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ENVIRONMENTAL DNA: HISTORY OF STUDIES, CURRENT AND PERSPECTIVE APPLICATIONS IN FUNDAMENTAL AND APPLIED RESEARCH

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❀ This review article is dedicated to a relatively young, actively developing approach to biodiversity assessment – analysis of environmental DNA (or eDNA). Current views on the nature of eDNA, a brief overview of the history of this approach and methods of eDNA analysis are presented. Major research directions, utilizing eDNA techniques, and perspectives of their application to the study of biodiversity are described. Key issues in development of eDNA approach, its advantages and drawbacks are outlined.

❀ **Keywords:** environmental DNA; metagenomics; biodiversity; biological monitoring; conservation biology; paleoecology; paleogenetics.

ДНК ОКРУЖАЮЩЕЙ СРЕДЫ: ИСТОРИЯ ИЗУЧЕНИЯ, СОВРЕМЕННЫЕ И ПЕРСПЕКТИВНЫЕ НАПРАВЛЕНИЯ В ФУНДАМЕНТАЛЬНЫХ И ПРИКЛАДНЫХ ИССЛЕДОВАНИЯХ

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❀ Статья посвящена сравнительно молодому и активно развивающемуся подходу в исследовании биоразнообразия — анализу ДНК окружающей среды (environmental DNA — eDNA). В ней изложены современные представления о природе eDNA, краткая история ее изучения, охарактеризованы основные методы анализа. Описаны основные направления современных исследований, использующих методы eDNA, и перспективы их использования для изучения биоразнообразия. Обсуждаются достоинства, недостатки и ключевые проблемы в развитии этого подхода.

❀ **Ключевые слова:** ДНК окружающей среды; метагеномика; биоразнообразие; биологический мониторинг; природоохранная биология; палеоэкология; палеогенетика.

INTRODUCTION

The study of environmental DNA (eDNA) is an actively developing field that may lead to revolutionary changes in biodiversity studies [1]. eDNA is genetic material (nuclear, chloroplast, mitochondrial) isolated from samples of different natural substrates (such as soils, sediments, sea, and fresh waters) [2]. Since the mid-2000s, the number of publications focused on eDNA studies has increased by more than 60 times with the development of next generation sequencing technologies [3].

ENVIRONMENTAL DNA NATURE

Despite the active development and interest in eDNA, the origin of this genetic material remains underinvestigated [4]. The most common routes of eDNA entry into the environment include natural desquamation of the skin epithelium, tissue trauma, release of metabolic products and reproductive cells, and decomposition of dead organisms [2]. After these processes, eDNA can be transported and stored in various substrates from several weeks in water bodies of the temperate zone [5] to hundreds of thousands of years in permafrost [6].

Concentration of eDNA in the medium and its preservation in various substrates depend on several biotic and abiotic factors [7]. The volume of genetic material released into the environment correlates with the biomass of organisms [8, 9]. It also depends on their physiological and ecological characteristics, age, and life cycle stage [10]. Numerous field [10] and laboratory [11, 12] studies have demonstrated that the rate of eDNA release into the environment varies in different species under similar conditions, even within the same genus [13]. Exposure to stress factors also significantly affects the rate of eDNA release from living organisms by changing metabolic intensity and immune responses, thereby increasing the division rate of epithelial cells and stimulating mucus secretion [7]. Seasonal fluctuations in the eDNA concentrations in fish, amphibians, and reptiles are related to seasonal changes in their behavioral activity [14]. The amplification of eDNA signals during the breeding season is associated primarily with the release of reproductive cells and tissues into the environment [15, 16]. Thus, eDNA can be used to clarify the breeding sites of various taxa.

Abiotic factors, such as temperature, salinity, acidity, and oxygen concentration, affect the eDNA content in the environment indirectly and directly by affecting behavior, physiology, growth and development of organisms and by determining the rate of eDNA degradation, respectively [7]. For example, M. Seymour et al. [17] demonstrated that amount of eDNA under alkaline conditions can exceed that under acidic conditions by 1–2 orders of magnitude. However, whether or not this phenomenon is due to a change in the rate of degradation or the release of eDNA into the medium remains unclear. The authors revealed that eDNA can be transported quickly in rivers as the eDNA signal propagates tens of kilometers from the source, which must be considered when conducting research and interpreting the results. eDNA persists for a relatively shorter period of time (usually measured in days) in aquatic environments than in soils and ice, where it can persist for years. For this reason, monitoring the current state of ecosystems using eDNA usually involves sampling water rather than other substrates. The rate of eDNA degradation in sediments and soils is influenced by the nature and proportion of clay minerals, organic substances such as humic acids, and charged particles that can adsorb eDNA fragments and protect them from further destruction. For example, montmorillonite can absorb more DNA than its own mass and protect it from DNAses [18]

Thus, the intensity of the eDNA signal depends on the rate of release of genetic material by organisms and on the stability of eDNA in the environment (the duration of the existence of DNA fragments after removal of their source from the system) [5]. This indicator is related to the population density of a species, the size of its individuals, and the ratio between the DNA released into the environment and the DNA degraded for a given species. In addition, it depends on abiotic factors influencing the degradation and transport of eDNA. It varies from 15 days to 30 days for freshwater fish and amphibians [5, 10, 19, 20], from 0.9 day to 7 days for marine mammals [21], and is about 14 days for reptiles [22]. In stagnant freshwater reservoirs, eDNA persists for up to 30 days [20] and about 7 days in marine environments [12, 21]. The size of eDNA fragments is also an important factor determining their stability; fragments 300–400 bp in length persist in aquatic environments for one week [23, 24], whereas short fragments (about 100 bp) can persist for years [6, 25].

Issues related to the influence of various biotic and abiotic factors on the stability and migration of eDNA in ecosystems must be clarified to expand the capabilities of eDNA technologies for

reliable diagnosis of the presence or absence of taxa of interest in the study area and for assessing their abundance.

SUMMARY OF THE DEVELOPMENT OF THE eDNA APPROACH TO STUDYING THE BIODIVERSITY OF MULTICELLULAR ORGANISMS

The eDNA approach originated from metagenomics which, since the mid-1980s, has been studying the diversity of natural microbial communities from water, sediment, or soil samples [26, 27]. For the first time, the concept of eDNA was used in microbiological studies of marine sediments [26]. In the 1990s, eDNA technologies were used to monitor phytoplankton blooms [28], but only at the turn of the XX–XXI centuries were metagenomic methods found to be extremely effective for studying the diversity of multicellular organisms. The first work studying the macroorganism DNA fate in the environment was published in 1998; it demonstrated that the DNA of transgenic tobacco remains in the soil for up to 3 years [29]. The real impetus for the further development of this field was the research by E. Willerslev et al. [30], who applied the eDNA method in paleoecology to reconstruct ancient communities. Thus, in 1999, they isolated 57 taxa of fungi, plants, algae, and protista in two ice cores from Northern Greenland, dated at 2000 and 4000 years, by using molecular methods.

The eDNA analysis of multicellular organisms in water samples was first performed in 2005 [31] to identify the source of fecal contamination of surface waters through the DNA of humans, cows, pigs, and sheep. An article on the detection of the invasive species of the American kaloula *Rana catesbeiana* Shaw 1802 in swamps of France was one of the first works that showed the promising nature of the eDNA approach in biodiversity monitoring [19]. Compared with traditional methods, the eDNA method showed higher sensitivity and rapidity in detecting invasive species, which is important for the timely containment of invasions. C.S. Goldberg et al. were the first to use the eDNA approach to detect endangered organisms, including two representatives of tailed amphibians (*Dicamptodon aterrimus*, Cope, 1867) and tailless amphibians (*Ascaphus montanus* Nielson, Lohman and Sullivan, 2001) [10]. In the sea, eDNA methods were first used in 2012 for monitoring marine mammals [21] and assessing fish diversity [12]. The first attempts to assess the relationship between the abundance of organisms and the concentration of eDNA date back to the same time [5].

To date, many review articles have focused on various aspects of eDNA research. The earliest such works date back to 2012 [32, 33]. Since then, reviews have been published, covering approaches to the use of eDNA in conservation biology [2, 19, 34–38], eDNA ecology [4, 7], and the study of ancient eDNA [39].

METHODOLOGICAL FOUNDATION OF eDNA RESEARCH

The approach to eDNA research is similar in its idea to DNA barcoding, in which the sequence of the mitochondrial cytochrome oxidase 1 (*COI*) gene of about 650 bp is used in animals as a marker of the taxonomic affiliation of biological material. Fragments of eDNA are usually shorter (about 100 bp), and sequences of other genes (usually mitochondrial, chloroplast, or ribosomal RNA genes) aside from *COI* are used in analysis [19]. In cases of unknown taxa, target sequences are usually grouped not by real taxa but by the so-called "molecular operational taxonomic units (MOTUs)." The sequence of procedures used in eDNA studies is presented in Fig. 1. Prior to conducting the main study, a pilot project is recommended to be developed to determine the probability of target taxa detection using specific sampling and analysis protocols, considering that many factors, including substrate chemical property and temperature, influence this probability [40].

Due to the variety of substrates from which eDNA can be extracted (ice and permafrost, lake sediments, soils, cave sediments, air and water from stagnant water bodies, rivers, brooks, and oceans), sampling methods for eDNA studies are variable [39, 41]. Thus, in the case of water sampling, different filtration methods or DNA precipitation with ethanol can be used [42]. The method of sampling and the volume and the number

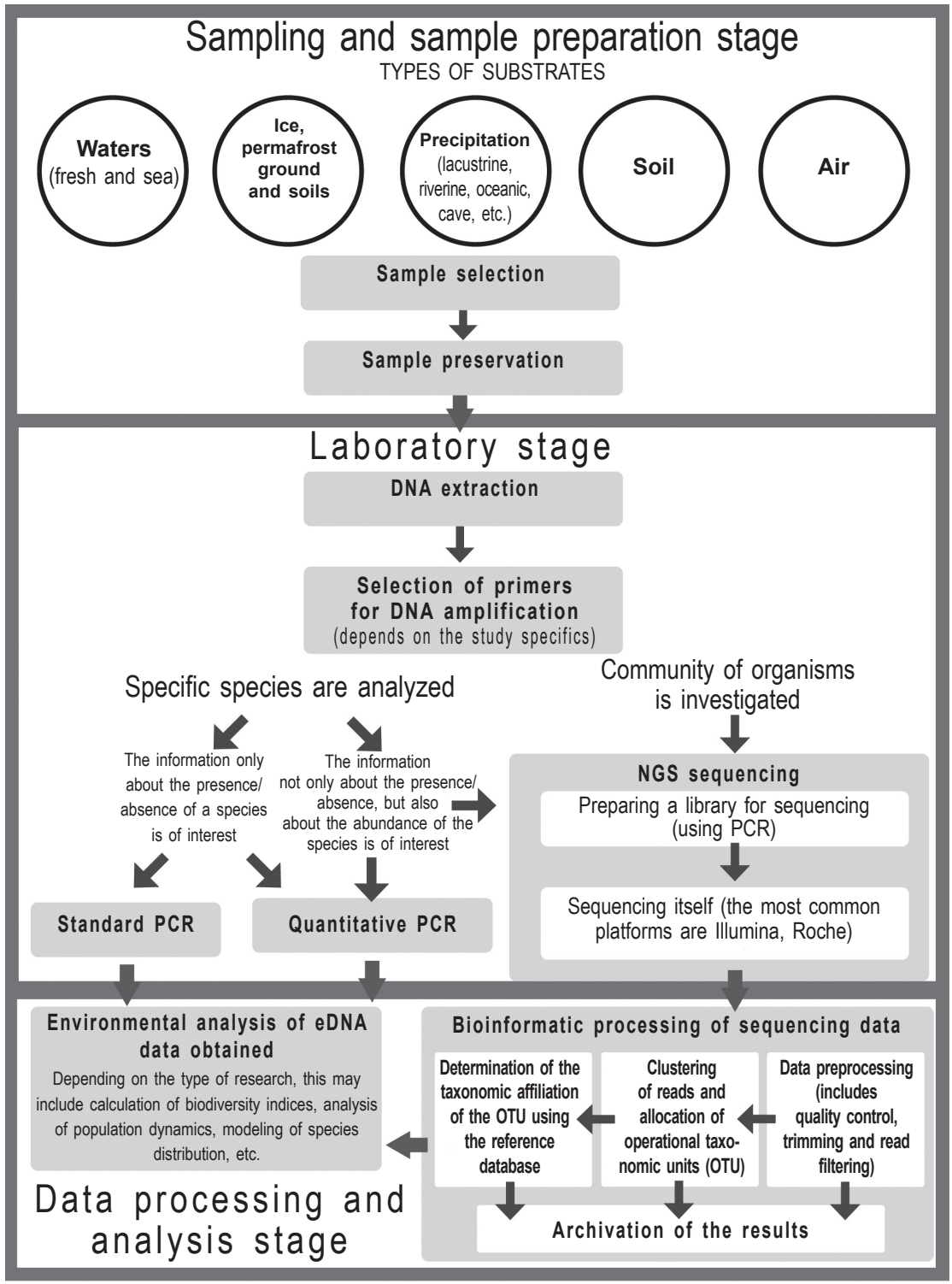

Fig. 1. The main general steps of environmental DNA analysis

of samples taken depend not only on the type of substrate but also on the specificity of the taxa of interest and the environment heterogeneity [37, 41]. For example, in soil or sediment sampling, large volumes and areal coverage are required to study large organisms, and sampling from more locations is needed to compensate for their heterogeneity and obtain a reliable description of the territory biodiversity [37]. The creation of negative control samples is also important because contamination is a common problem of eDNA analysis [40]. After sampling, the samples

are preserved by freezing at −20°C, in 100% ethanol, or in a cell lysis buffer [40, 41].

The stage of laboratory work depends less on the type of samples and is determined by the study objectives. DNA extraction is usually performed using special kits, the choice of which depends on the nature of the study and the taxa of interest; in some cases, conventional phenol-chloroform extraction is the most effective [37, 41].

At present, eDNA studies have two main types: targeted (species-specific) and semitarized (aimed at studying entire communities) [36] (Fig. 1). The first type involves the use of protocols designed for the detection of specific species from species-specific DNA fragments in samples. In general, the presence or absence of a species is determined using a standard polymerase chain reaction (PCR), but quantitative or digital PCR approaches that can assess the abundance of species have been actively developed recently. The second type of eDNA research is aimed at the characterization of entire communities. Laboratory methods in this type of studies are diverse and involve the use of a wide range of new-generation sequencing technologies, including metabarcoding and shotgun sequencing. They are aimed to determine the species identity of all DNA fragments in a sample, the success of which largely depends on the quality of existing databases. As a rule, for macroorganisms, they are still incomplete, which limits the taxonomic resolution of such an analysis. Studies of the second type are considered by many authors to be advanced in the field of eDNA and the most promising for the development of interdisciplinary research [36].

The choice of PCR primers is a crucial issue that must be considered in the design of the laboratory stage of both types of eDNA studies. In eDNA metabarcoding, the primers should be short enough to apply fragments of degraded DNA to assess the diversity of the community. The standard diagnostic region in barcoding for animals is the mitochondrial gene for subunit 1 of *COI*, those for plants are plastid genes of ribulose biphosphate carboxylase (*rbcL*) and maturase K (*matK*), and those for fungi are internal transcribed spacers of nuclear genes of ribosomal RNAs [37]. Different primers and regions differ in coverage, resolution, and affinity for different taxa.

In recent years, Illumina is the most common sequencing platform in eDNA metabarcoding [3]. Third-generation sequencing technologies have also been applied. The PacBio platform is especially effective when barcodes of more than 500 bp are required and the diversity of organisms is relatively small for precise taxonomic identification [43]. In addition, nanopore sequencing may simplify significantly the molecular detection of invasive species in aquatic ecosystems and widen its availability [44].

The final stage of eDNA studies is bioinformatic analysis of the data obtained. For its implementation, standardized pipelines that are customized for a specific study, depending on the sequencing technology, software used, and research tasks, are used. The main stages of bioinformatics analysis using the Illumina platform for eDNA metabarcoding have been discussed [45]. In many studies, special databases for metabarcoding were created, which include only the taxa of interest and indicator sequences from various sources, supplemented by their own data. For example, J. Axtner et al. [46] developed a database for metabarcoding of tetrapods, including various markers, such as fragments of the *16S* rRNA and *12S* rRNA genes, cytochrome B, and COI. The taxonomic composition of a sample is determined by comparing molecular taxonomic units or directly reads after quality filtration with a reference database. Many approaches have been proposed to implement this procedure, including the use of sequence alignment programs, Markov models (JM-MOTU), machine learning (TACOA), and probabilistic determination of taxonomic identification. The choice of a particular method depends on the markers used and completeness of the reference databases. If metagenomic studies of microorganisms have a significantly long history and many software packages and web services have already been created so that specialists, even without significant computer experience, can analyze simple metagenomic data, then such projects are just beginning to appear in the field of eDNA. For instance, SLIM is an open source

web application that simplifies metabarcoding data processing with an intuitive graphical interface [47].

FIELDS OF RESEARCH USING eDNA METHODS

EDNA methods are actively used in fields related to ecology and environmental protection. They are mainly applied in paleogenetic studies, the study of ancient communities; biomonitoring, the detection of invasive and rare species and the identification of "hidden biodiversity"; monitoring of environmental pollution and establishing new environmental indicators of its state; and tracking the spread of infectious diseases. The eDNA approach is also used in medicine, such as in monitoring the quality of water and air and in searching for pathogenic organisms. In agriculture, eDNA methods are used to detect phytopathogens. The main fields where eDNA analysis is actively used are presented in Fig. 2. Examples of case studies in these fields are discussed below.

Paleoecology. The application of eDNA methods for studying macroorganisms started with paleogenetic studies. E. Willerslev et al. [6] showed that the DNA of plants and animals can persist for a long time in permafrost soils and in sediments of the temperate zone, which enables to study the biodiversity of ancient communities even in the absence of macrofossils in sediments. The authors found 19 taxa of plants and representatives of megafauna (mammoths, bison, horses) in permafrost sediment core samples from Siberia aged 400,000 to 10,000 years and 29 taxa of plants and representatives of fauna, such as moa, in cave sediments of New Zealand; in addition, the biota of the island before human settlement was characterized. In 2007, E. Willerslev et al. [25] analyzed the basal parts of ice core samples from deep wells in Greenland by using eDNA technologies. At the high latitudes of this island, now buried under more than 2 km of ice, forests with a variety of conifers and insects (can be more than 450 thousand years old) existed. The authors suggested that many deep continen-

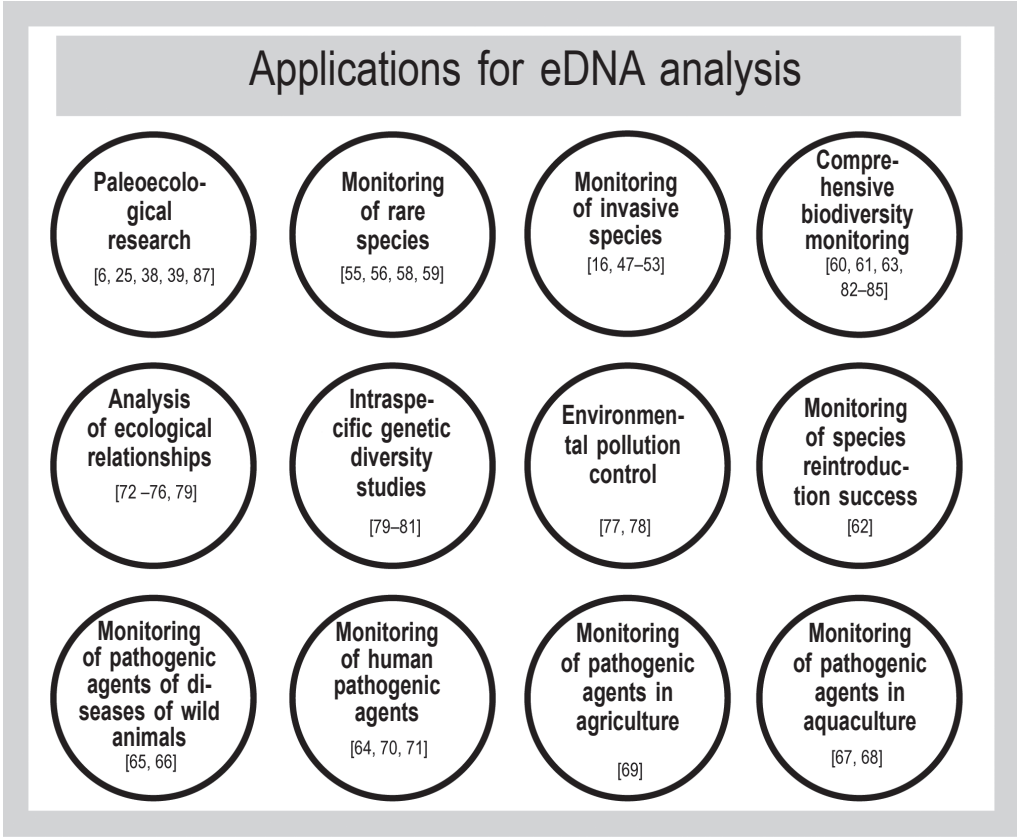

Fig. 2. The main areas in which DNA analysis of the environment is used, and links to examples of research in these areas

tal ice core samples may contain traces of buried paleoecosystems. Studies of ancient eDNA reconstructed in detail the evolution of ecosystems over time. M.W. Pedersen et al. [89] described the results of an eDNA analysis of lacustrine sediment core samples from the narrowest part of the corridor in Beringia, which was cleared by a glacial retreat. Their study aimed to establish the time when this region was colonized by flora and fauna to the extent that human migration became possible through it. After the glacier, steppes with mammoths and bison were the first communities to form in this area and leave traces in the form of eDNA. They date back about 12.6 thousand years ago. About 11.5 thousand years ago, they were replaced by sparse small-leaved forests inhabited by elks, deer, and bald eagles; about 10 thousand years ago, boreal forests formed in this area. Therefore, the first Americans who inhabited the continent earlier than 12.6 thousand years ago could not migrate to North America along this path.

The long-term (on a scale of hundreds and thousands of years) ecological dynamics of communities and ecosystems is significant for explaining the ecological processes currently noted. Temporal data (for example, on the state of ecosystems before anthropogenic interference and their further transformation) must be used when setting conservation aims taking into account the historical context [38]. eDNA methods have high potential for solving such problems, emphasizing that eDNA enables to obtain data on biodiversity dynamics over long periods of time for a wider range of taxa and in a wider variety of geographical contexts than other sources of temporal data in ecology (such as long-term monitoring, historical documents, paleoecology) [38]. In this regard, eDNA occupies a special niche, filling the gap between paleontology and paleoecology, long-term experimentation, and meta-analysis of contemporary research.

Monitoring of invasive species. Invasive species represent one of the main factors of biodiversity reduction, and the eDNA approach, given its sensitivity, has already established itself as one of the most effective methods for controlling the distribution of these taxa [49]. The use of sterile and disposable sampling tools in this type of research reduces the risk of the transfer of invasive species and pathogens during fieldwork [50]. One of the studies that demonstrated the efficiency of eDNA for the detection of invasive species was the study by E.A. Brown et al. [51], who aimed to identify non-endemic zooplankton species in 16 major ports in Canada. A total of 379 zooplankton species were identified, 24 species of them were found to be alien, and 11 allochthonous taxa were found in locations where they had not been previously recorded. Currently, protocols for eDNA analysis of invasive species of various groups of organisms are being actively developed. In addition to numerous works on fish and amphibians, methods have been proposed for the detection of invasive species of freshwater mollusks from Europe [52], the Burmese python in Florida, and even the algae *Codium fragile* Suringar, Hariot 1889 [53].

Monitoring programs for invasive species using eDNA are already implemented at the national level in several countries. Since 2013, the Asian Carp Regional Coordinating Committee, Ontario, together with the Upper Midwest Environmental Sciences Center and several private companies, has been implementing an eDNA-based monitoring program for Asian carp, a group of invasive species displacing native fish species in Mississippi, Ohio and other water bodies of North America [54, 55]. Results revealed that eDNA can be used not only for detecting but also for assessing the abundance of Asian carp [16].

New developments in eDNA technologies enable to perform all stages of analysis in the field. A number of authors [56] have already tested systems of sampling, filtration (Smith-Root eDNA Sampler), extraction of DNA, and a mobile qPCR platform (Biomeme) for a full cycle of eDNA analysis for the rapid and highly efficient detection of invasive aquatic organisms in the field. It requires further optimization, especially in terms of the extraction process, to avoid inhibition of PCR. In the current state, eDNA is still a reliable tool for detecting species.

Monitoring of rare and endangered species. The use of eDNA in monitoring rare species is no less effective because monitoring programs using eDNA are actively being introduced into

the practice of environmental organizations. For example, in 2014, Nature England (a non-governmental organization sponsored by the UK Department of Environment, Food, and Rural Affairs) approved the eDNA analysis protocol [57] for the detection of the crested newts *Triturus cristatus*, Laurenti, 1768, which is listed in the international Red Book. The testing of this protocol has demonstrated its higher efficiency compared with traditional methods (including visual search in the daytime, searching for spawn, the use of bottle traps, etc.). Thus, the time spent has been reduced by more than 10 times, whereas the financial cost for the study was 6–10 times lower. The presence of crested newts during this pilot project was identified in 99.3% of cases [58].

Most of the protocols for rare species eDNA research have been designed specifically for fish and amphibians. Applying this approach to reptiles and mammals has certain difficulties. Organisms with hard, keratinized cover tissues secrete significantly less DNA into the environment than organisms that form mucus [59]. Nevertheless, eDNA is often a reliable approach to monitoring these groups, as has been demonstrated for the crocodile lizard (*Shinisaurus crocodilurus* Ahl, 1930) [60]. eDNA methods can increase significantly the efficiency of monitoring rare representatives of terrestrial vertebrates in winter. They were successfully used to detect rare species of mammals, namely, Canadian lynx (*Lynx canadensis* Kerr, 1792), woolang (*Pekania pennanti* Erxleben, 1777), and wolverine (*Gulo gulo*, Linnaeus, 1758), on the basis of snow samples taken from their tracks and in locations where the animals were photographed [61].

One of the advantages of eDNA methods for monitoring biodiversity is the ability to involve amateur volunteers in research and thus familiarize a wide range of people with environmental problems. During the above-mentioned test monitoring of the crested newts, the volunteers took samples from 239 ponds in England, Wales, and Scotland. A large-scale, volunteer-based biodiversity monitoring program using eDNA was established by the University of California in 2017 [62]. During this program, volunteers collected soil and sediment samples and researchers analyzed eDNA and shared their results on a specially designed website that, given its user-friendly interface, is convenient for researchers and the general public, and contains educational and analytical modules [62, 63]. Within these works, more than 1679 locations have already been studied [63].

EDNA studies are also significant in monitoring the success of reintroduction of various species, as demonstrated in the example of the muddler (*Cottus rhenanus* Freyhof, Kottelat and Nolte, 2005) in West Germany [64]. Given their sensitivity, eDNA methods can be effectively used to determine the so-called hidden biodiversity in cases where the species of interest are not found by traditional methods in their habitats. In this respect, G. Boussarie et al. [65] detected 44% more shark species in New Caledonia by using eDNA methods than using traditional methods. In some areas exposed to anthropogenic pressure, some species were identified for the first time. Researchers have concluded that large-scale monitoring programs using eDNA methods are urgently required to identify rare species and develop programs for their protection.

Control of the spread of parasitic organisms. Another field of eDNA application is the control of the spread of infectious diseases and parasitic organisms. Thus, a system has been developed for the detection and monitoring of the causative agent of schistosomiasis (*Schistosoma mansoni* Sambon, 1907) by using eDNA from water samples [66]. The eDNA detection of these parasites was successful already at a number of 10 cercariae per liter of water under laboratory conditions. In field trials in Kenya, this approach identified schistosome in two locations undetected by traditional methods. Thus, eDNA analysis may be an important element in the control and complete elimination of schistosomiasis. eDNA studies can also facilitate the monitoring of pathogens in wild animals, and its non-invasiveness is an important advantage of this approach. eDNA methods can be used to detect pathogens causing massive death of amphibians, ranaviruses [67], and chytrids (*Batrachochytrium dendrobatidis* Longcore Pessier & D.K. Nichols, 1999) [68] before they lead to lethal consequences. eDNA methods are being actively implemented in monitoring patho-

gens and in aquaculture. Diseases result in about 40% loss in aquaculture productivity because of the difficulty in early detection of pathogens. eDNA analysis allows this assessment to be performed quickly and prevents the spread of infections. eDNA enables to detect efficiently the protozoa *Chilodonella* in barramundi aquaculture *Lates calcarifer*, Bloch, 1790 [69]. EDNA may also provide a new, reliable, low-cost pathogen tracking tool in salmon aquaculture. L. Peters et al. [70] demonstrated the possibility of identifying such parasites as *Lepeophtheirus salmonis* (Krøyer, 1837) and *Paramoeba perurans* (Young et al. 2007) to the species, as well as microalgae (*Prymnesium parvum*, *Pseudonitzschia seriata, and Pseudonitzschia delicatissima*) to the genus (they were based on the sequencing of amplicons of the 18S SSU region of rDNA v9). Moreover, the use of the cheaper Ion Torrent sequencing platform was more sensitive than the Illumina method.

Detection of plant pathogens. The eDNA method is used not only to detect animal pathogens but also to identify plant pathogens in agriculture. Thus, the Precision Biomonitoring campaign [71] provides a service for the detection of a wide range of microorganisms (bacteria and fungi) that pose a threat to humans and plants in *Cannabis* samples. The whole process takes about 80 min and is based on the qPCR system. It can be performed entirely at the sampling site, *in situ*, thereby saving time significantly compared with the traditional approach based on the cultivation of microorganisms.

Healthcare. The eDNA method is promising in healthcare for monitoring fungi, whose spores and mycelium fragments, when in the air, can cause allergic reactions and become sources of infection. The use of metabarcoding can increase the taxonomic coverage of fungi present in the air by tenfold compared with microscopy [72]. X. Tong et al. [73] demonstrated that such analysis of fungal diversity in air samples in hospitals can be an essential element for preventing infections and for choosing the optimal approach to disinfection.

Theoretical biology. The eDNA analysis is actively used to test ecological hypotheses, such as in studies of the ecology of pollination, which are especially relevant because of the global decline in pollinator populations and the spread of bee colony collapse syndrome. Honey and pollen samples can be analyzed using the eDNA approach to determine the preferences of bees for pollination of certain plant species. A. Valentini et al. [74] performed an eDNA analysis of honey to determine its geographical origin. N. de Vere et al. [75] analyzed honey samples from the National Botanic Garden of Wales and found that bees prefer native plants found in undisturbed forest communities and green hedges. Maintaining such communities and their protection against invasive species are essential for the wellbeing of the bees. Another group of authors [76] showed that DNA metabarcoding of pollen from pollinators can reveal 2.5 times more interactions of plants with pollinators compared with the traditional approach. Using the same method, A. Lucas et al. [77] revealed an individual seasonal specialization in the selection of plants by hoverflies. Another group of authors [78] showed the possibility of transcontinental pollination by revealing pollen from African taxa in butterflies on the European Mediterranean coast. Thus, eDNA analysis can also be used to identify the geography of pollinator migrations.

Indication of the state of water reservoirs. eDNA methods can be an effective tool for monitoring the pollution of aquatic ecosystems and establishing new environmental indicators. F. Li et al. [79] used metabarcoding for a comprehensive assessment of changes in the structure of river communities in the Yangtze river estuary (including both pro- and eukaryotes) in response to stress factors associated with anthropogenic activity. Their tasks were to identify factors that negatively influence the structure of communities and to assess the capabilities of the eDNA approach for determining the status of river pollution. The results revealed that an excess of nutrients (including DO, NO_3^-, NH_4^+, TN, and TP) is such a factor. The authors also identified molecular taxonomic units that can be indicators of the status of river water pollution with these components. Another study showed that analyzing the temporal profiles of bacterial communities in rivers based on *16s* rRNA sequencing data can be used

to predict the cyanobacterial bloom date more accurately than analyzing environmental parameters (with a probability of 78%–92%) [80].

PROSPECTIVE FIELDS FOR APPLICATION OF eDNA IN THE FUTURE

One of the emerging trends in eDNA analysis is its use in population genetic studies to investigate the genetic structure of populations, which enables to reconstruct their history, assess the current state, and predict future prospects for planning environmental protection measures. Traditional research of this kind involves the collection of samples of blood or tissues of organisms, which leads to their stress or even lethal consequences. This process can be expensive, dangerous (for example, when working with poisonous organisms), laborious, and time consuming for researchers. Although this field is only emerging, a number of studies used eDNA to obtain intraspecific population genetic data. Thus, seawater eDNA was used to study the diversity of mtDNA haplotypes in the whale shark *Rhincodon typus* to determine their population structure and trophic relationships. The recently established Qatari population of these sharks has been shown to be closer to Indo-Pacific than to Atlantic representatives of the species. For over 2 years, a positive correlation was traced between the number of eDNA copies of whale sharks and kawakawa (*Euthynnus affinis* Cantor, 1849) possibly because of their trophic links (tunas are included in the diet of these sharks) [81]. Thus, eDNA can be used not only to determine already established haplotypes but also to isolate new ones. Previously unknown haplotypes for the mtDNA control region were revealed in the harbor porpoise (*Phocoena phocoena* Linnaeus, 1758) during the analysis of eDNA from seawater samples [80]. Moreover, eDNA metabarcoding enables to study haplotypes not only for one but for many species simultaneously, as demonstrated for arthropods from freshwater bodies [83]. Details on this line of research are presented in the review article by C.I. Adams et al. [59].

Creation of eDNA national or international biobanks has been proposed to systematize community biomonitoring using eDNA [3]. Thus, obtaining eDNA time series allows the documentation of changes in ecosystems over time. It also enables to analyze eDNA material from previously published papers using new methods. Currently, one problem in eDNA metabarcoding is the lack of standards that would allow comparing eDNA data obtained in different studies using various technologies, and the creation of eDNA biobanks is proposed to solve this problem.

Some studies used eDNA methods not only to detect species but also to determine their abundance. Many articles considered the relationship of eDNA concentration with the abundance or biomass of certain species of aquatic organisms (for this, quantitative PCR, qPCR, or dd PCR is used). The results usually show a moderate degree of correlation between them. Errors in assessing the abundance can be determined by numerous factors affecting the concentration and transfer of eDNA in water and by the specifics of analytical protocols, including the selection of primers. Current studies are focused on evaluating the possibilities of using eDNA metabarcoding to determine the relative abundance of many taxa by the number of reads belonging to different molecular taxonomic units. The first work to demonstrate the potential of eDNA metabarcoding for quantifying large-scale variations in the structure of fish communities in a large river was the article by D. Pont et al. [84], who analyzed water samples taken along 500 km of the Rhone River. They found a significant (moderate) correlation between the relative abundance of fish species and the number of standardized reads, and concluded that metabarcoding provides important information about the relative abundance of species (although it does not estimate accurately their abundance or biomass). Samples were taken during one 12-day expedition, and the data obtained as a result of such monitoring were comparable to those obtained during 10-year monitoring with the traditionally used electro-fishing (about 300 days of field work).

CONCLUSION

Since their active application to eukaryotes in the 2000s, eDNA methods have been used successfully in many lines of research. The main ones

are paleogenetic studies (aimed at studying the biodiversity of ancient communities and reconstructing the evolution of ecosystems in time); monitoring invasive and rare species and identifying "hidden biodiversity"; controlling the spread of infectious diseases and parasites (which is used in health care, aquaculture, agriculture, and environmental protection); testing ecological hypotheses and studying biotic relationships; and monitoring environmental pollution and establishing new environmental indicators. In the future, the improvement of eDNA technologies will enable to use them not only for the detection of species but also for the study of intraspecific genetic diversity (in population genetic studies), as well as for the quantitative characterization of the structure of entire communities (not only for assessing the abundance of individual species).

The main field in the practical application of eDNA methods is monitoring invasive and rare species in aquatic ecosystems. eDNA technologies can supplement significantly to traditional monitoring methods because of their several advantages, including high sensitivity (which is especially important in the case of small species), less stressful impact on organisms due to non-invasiveness, and low research costs (especially in large-scale programs), thereby reducing the time and labor costs of research and the risk for specialists in sampling. Such methods allow amateur volunteers to join monitoring programs and various people to be familiarized with biodiversity problems (due to the simplicity of the field stage of such works). They also facilitate automated monitoring; in fact, portable stations for conducting eDNA analysis in the field already exist and are being actively improved. Many advantages of eDNA methods make them promising for conducting large-scale studies of biodiversity in hard-to-reach regions, such as in the Arctic [85] and Antarctic waters [86], for tracking the consequences of climate change (such as the development of invasions) and anthropogenic activities. A project has been launched to develop a system for identifying species in the disphotic zone of the ocean using eDNA (Woods Hole Oceanographic Institution ocean twilight zone project) [87].

Despite several advantages, many problems associated with the use of eDNA technologies remain. Many review articles (e.g., [1, 2, 37]) discussed that the limitations of eDNA techniques are the high risk of contamination and, accordingly, false positive results (to combat this, comparison samples must be used at all stages of work and other precautions [2]); errors associated with the inhibition of polymerases by humic acids and other substances present in the samples at the PCR stage, which can lead to false negative results; errors at the PCR or sequencing stage, including the formation of chimeric molecules; incompleteness of reference databases and their taxonomic and geographical coverage; and difficulties in interpreting the results associated with the impossibility of distinguishing the eDNA signal from living and deceased organisms and different stages of the life cycle, as well as hybrid species (in the case of using a mitochondrial marker region). The difficulties in assessing the temporal and spatial coverage of the eDNA signal are especially noteworthy. DNA degrades in surface waters from several hours to several days [5]; therefore, their eDNA reflects the current state of the community; however, it can persist much longer in soils and sediments. Moreover, in certain types of ecosystems (e.g., rivers), eDNA can be transported quickly, which hinders the determination of its geographical origin. For example, J. Pansu et al. [88] identified the DNA of communities of land plants and livestock in samples of lake sediments. Water bodies accumulate DNA from the entire catchment basin. Thus, the influence of various factors (biological, physical, chemical) on the transport of eDNA in the landscape and the rate of its degradation requires an intensified study [1].

Most studies that use the eDNA approach are currently aimed at studying the diversity of eukaryotic organisms, and their number has been steadily increasing in recent years [90]. Despite the listed difficulties, the use of eDNA is a promising technology that is already leading to revolutionary changes in nature conservation and opens up new horizons in the study of biodiversity of our planet and its development.

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