 3). For EIA, as we have discussed, the receptor-oriented process of impact assessment cannot cope with the disconnection between DNA released from a receptor (Sections 4 and 5). But, from the point of view of the presence/absence probabilities, a solution can be found for which a change in probability becomes detectable (Section 6). It would be necessary, however, to rethink parts of the EIA process and potentially this could push EIA in new directions where concepts like ecological communities, cumulative impacts and rarity are revised.

Fundamentally, eDNA changes the traditional empirical paradigms of ecology and biology. Until the late 1990s, biology and ecology focused on studying organisms that could be “seen” [122]; this means species that could be cultured, grown and observed under controlled conditions. Darling [123] called this “catch and look” in their recent perspective. After the Linnean revolution in systematics, this criteria of needing to be visible organized how all organisms are named, classified and studied as well as how their roles have been framed within ecosystems (e.g., [124]).

One example relevant to EIA is that ecosystem functions cannot be assessed if the molecular method does not also include means to determine if the organism detected is alive or dead [125]. Other problems are related to the compatibility of eDNA information with existing methods used for assessing ecosystem responses. Many long-term trends in populations and communities have been evaluated with approaches from historical sciences (e.g., for fisheries, see [126]). The backwards compatibility of eDNA results with these existing resources is only beginning to be examined [30,127].

Table 3. Opportunities, limitations and challenges of using e-DNA in the EIA process.

EIA Stage	Opportunities	Limitations	Challenges
Screening	More ecological components (e.g., species, assemblages) detected	Conditioned by availability in data libraries	When unknown, eDNA shedding dynamics should be characterized with preparatory studies
Scoping	Better characterization of ecological system structure	Requires access to complementary information about receptor(s)	Developing a quantitative index of cumulative impact accounting
Baselines	Increased spatio-temporal resolution of species presence; more efficient detection and databanking of species present	Need for reference samples of known compositions for inter-laboratory comparisons	Standardization and normalization of analysis protocols; ensure backwards compatibility; develop formal sampling plans using statistical principles
Impact assessment	Probabilistic prediction of impact on species assemblages; sensitive detection of rare species presence changes	Need to link discrepancies between eDNA and receptor(s) changes	Integrate quantification of eDNA fate and transport over region of interest
Monitoring	Cost effective field surveys with higher taxonomic resolution and coverage	Could require long-term access to cold storage and analysis facilities	Determination of mitigation effectiveness could require formal sampling plans using statistical principles

7.1. New Solutions That May Facilitate eDNA Use within EIA

The opportunities listed in Table 3 are leading to new and innovative solutions for exploiting eDNA information for EIA. In the previous sections we identified three issues:

- the disconnection between the receptor state and the corresponding eDNA signal;
- ensuring that a sampling strategy imposed for a given environment will also sample the eDNA targeted; and
- the statistical analysis of eDNA information for which only probabilities can be estimated and not certainties.

Environmental DNA studies are advancing quickly to address and remedy many of these questions. However, at this time, eDNA is not conclusively able to be used to report abundances of living organisms in environments outside the laboratory. This effectively limits eDNA to applications that can exploit presence/absence data, of which several are important to EIA, including: mapping habitat suitabilities [128], detection of presence of rare and endangered species [26,129], and for background determinations.

Many new proposals suggest how to improve the quality of eDNA information, including adding benchmarking steps, using more replication and machine learning. With respect to the methodological changes needed to improve data quality, Yang et al. [130] proposed a new approach to reduce false negative and false positive results with a modified qPCR technique, replication of detection analyses for each sample, and both positive and negative controls. Forster et al. [111] has suggested testing the efficiency of sets of markers of both ciliate eDNA and eRNA to strengthen monitoring of salmon aquaculture impacts

on benthic ecosystems. Cordier et al. [131] focused on data analysis by testing machine learning techniques' capacity to improve the calculation of biodiversity-based indices of marine environmental quality [132]. Dully et al. [33] presented a similar approach to improve the reliability of benthic bacterial community diversity assessments for salmon aquaculture, while Stoeck et al. [133] on the same topic, focused on the importance of relevant temporal resolutions to monitor changes of environmental quality.

There is also a need to develop suitable markers to assess the biodiversity in particular environments such as the deep sea, for which species are both largely undescribed and difficult to classify [134]. It has been suggested that eDNA-based indicators of changes in deep-sea communities are the only viable solution for monitoring impacts during forthcoming abyssal seafloor mining projects (e.g., [95,135,136]). In these areas, molecular operational taxonomic units (MOTU) tend to replace traditional species classifications, because they are a means for rapid assessment of spatio-temporal distribution patterns [99]. Cowart et al. [107] argue that eDNA metabarcoding is valuable as a reproducible method for describing baseline biological metazoan diversity in hydrothermal vent field environments. However they also recommended when working at small spatial scales (tens of meters), that eDNA be used in compliment to other data. High throughput sequencing of benthic microbial diversity has been tested to monitor impacts during offshore platform operations using community-level changes [31]. Molecular information is inspiring new discussions about how to decide the level of description of a species for spatial planning questions [19]. The main challenge for EIA will be to decide how well the classification flexibility characteristic of molecular taxonomies (e.g., using OTUs, MOTUs) copes with existing practices in environmental quality management and species conservation.

A promising new use of eDNA in EIA is for characterizing changes in community structures [31,137–140]; however, community-level changes are detected based on exploratory statistics (multidimensional data analysis) rather than inference. Interestingly, even if it does not rest on a strict application of decision theory, Kelly et al. [141] explore inferences based on rarefaction curve and correlation with environmental properties for different levels of diversity along a gradient of disturbances in Puget Sound, Washington, USA. They pointed out the difficulty to address changes in biodiversity because of the multiplicity of spatio-temporal scales at which it should be assessed, but also that an ecosystem-based approach which includes adaptive patterns (changes in life history-traits) will be a key component for future developments in EIA. Another interesting idea is to combine probability estimates with spatial distribution modeling [142] to infer if a distribution has changed significantly before and after impact. This type of inference can be performed from spatial estimate distributions in the limited project area using appropriate covariance functions.

Finally, there is the potential with advancements in sequencing technologies to understand population level genetic interactions, demographics history, and selection pressures [143]. Genetic material has been used to assess the health and trends of populations and individuals for decades [144] and typically the mitochondrial genome has been utilized for these [145,146]. Recently, the d-loop region of haplotypes from eDNA samples have been utilized to distinguish phylogenetic relationships in populations [147–149]. Two recent studies attempted to capture nuclear eDNA for population genetic analyses: one was unsuccessful [108], with low initial eDNA concentrations, while a second [150], was successful. This remains an interesting area for further advancements of the use of eDNA in ecology, but its applicability for EIA would require much more advanced DNA capture and probe matching techniques to make it applicable.

7.2. Persistent Need for Taxonomic Expertise

It has been suggested that eDNA sequencing with a barcode system would eventually replace taxonomic expertise as the number of banked sequences increased. However, a very large proportion of species have not yet been identified and described, *a fortiori* sequenced [151,152]. Significant gaps exist in public barcode collections for a variety of reasons [153] and a new open-access tool aims to solve this problem by mapping where

these gaps exist [154]. Given the large uncertainties in the estimates of numbers of species on Earth, it is difficult to even provide a rough estimate of how large these gaps are, in the absolute sense, for understudied environments like the deep-sea.

The barcode concept presumes all the individuals that make up a species population can be described by a relatively short (in terms of numbers of base pairs), unique, invariant sequence [155]. Poor marker selections are a source of cross-amplification errors when similar species are present in the same sample [156], an issue which can be remedied with methodological changes (see above, Section 7.1). Fragments of rare taxa may result in false negatives for a species of interest. In addition, cryptic diversity can inflate biodiversity estimates because the DNA of one species can separate into distinct molecular sequences [157].

Given these challenges and the acknowledged weaknesses in laboratory protocols [4,36,158], some authors have suggested that eDNA be viewed as a complement to classic methods of species identification [108,159,160]. This will certainly change quickly for some applications. One example of this is how rapidly the conversion of a marine biotic index was achieved. Benthic macroinvertebrate communities have been used for biomonitoring programs for many years because these groups exhibit strong responses to sustained environmental changes [161]. The biotic indices developed are applied to assess the health of benthic systems (e.g., AZTI's Marine Biotic Index or AMBI [162]). However, even if Aylagas et al. [163] and Darling et al. [164] suggested that indices adopt a barcode-type system in 2014, species identifications are difficult to complete without efficient access to an array of taxonomic expertise [165]. In later testing, four years after the initial suggestion, it was reported that similar results on environmental quality were given with either a genetics-based or morphological-based index [166].

Nonetheless, there are areas of EIA and ecology that will continue to need taxonomic expertise and actual detection of the living organism. For instance, in marine environments where broad surveys would be expected to be used in unstudied areas, more traditional whole organism-oriented taxonomic expertise will be important for tasks like background and baseline surveys. Environmental impacts can also involve interactions that alter behaviors, like acoustic energy impacts on animal behavior [167,168], and this too will continue to require other approaches. Finally, many authors have tested the correspondences between other non-invasive methods, such as underwater video, with eDNA (e.g., [169–171]). These have shown that eDNA is insufficient by itself, but does help reach a higher taxonomic resolution [170].

8. Conclusions

Environmental DNA has been characterized as a “visionary solution” by Lodge et al. [172] and it has been suggested that the impact of DNA technology on science will be on par with that of the microscope [173]. These are unusual statements for the scientific literature, but as we have seen, the rapid technological growth and successes with introducing the technique for very different types of study questions, cannot be ignored.

Currently, there are no eDNA methodological developments that are specific to EIA and only a handful of research articles discuss this topic with case studies. Our review leads to the conclusion that eDNA has an undeniable potential for EIA. It offers the advantages of being rapid to implement, is complementary to other sampling techniques and is extremely sensitive for the detection of species. However, as with any molecular analysis, there are important technical limitations to be accounted for that may vary by species, species life stage, season and location.

The overarching challenge in EIA is that eDNA does not directly measure the state of the receptor, but is instead a proxy for the presence or absence of receptors. This means that eDNA results have a variable reliability that must be modeled statistically to be compatible with the decision theory used in environmental and risk assessments. Hence, if doubts about the reliability of results cannot be decreased, managers and decision-makers will require ancillary information before to settle on a plan of action.

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Appendix A. Topical Bibliography

In this appendix, we provide some complementary background information and references important to the epistemic development of eDNA for environmental sciences research and environmental impact assessment studies.

Appendix A.1. Search Method Used with Web of Science Database

We searched the Web of Science (WoS) database during November 2021. For a search string of “eDNA” (Topic) or “environment* DNA” (Title) and publication date > 1970, the total number of articles returned was 3035, spread among 144 topics. This information agrees an earlier scientometric analysis of environmental DNA research published in 2017 by Jiang and Yang [16].

Unfortunately, the new journal, Environmental DNA (published by Wiley Science since 2019), is not indexed by WoS. As of December 2021, 224 articles have appeared in this journal, including 6 review articles, 24 additional application type articles and 48 more studies in marine systems. These articles are not included on the histogram of Figure 1.

Appendix A.2. Historical Overview of Important Conceptual Breakthroughs

As mentioned in the Introduction (Section 1), key concepts for eDNA originated in two areas: biological oceanography and microbial ecology. Starting in the 1960s, there was a concerted effort among biological oceanographers to measure dissolved and particulate DNA. The aim of these studies was to determine if there were identifiable patterns in extracellular DNA and if these could be used as a proxy for sediment and water column phytoplankton productivity and bacterially-mediated cycles of organic matter degradation. The works cited [2,3,92], discuss how they approached the topic as a problem in ocean biochemistry, that is of molecular reactivity and framing their studies like for any other molecule of interest. Other articles published in this period (1960–1990) consider the reactivity of DNA in environmental samples experimentally, such as:

- Aardema, B.W.; Lorenz, M.G.; Krumbein, W.E. Protection of sediment-adsorbed transforming DNA against enzymatic inactivation. *Applied and Environmental Microbiology* **1983**, *46*, 417–420. doi:10.1128/aem.46.2.417-420.1983.
- DeFlaun, M.F.; Paul, J.H.; Jeffrey, W.H. Distribution and molecular weight of dissolved DNA in subtropical estuarine and oceanic environments. *Marine Ecology Progress Series* **1987**, *38*, 65–73.
- Lorenz, M.G.; Wackernagel, W. Adsorption of DNA to sand and variable degradation rates of adsorbed DNA. *Applied and Environmental Microbiology* **1987**, *53*, 2948–2952. doi:10.1128/aem.53.12.2948-2952.1987.
- Paul, J.H.; Jeffrey, W.H.; David, A.W.; DeFlaun, M.F.; Cazares, L.H. Turnover of extracellular DNA in eutrophic and oligotrophic freshwater environments of southwest Florida. *Applied and Environmental Microbiology* **1989**, *55*, 1823–1828.

Environmental DNA as understood today did not really exist prior to the invention and adoption of PCR amplification and automated sequencing. The period 1986–1989 was a watershed period for these technologies. During this short interval the PCR amplification method was presented to the scientific community and the first important peer-reviewed publications appeared:

- Mullis, K.B.; Faloona, F.A.; Scharf, S.J.; Saiki, R.; Horn, G.T.; Erlich, H.A. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harb. Symp. Quant. Biol.* **1986**, *51*, 263–273. doi:10.1101/sqb.1986.051.01.032.
- Mullis, K.B.; Faloona, F.A. Specific Synthesis of DNA in vitro via a Polymerase-Catalyzed Chain Reaction. *Methods in Enzymology* **1987**, *155*(F), 335–350. doi: 10.1016/0076-6879(87)55023-6.
- Saiki, R.; Gelfand, D.H.; Stoffel, S.; Scharf, S.J.; Higuchi, R.; et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **1988**, *239*, 487–491. doi:10.1126/science.2448875.
- Verlaan-de Vries, M.; Bogaard, M.E.; van den Elst, H.; van Boom, J.H.; van der Eb, A.J.; Bos, J.L. A dot- blot screening procedure for mutated ras oncogenes using synthetic oligodeoxynucleotides. *Gene* **1986**, *50*, 313–320. doi:10.1016/0378-1119(86)90335-5.

Afterwards, and for nearly two decades, PCR amplification and sequencers became standardized, automated and accessible, which produced numerous new approaches for data analysis and a new field, bioinformatics, emerged. Microbiologists developed new culture-independent methods and assays to sequence DNA extracted from environmental samples to study microbial groups that could not be cultured. However, this did not necessarily involve considering other environmental factors relevant for aquatic systems, such as transport, partitioning and reactivity of extracellular DNA. Indeed, a truly marine microbial ecology only took shape during the 1990s, and the first large-scale oceanic surveys were organised post 2000 [174]. Since the publication of these surveys (ca. 2004–2007) questions about how environmental conditions affect the quality of sequencing and limit possible interpretations are increasingly present under the eDNA paradigm:

- Bhadury, P.; Austen, M.C.; Bilton, D.T.; Lambshead, P.J.D.; Rogers, A.D.; Smerdon, G.R. Molecular detection of marine nematodes from environmental samples: overcoming eukaryotic interference. *Aquatic Microbial Ecology* **2006**, *44*, 97–103. doi:10.3354/ame044097.
- Strickler, K.M.; Fremier, A.K.; Goldberg, C.S. Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. *Biological Conservation* **2014**. doi:10.1016/j.biocon.2014.11.038.
- Buxton, A.S.; Groombridge, J.J.; Griffiths, R.A. Is the detection of aquatic environmental DNA influenced by substrate type? *PLoS One* **2017**, *12*, e0183371. doi: 10.1371/journal.pone.0183371.
- Guillera-Arroita, G.; Lahoz-Monfort, J.J.; van Rooyen, A.R.; Weeks, A.R.; Tingley, R. Dealing with false- positive and false-negative errors about species occurrence at multiple levels. *Methods in Ecology and Evolution* **2017**, *8*, 1081–1091. doi:10.1111/2041-210X.12743.
- Corsaro, D.; Venditti, D. An apparent *Acanthamoeba* genotype is the product of a chimeric 18S rDNA artifact. *Parasitology Research* **2018**, *117*, 571–577. doi:10.1007/s00436-017-5690-9.
- Kelly, R.P.; Shelton, A.O.; Gallego, R. Understanding PCR Processes to Draw Meaningful Conclusions from Environmental DNA Studies. *Scientific Reports* **2019**, *9*. doi:10.1038/s41598-019-48546-x.
- Furlan, E.M.; Davis, J.; Duncan, R.P. Identifying error and accurately interpreting environmental DNA metabarcoding results: A case study to detect vertebrates at arid zone waterholes. *Molecular Ecology Resources* **2020**, *20*, 1259–1276. doi:10.1111/1755-0998.13170.
- Sales, N.G.; McKenzie, M.B.; Drake, J.; Harper, L.R.; Browett, S.S.; Coscia, I.; Wangenstein, O.S.; Baillie, C.; Bryce, E.; Dawson, D.A.; Ochu, E.; Haenfling, B.; Handley, L.L.; Mariani, S.; Lambin, X.; Sutherland, C.; McDevitt, A.D. Fishing for mammals: Landscape-level monitoring of terrestrial and semi- aquatic communities using eDNA from riverine systems. *Journal of Applied Ecology* **2020**, *57*, 707–716. doi:10.1111/1365-2664.13592.

Finally, the concept of barcoding was the third and pivotal concept that really encapsulated the promise and potential of eDNA for research. Barcoding implied a new capacity to automate and speed up species identifications. Hebert et al. study was published in 2003 [10]. The idea set off a wave of opposition among taxonomists, which in retrospect was partly justified, because both the methodology and statistical analyses, were not validated for all the uses preconized [4]. The acceptability of bar-coding remains an issue for many reasons, among which are: unfamiliarity with molecular methods, distrust of non-visual identifications, misidentified organisms in barcode databases, and database incompleteness. There is a small literature on this issue (see articles cited in Section 7), and:

- Pawlowski, J.; Lejzerowicz, F.; Apothéloz-Perret-Gentil, L.; Visco, J.; Esling, P. Protist metabarcoding and environmental biomonitoring: Time for change. *European Journal of Protistology* **2016**, pp. 12–25. doi:10.1016/j.ejop.2016.02.003.

At present, the concept of environmental DNA, *per se*, has become widely known through articles appearing in high profile journals since 2003. As the approach has undergone tests in a wider variety of circumstances, eDNA began its transition to becoming a method with real-world applications. A 2017 bibliometric analysis [16], identifies several highly co-cited articles, like Jerde et al. 2011 [14], Ficetola et al. [12], and two others:

- Taberlet, P.; Coissac, E.; Hajibabaei, M.; Rieseberg, L.H. Environmental DNA. *Molecular Ecology* **2012**, 21, 1789–1793. doi:10.1111/j.1365-294X.2012.05542.x.
- Thomsen, P.F.; Kielgast, J.O.S.; Iversen, L.L.; Wiuf, C.; Rasmussen, M.; et al. Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology* **2012**, 21, 2565–2573. doi: 10.1111/j.1365-294X.2011.05418.x.

All of which have co-citation frequencies of more than 80. Hence, the ideas and suggestions in these articles are presumed to have contributed to the rapid spread and adoption of eDNA.

Appendix A.3. Methodological Issues Relevant for EIA

As we have seen, eDNA s based on validated, mature techniques of analysis for genetic material. However, as we have discussed in the article, the “environmental” part of eDNA, is still being perfected (Sections 3 and 5). There are a number of areas of active research on using and interpreting eDNA data for environmental studies, like EIA. These include: sampling strategies, evaluation of errors and sensitivity of detection, and the reliability of a species identification. A small selection of these articles is given below:

Evaluating Sampling Strategies

- de Souza, L.S.; Godwin, J.C.; Renshaw, M.A.; Larson, E. Environmental DNA (eDNA) detection probability is influenced by seasonal activity of organisms. *PLoS One* **2016**, 11, e0165273. doi:10.1371/journal.pone.0165273.
- Cornman, R.S.; McKenna, James E., J.; Fike, J.; Oyler-McCance, S.J.; Johnson, R. An experimental comparison of composite and grab sampling of stream water for metagenetic analysis of environmental DNA. *PeerJ* **2018**, 6. doi:10.7717/peerj5871.
- Abrams, J.F.; Hoerig, L.A.; Brozovic, R.; Axtner, J.; Crampton-Platt, A.; Mohamed, A.; Wong, S.T.; Sollmann, R.; Yu, D.W.; Wilting, A. Shifting up a gear with iDNA: From mammal detection events to standardised surveys. *Journal of Applied Ecology* **2019**, 56, 1637–1648. doi:10.1111/1365-2664.13411.
- Fukaya, K.; Kondo, N.I.; Matsuzaki, S.I.S.; Kadoya, T. Multispecies site occupancy modelling and study design for spatially replicated environmental DNA metabarcoding. *Methods in Ecology and Evolution*. **2021**, doi:10.1111/2041-210X.13732.

Evaluating the Quality and Limits of Species Identifications

- Galtier, N.; Nabholz, B.; Glémin, S.; Hurst, G.D. Mitochondrial DNA as a marker of molecular diversity: a reappraisal. *Molecular Ecology* **2009**, 18, 4541–4550. doi:10.1111/j.1365-294X.2009.04380.x.

- Yao, H.; Song, J.; Liu, C.; Luo, K.; Han, J.; Li, Y.; Pang, X.; Xu, H.; Zhu, Y.; Xiao, P.; Chen, S. Use of ITS2 region as the universal DNA barcode for plants and animals. *PLoS One* **2010**, *5*, e13102. doi: 10.1371/journal.pone.0013102.
- Pilgrim, E.M.; Jackson, S.A.; Swenson, S.; Turcsanyi, I.; Friedman, E.; Weigt, L.; Bagley, M. Incorporation of DNA barcoding into a large-scale biomonitoring. *Journal of North American Benthol. Society* **2011**, *30*, 217–231. doi:10.1899/10-012.1.
- Costa, F.; Landi, M.; Martins, R.; Costa, M.; Carneiro, M.; Alves, M.J.; Steinke, D.; Carvalho, G. A ranking system for reference libraries of DNA barcodes: Application to marine fish species from Portugal. *PLoS One* **2012**. doi:10.1371/journal.pone.0035858.
- Bourlat, S.J.; Borja, A.; Gilbert, J.; Taylor, M.I.; Davies, N.; Weisberg, S.B.; et al.. Genomics in marine monitoring: new opportunities for assessing marine health status. *Marine Pollution Bulletin* **2013**, *74*, 19–31. doi:10.1016/j.marpolbul. 2013.05.042.
- Stoeck, T.; Breiner, H.; Filker, S.; Ostermaier, V.; Kammerlander, B.; Sonntag, B. A morphogenetic survey on ciliate plankton from a mountain lake pinpoints the necessity of lineage-specific barcode markers in microbial ecology. *Environmental Biology* **2013**, *16*, 430–444. doi:10.1111/1462-2920.12194.
- de Barba, M.; Miquel, C.; Boyer, F.; Mercier, C.; Rioux, D.; Coissac, E.; Taberlet, P. DNA metabarcoding multiplexing and validation of data accuracy for diet assessment: application to omnivorous diet. *Molecular Ecology Resources* **2014**, *14*, 306–323. doi:10.1111/1755-0998.12188.
- Deagle, B.E.; Jarman, S.N.; Coissac, E.; Pompanon, F.; Taberlet, P. DNA metabarcoding and the cytochrome c oxidase subunit I marker: not a perfect match. *Biology Letters* **2014**, *10*, 20140562.
- Schnell, I.B.; Bohmann, K.; Gilbert, M.T.P. Tag jumps illuminated - reducing sequence-to-sample misidentifications in metabarcoding studies. *Molecular Ecology Resources* **2015**, *15*, 1289–1303. doi:10.1111/1755- 0998.12402.
- Bhattacharya, M.; Sharma, A.; Patra, B.; Sharma, G.; Seo, E.; Nam, J.; Chakraborty, C.; Lee, S.S. DNA barcoding to fishes: current status and future directions. *Mitochondrial DNA Part A* **2016**, *27*, 2744–2752. doi: 10.3109/19401736.2015.1046175.
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Appendix A.4. Future Developments

As technological and analytical improvements accumulate, many new ideas are being discussed about how traces of ecological processes could be captured with eDNA. For instance, a variety of automated technologies (e.g., [112]) are under development. Other groups are considering how to sample and interpret the DNA collected by organisms during

their normal feeding activities. Some filter-feeders like sponges, are being re-examined as “natural” collectors of DNA. Examples of these new topics of research include:

New Sampling Approaches and Uses

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New Technologies

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